Chapter 1

Ticks and micro-organisms associated with ticks

1.1 Biology of ticks

Ticks are blood feeding ectoparasites of mammals, birds, reptiles and also amphibians. Approximately 870 species have been described worldwide (Furman and Loomis, 1984), but investigations in wild areas are leading to the description of new species, and further novel species might be revealed in the future also by molecular tools, capable of proof the existence of cryptic taxa. Ticks are arthropods of the order Parasitiformes (suborder: Ixodida) belonging to class Arachnida (subclass: Acarina), which includes spiders, scorpions, and mites, in the subphylum Chelicerata (Sonenshine, 1991). Ticks, which are among the oldest and most successful group of this class, are chelicerate arthropods characterized by the presence of two chelicerae, highly modified structures which bear laterally directed cutting edges, used for grasping, piercing, cutting and other functions associated with feeding and also sexual reproduction. Within the Arachinida, spiders (Araneae) and scorpions (Scorpiones) present a division of the body in two major regions, the anterior prosoma, constituted by 6 somites, bearing the pedipalps (modified second pair appendages) and four pairs of walking legs, and the posterior opisthosoma, abdominal region constituted by 12 somites. In ticks occurred an evident modification that brought to the fusion of prosoma and opisthosoma to form the idiosoma, lacking any visible segmentation. The mouthpart of tick (capitulum) bearing chelicerae and palps is located in the frontal (Ixodidae) or ventral (Argasidae) body region. This structure, homologous to the gnathosoma of other acarines, presents an unpaired ventral appendage, under the chelicerae, the hypostome, a sort of scalpel covered with curved denticles, used for attachment to the vertebrate host skin. The genital aperture is normally located at the level the third/fourth coxae, while the anal aperture in located in the ventral posterior part of the idiosoma. The central nervous system is concentrated into a single nerve mass, the synganglion. Host searching in ticks is accomplished by highly efficient sensory system for detecting odors, vibrations, temperature changes and other environmental parameters. Ticks use their forelegs in a manner similar to insect antennae, exposing the olfactory, gustatory, mechanoreceptor and thermoreceptor sensillae of the Haller's organ, located on each tarsus, to the air stream for obtaining information. The respiratory system consists of two paired spiracles (stigmata), opening within a spiracular plate, located on the lateral surface of the idiosoma immediately posterior to the IV coxa (the taxonomic tick category Metastigmata derives from this characteristic), the site of departure of convoluted tracheae. Ticks use tracheae, a system of interconnected tubes similar to that used by insects, to convey atmospheric oxygen to their tissues. The excretory system is composed by Malpighian tubules

that open in the rectal sac in the proximity of the genital aperture. Ticks have numerous muscle groups; striated muscle fibers are present in the body cavity, the capitulum and the legs, while smooth muscle fibers from the external wall of the midgut and cover the organs of reproductive system and other internal organs. External morphological features of ixodid ticks are illustrated in Fig 1.1.

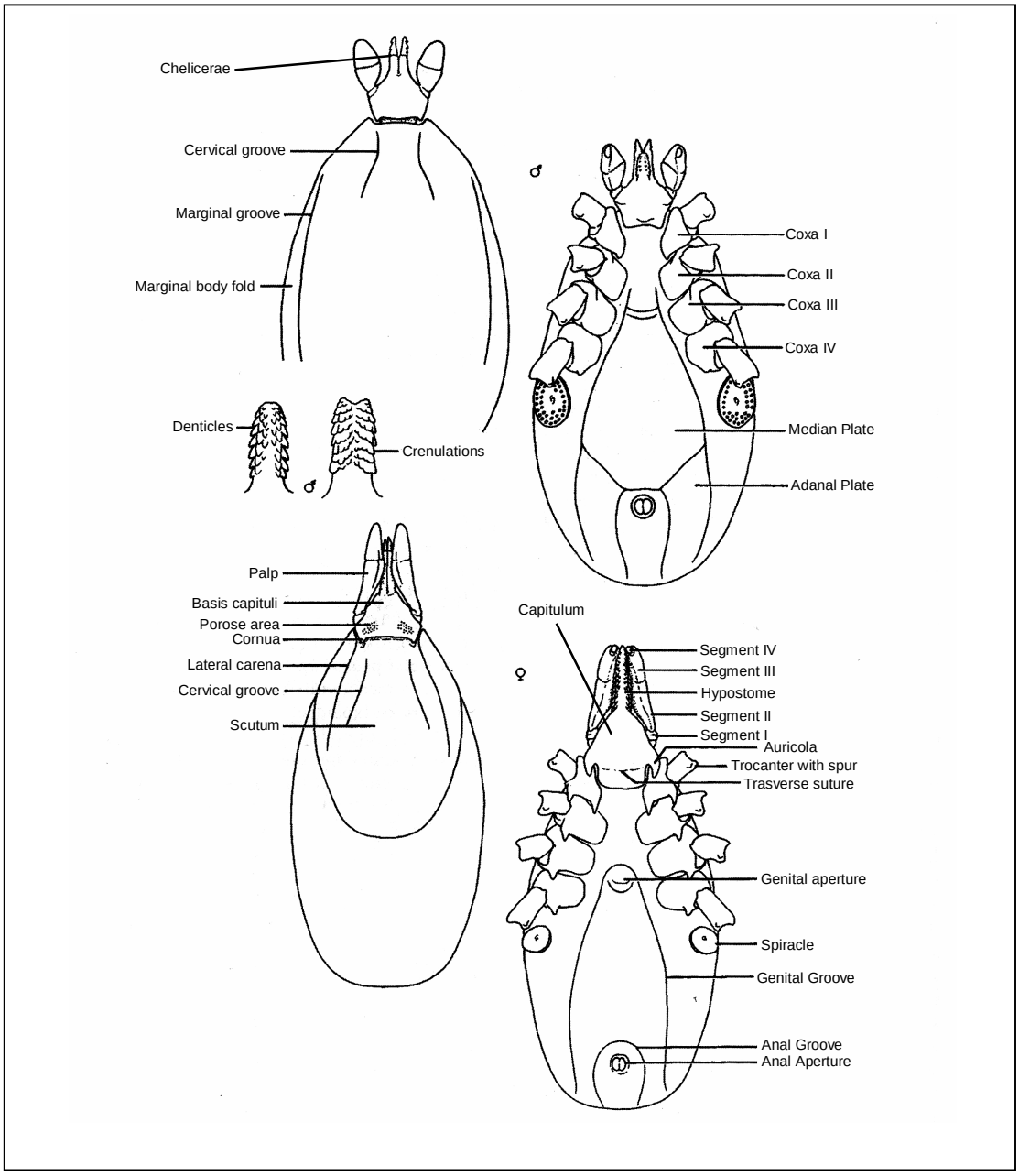


Fig 1.1 – Drowings of an ixodid tick (Ixodes sp.) showing dorsal and ventral morphological features including details of two types of male hypostome with denticles or crenulations (derived from Keirans and Clifford, 1978).

The following paragraph is based on the book 'Biology of Ticks' (Sonenshine, 1991) and references therein. Three families of ticks are recognized, two of them, hard ticks (Ixodidae) and soft ticks (Argasidae) are important vectors of disease-causing agents in humans and animals. The third family (Nuttalliellidae) is represented by a sole species, *Nuttalliella namaqua*, a South African tick with peculiar characteristics. This species can be distinguished from ixodid and argasid ticks by a combination of characters including the position of the stigmata, lack of setae, presence of strongly corrugated integument, and form of fenestrated plates. Argasidae present a 'leathery' body surface without any hard dorsal plate (scutum), several nymphal instars (even 7/8) before molting to adults, need numerous meals that last for only short periods (even less than 1 h). Argasidae differ from hard ticks even in other biological features, e.g. in the internal anatomy; soft ticks will not be object of this PhD dissertation.

In the following text, the 'typical' biology and life cycle of Ixodidae is described. Remarkably, the life cycle of hard ticks is uniformly conserved throughout the family. All ixodid ticks have a single nymphal stage. However, few species in the family can exhibit ecological, biological or behavioral variations from the characters shared by the majority of the members of the family.

The family Ixodidae can be divided in two groups: Prostriata and Metastriata. The Prostiata ticks (only genus *Ixodes*) are easily distinguished from Metastriata (genera *Amblyomma*, *Aponomma*, *Haemaphysalis*, *Hyalomma*, *Dermacentor*, *Cosmiomma*, *Nosomma*, *Rhipichephalus*, *Anomalohimalaya*, *Rhipicentor*, *Boophilus* and *Margaropus*) for the presence of an anal groove extending anterior to and around the anal aperture. The Ixodidae are characterized by the presence of a tough, sclerotized plate on the dorsal body surface (scutum), that covers the entire dorsal body in males, while is limited to the anterior dorsal body region in females, nymphs and larvae. The scutum is the site of attachments of dorso-ventral muscles, cheliceral retractor muscle and many other muscular groups. Females, with limited scutum coverage can engorge hugely as a result of the new synthesis of body cuticle to accommodate the blood meal; nymphs and larvae also increase in size after the blood meal, while plate-armored males experiment engorgement constrains. Due to scutum coverage, sexual dimorphism is evident in the adult stage (Fig. 1.1), while ticks at the larval and nymphal stage are not easy (or are impossible) to sex. Eyes, when present, are located on the postero-lateral margin of the scutum. The mouthpart, highly efficient in the blood-sucking function and visible from dorsal side protruding anterior to the scutum, presents three visible components. The capitulum is composed by a basal structure (basis capituli) where two pyramidal 4-segmented appendages, the palps, are attached; between these it is located a pair of 2-segmented chelicerae, which protect the center rod-shaped structure, the hypostome. The palps move laterally while the tick is feeding and do not enter the skin of the host. The rough hypostome has many beak-like projections (denticles), but in males of few species these denticles are not present. This is the structure which plunges into the skin of the host while feeding. The backward directed denticles prevent easy removal of the attached tick. Most hard ticks secrete a cement-like substance

produced by the salivary glands to fasten themselves to the host; that dissolves after feeding is complete. In addition, several tick species secrete anticoagulant, immunosuppressive, and anti-inflammatory compounds into the area of the tick bite. These substances presumably help the tick to obtain a blood meal without triggering the anticoagulant and inflammatory reaction of the host. The same substances also might facilitate pathogens to infect the host.

Ixodidae have four distinct life stages, the embryonated egg is followed by other three active stages. Six legs larvae emerge from the egg and start searching actively for a suitable vertebrate host. After obtaining a blood meal, they molt to the eight-legged nymphal stage. Nymphs feed and molt to the next and final stage, the adult. After feeding once more, the adult female, after mating, lays one batch of eggs (350 – 22.000 depending from species) and dies soon after deposition. Only one blood meal is required during each of the three life stages. The time to completion of the entire life cycle may vary from less than a year in tropical regions to over three/four years in temperate areas, where larvae, nymphs and adults may enter winter diapause until hosts are again abundant in the next season. Certainly, the local micro-ecological conditions can influence the length of the life cycle. Ixodidae seek hosts by a typical behavior called "questing", in which they crawl up the stems of grass or on the edges of leaves of plants in a typical posture with the front legs extended, especially in response to a host (Fig. 1.2).

Fig 1.2 – Unengorged female of *Ixodes ricinus*, in the process of searching for vertebrate hosts, moving front legs in 'questing' activity on a grass blade.

Biochemical volatile compounds, such as carbon dioxide, ammonia and other host odorous substances, as well as heat and movement serve as stimuli for questing. In this way, ticks climb on to any potential host which passes against their extended front legs. The majority of hard ticks wait on grass and low vegetation with the first pair of legs actively questing for a susceptible host on which they can attach and feed. At least one species, *Hyalomma dromedarii*, the desert camel tick, is known to actively search for host. This capacity is even more remarkable for the fact that evolved in Northern African semi-desert areas, with scarce vegetation low humidity rate and high temperatures. Hard ticks are most commonly collected for research purpose by the use of "flags" or "drags" which are pieces of roughly textured tissues such as fleece or flannel attached to a rod handle. The flags are slowly dragged across the surface of vegetation to collect questing unengorged (no ingested blood-meal) ticks, while engorged specimens can be collected only on the hosts.

Once on the host the tick crawls to a feeding predilection site where it splits the skin with the two chelicerae and inserts the hooked hypostome that secure the tick while feeding. Hard ticks feed for extended periods of time on their hosts, varying from several days to weeks (larva, 2–3 days; nymph, 4–5 days; adult female, 7–14 days), depending on such factors as life stage, host type, and species of tick. In feeding females, active growth of gut and cuticle occur in order to contain the huge blood meal, most of which will be acquired in the final 24 hours of engorgement (Sonenshine 1991). The adult male rarely feeds and never engorges. In *I. ricinus*, males present a modified hypostoma with scales not suitable to pierce the skin (see below).

In general, hematophagous arthropods feed by sucking blood directly from a small venule or other vessel (e.g. mosquitos), a process known as solenophagy. Hard ticks do not neatly pierce blood vessels but create a feeding pool by cutting capillaries and other blood vessels generating an expanding hemorrhage as blood flow into the wound site (telmophagy). The feeding lesion expands as a result of the anticoagulant and antihemostatic activity of the salivary compounds, vasoactive mediators and immunomodulators injected into the wound (Grubhoffer, 1999). During feeding the blood meal is concentrated by the extraction of water which is then secreted back into the host by specialized salivary gland cells (Type I acini) and this is an important means by which also tick-borne pathogens invade the host. Once fully engorged the tick withdraws its hypostome and tumbles to the ground where it begins digesting the blood meal and developing to the next instar or prepare for oviposition (females). The digestive process consists of liquid pinocytosis (microphagocytosis) and endocytosis of blood components by cells lining the gut, followed by intracellular digestion rather than intralumenal enzymic digestion as occurs in most other haematophagous arthropods. Only the lysis of erythrocytes takes place in the gut lumen. The lack of digestive enzymes in the tick gut favors the survival of ingested microorganisms and may explain why ticks transmit the greater variety of pathogens compared to other groups of arthropod vectors.

Hard ticks apply different strategies in order to increase their chance of contact with an appropriate host and ensure survival. Some ticks feed on only one host throughout all three life stages (one-host ticks). This type of tick remains on one host during the larval and nymphal stages, until they become adults, and females drop off the host after feeding to lay eggs. Other ticks feed on two hosts during their lives and they are called two host ticks. This type of ticks feeds and remains on the first host during the larval and nymphal stages, and then drops off and attaches to a different host as an adult for its final blood meal. The adult female then drops off after feeding to spawn. Finally, many ticks feed on three hosts, one during each life stage, and are appropriately named three host ticks. These ticks drop off and reattach to a new host during each life stage, until finally the adult females lay their batch of eggs. In each case, the fed adult stage is terminal, that is, after laying one batch of eggs the female dies, and after the male has reproduced, he dies as well. Multi-hosts ticks present higher potential in disease transmission.

1.2 Ticks as vector of diseases

Tick-borne diseases (TBDs) are considered emerging threats to public health. Ticks transmit the widest variety of pathogens compared with any other blood sucking arthropod, including bacteria (Lyme disease spirocheates, rickettsiae, ehrlichiae, *Francisella* spp., etc) nematods (*Acanthocheilonema* spp.) protozoa (*Babesia* spp. and *Theileria* spp.) and viruses (flaviviruses, coltiviruses and nairoviruses). With the only known exception of relapsing fever caused by *Borrelia duttonii*, tick-borne infections of humans are zooanthroponoses, diseases of animals transmissible to human beings that represent incidental dead-end hosts.

The first impulse to systematic research, biology, ecology and geographical distribution of Ixodidae has been given by the initial findings on the pathogenic role played by some species in relation to farm animals, especially those of medium and large size, when decimated by so-called piroplasmosis or babesiosis. The micro-organisms or viruses, usually acquired from ticks through the blood meal, are often transmitted from one stage to another (transtadial transmission) and in a number of cases from females to eggs (transovarian/vertical transmission). Ticks can, thus, be regarded as reservoirs and multiplicative stations of such organisms, ensuring their survival over time and favouring the spread through the different host species. Vertical transmitted pathogens can survive and spread through the abundant offspring, forced to take blood in each stage. These ways of transmission ensure the expansion of the spectrum of potential hosts who may become infected. The tick host specificity and the vagility of the hosts are key factors to consider in the epidemiology of TBDs.

Hosts might behave as rings of transmission for other ticks and other guests, like migratory birds, that may also contribute to TBDs spreading as simple vehicles (Cringoli et al., 2005). The man, as regular host, can be attacked by ticks in natural habitats, but also indoor trought pets or domestic animals.

Ticks are second only to mosquitoes as vectors of human diseases and also can transmit toxic compounds. In the United States and Europe, ticks are the most common arthropod vectors of diseases. From the perspective of disease transmission to vertebrates and humans, the essential characteristic of ticks is their need to ingest a blood meal to achieve their next stage of development or to give birth to their offspring. As mentioned before, they parasitize and ingest blood from all classes of vertebrates, with the exception of fish. Ticks with low host specificity, able to feed on a wide range of animal species, present higher risk to transmit vector-borne diseases. During the blood meal, a single tick can transmit multiple pathogens, a phenomenon that has led to atypical presentations of classic tick-borne diseases. Secondary infections and allergic reactions to proteins in tick saliva are also possible. Anyway, one study suggests that repeated tick bites may actually protect against Lyme disease, possibly due to developed hypersensitivity from the prior bites of uninfected ticks. Around thirty tick species are known to feed frequently on humans. The pathogen of greater importance transmitted by ticks is probably *Borrelia burgdorferi*, a spirochete that is the etiologic agent of Lyme disease (Hovius et al.,

1998). This is a multi-organ disease that can affect the skin, heart, joints and central nervous system, but also the peripheral nerves (neuroborreliosis). The disease is caused by both direct invasion of these bacteria, and by the violent response and possibly autoimmune immunopathology that can trigger. Serious human diseases of current interest in the United States and in Europe caused by tick-borne pathogens include: Lyme disease, human monocytotropic ehrlichiosis (HME), human granulocytic anaplasmosis (HGA), babesiosis, rickettsiosis (respectively Rocky Mountain spotted fever in USA and Mediterranean spotted fever in Europe), tularemia, Q fever, and Tick-borne Encephalitis (TBE). In Europe, some disease like the Colorado tick fever does not occur, but other life threatening viral diseases are present, like TBE and Crimean-Congo hemorrhagic fever. The most common of these diseases will be further discussed in the following chapters.

Besides pathogenic agents, other microbes coexist in hard ticks, such as endosymbionts, commensals or microbes acquired from the blood meal on animal hosts (Noda et al., 2000; Epis et al., 2008)

For more information on tick borne diseases I suggest the monograph 'Ticks, biology, diseases and control' (Bowman and Nuttall, 2004).

1.3 *Ixodes ricinus*

The European tick, *Ixodes ricinus*, is the most common species biting humans in Europe. *Ixodes ricinus* is the primary vector of spirochetes of *Borrelia burgdorferi* sensu latu complex and of the TBE virus. This tick is also present in North Africa and in several Italian regions especially in thermo-mesophilous deciduous woods, mixed woodlands and shrubby habitats where a diverse array of hosts are present and the relative high degree of humidity allow the tick to complete the 3/4 years life cycle. After blood meal, digestion is slow, and development of the new instar takes several months in the temperate regions. *Ixodes ricinus*, presents three instars (larva, nymph and adult) and each of them wait on vegetation searching hosts trough questing. *Ixodes ricinus* females require a blood meal to complete oogenesis. The newly moulted (or hatched) unfed tick may remain quiescent for a time but will eventually ascend the vegetation to quest for a host and a blood meal. In *I. ricinus* a full year may separate the active feeding periods of successive instars. The free-living stages of these ticks are very sensitive to desiccation and cannot survive relative humidity of less than 80% for long time. This requirement restricts the ticks to habitat in which humidity at the base of vegetation rarely falls below this level, even in the summer. Ticks can acquire water from humid air by ingestion of hygroscopic material secreted by the salivary glands (Kahl & Knülle, 1988). This capability enables the unfed stages to make host-seeking excursions into the upper vegetation where they can wait for hosts for several days before needing to descend to the vegetation base in order to rehydrate. Ticks may also occur in open areas where there is high rainfall and diverse vegetal

substrates, such as rough hill-land in the UK and Ireland and the forests of the Scandinavian countries, where the main hosts for all instars are usually sheep, cattle, deer or wild ungulates.
Even if this species is known to parasitize almost 300 vertebrate species, the three instars of *I. ricinus* tend to occur in different proportions on different hosts. In most regions larvae feed most readily on rodents, nymphs on rodents, birds and medium-sized mammals, and adults on large hosts, such as deer. The different instars quest at different heights in the vegetation (Gigon, 1985, Mejlon & Jaenson, 1997), apparently in response to desiccation stress (Randolph & Storey, 1999). The stratified occurrence in the vegetation probably makes a major contribution to host specificity, but attachment and feeding preferences are also likely to play a part (Nilsson & Lundquist, 1978). All instars of *I. ricinus* bite humans, but the few studies on this aspect indicate that nymphs are involved more often than either larvae or adult females. As mentioned before nymphs preferably feeds on small rodents and since these small mammals are regarded as important reservoir hosts of diverse zoonotic pathogens, nymphs could play a major role in disease transmission. In fact, nymphs are primarily responsible for transmitting *B. burgdorferi* s.l. spirochetes to rodents and differences in nymphal infestations of these reservoir hosts may influence the regional prevalence of the pathogen.
Identification of the factors that determine *I. ricinus* abundance and vector potential for disease transmission is necessary in order to apply eradication campaigns and risk management projects. Tick density in any particular habitat is determined by factors such as vegetation cover, climate and weather, which affect the survival and development of the free-living phases, and by the success of host acquisition and feeding by the parasitic phases. The free-living phases are highly dependent on the year-round availability of a humid microclimate and adequate temperatures for development. The optimal habitats in these respects are deciduous woodlands in temperate climates, and such woodlands usually harbour diverse and numerous hosts so that the immature tick instars are rarely limited in their feeding opportunities. However, the adult ticks require large mammals such as deer to feed successfully and produce the next generation, and there are now ample data to suggest that the availability of such hosts has a major impact on the population density of ticks within tick-permissive habitats. Where the habitat is especially favorable for the free-living phases of the tick life cycle, relatively small numbers of deer can maintain very large tick populations (Robertson et al, 2000). An understanding of the factors that determine the density of tick populations has predictive value for the effects of such phenomena as climate change. In a recent study in Sweden the northward expansion and increased density of *I. ricinus* between the 1980s and 1990s, resulting in increased incidence of TBE (Lindgren et al., 2000) and these changes were related to warmer winter temperatures. Such a temperature rise could enhance tick densities and distribution in several different ways, including longer periods for tick development, increased vegetation growth thus extending tick-permissive habitats, increased host acquisition opportunities in autumn and winter, and better wintering of the main host for adult ticks (*Capreolus capreolus*). Additionally, roe deer

populations in Sweden are thought to have increased in the mid-1980s as a result of reduced predation following a scabies outbreak in the fox population. This study illustrates the complex dynamics underlying tick-transmitted zoonoses and the value of developing good mathematical models for their analysis. It is increasingly evident that a sound knowledge of the biological processes involved in the transmission of these diseases is vital for an understanding of their eco-epidemiology and the full exploitation of predictive models.

I. ricinus acts both as vector and reservoir for a series of zoonotic pathogens, in particular, the agents of Lyme disease, Tick borne encephalitis virus (TBEV), human monocytotropic ehrlichiosis (HGE) and anaplasmosis (HGA), which are emerging in most of Europe. Wildlife reservoir species play a central role in maintenance and persistence of these infections, (i.e. the small mammals *A. flavicollis* and *C. glareolus*). It is also important to consider the double effect of roe deer (*C. capreolus*) in maintaining tick population and act as reservoir for *A. phagocytophilum*, but is not a competent host for *B. burgdorferi* and TBE virus. More than 300 animal species have been reported as natural hosts for *I. ricinus* and 50 vertebrate species have been identified as reservoir hosts for *B. burgdorferi*.

1.4 Endosymbionts of ticks and other arthropods

Symbiotic associations between eukaryotes and prokaryotes has long arisen the interest of biologists because of the remarkable, intricate co-evolturionary adaptations that can occur between the partners. Bacteria inside eukaryotic cells are frequently encountered in insects and other arthropods. These microorganisms, belonging to different bacterial lineages, are encountered free in the cytoplasm or, more often, within host-membrane derived structures (phagosomes, endosomes, vacuoles, even in specialized cells called bacteriocytes). They seldom occupy other intracellular niches. Some bacteria of the genus *Rickettsia* are frequently reported free in the cytoplasm of the host cells and are also known for the ability to colonize even the nucleus (Raoult and Roux, 1997). Arthropod endosymbionts are classified in two broad categories: Primary (P) and Secondary (S). P-endosymbionts were associated with their insect hosts for many millions of years (from 10 to hundred millions), developing obligate associations, and display co-speciation with their hosts. S-endosymbionts exhibit an association established in more recent time, are sometimes horizontally transferred between hosts, do not live inside highly specialized structures and are not obligate. Among P-endosymbionts of insects, the pea aphid (*Acyrthosiphon pisum*) endosymbiont *Buchnera* sp., the tsetse fly *Glossina morsitans morsitans* endosymbiont *Wigglesworthia glossinidia brevipalpis* and the *Blattabacterium* spp. reside inside specialized cells (bacteriocytes) and have been widely investigated. In these cases, the symbiosis is obligate, meaning that neither the bacteria nor the insect is viable without the other. These bacteria are impossible to cultivate in lab conditions outside of the insect. With special nutritionally-enhanced diets, the insects can survive, but are unhealthy, and at best survive only a few generations. In some insect groups, as in the

cockroaches, the endosymbionts are maternally-transmitted (i.e. the mother transmits her endosymbionts to her offspring). In termites, a variety of endosymbionts reside within the hindgut and are transmitted through trophallaxis among colony members. P-endosymbionts help the host either by providing fundamental nutrients, or by metabolizing insect waste products into safer forms. For instance, the putative primary role of *Buchnera* spp. is to synthesize essential amino acids that the aphid cannot acquire from its natural diet of plant sap. Similarly, the primary role of *Wigglesworthia* sp. is probably to synthesize vitamins that the tsetse fly does not obtain from the blood meal. Bacteria benefit from the reduced exposure to predators and competitors, remain in a protected stable niche with plentiful supply of nutrients and relative environmental stability inside the host cell. Attacking obligate bacterial P-endosymbionts may present a way to control their insect hosts, many of which are pests or carriers of human diseases.

Less is known about S-endosymbionts. The pea aphid (*Acyrthosiphon pisum*) is known to contain at least three secondary endosymbionts, *Hamiltonella defensa*, *Regiella insecticola*, and *Serratia symbiotica*. *H. defensa* seems to have a role in defending the insect from parasitoids. *Sodalis glossinidius* is a secondary endosymbiont of tsetse flies that lives inter- and intracellularly in various host tissues, including the midgut and hemolymph. As expected, phylogenetic studies have not indicated a co-evolution between *S. glossinidius* and the tsetse fly. Unlike tsetse P-endosymbiont *Wigglesworthia* sp., though, *S. glossinidius* has been cultured in vitro.

Endosymbionts are important sources of evolutionary novelty for their eukaryotic hosts. In general, their close association is extended so far as the development of complementary metabolic pathways with their eukaryotic hosts. The most amazing action showed by some endosymbionts might be their ability to act as reproductive parasites, developing strategies to convert non-transmitting male hosts into transmitting females through feminization of genetic males and parthenogenesis induction. Recent investigations have also highlighted that endosymbionts can impact upon host sex-determination more subtly through genetic conflicts, resulting in selection of host nuclear genes resisting endosymbiont effects. Paradoxically, it is because of their selfish nature that reproductive parasites are such powerful agents of evolutionary change in their host sex-determination mechanisms. They might therefore represent excellent models for studying transitions between sex-determining systems and, more generally, the evolution of sex-determination mechanisms in eukaryotes.

The most frequent endosymbiont of arthropods is *Wolbachia*. These Gram-negative bacteria maintain intracellular inherited infections in members of several insect orders and in many other invertebrates, including spiders, mites and even nematodes. At present, the limits of the host range of *Wolbachia* are not fully appreciated, and the success of these bacteria can be attributed to the diverse phenotypes that result from infection. These range from classical mutualism to reproductive parasitism as characterized by the ability of *Wolbachia* to overrule

chromosomal sex determination. *Wolbachia* is able to induce parthenogenesis, selective male-killing, influence sperm competition and generate cytoplasmic incompatibility in early embryos. The unique biology of *Wolbachia* has attracted a growing number of researchers interested in questions ranging from the evolutionary implications of infection to the use of this agent for pest and disease control.

Genome sequencing of obligate bacterial endosymbionts reveals that they present the smallest known independent genomes (0.5-1.2 Mb) with the lack of many genes that are commonly found in closely related bacteria. Presumably some of these genes were not necessary in the environment of the host insect cell. A complementary theory suggests that the relatively small numbers of bacteria inside each insect decrease the efficiency of natural selection in 'purging' deleterious mutations and small mutations from the population, resulting in a loss of genes over many millions of years. Research in which a parallel phylogeny of bacteria and insects was inferred supports the belief that the primary endosymbionts are transferred only vertically (i.e. from the mother), and not horizontally (i.e. by escaping the host and entering a new host).

Several tick species (both Argasidae and Ixodidae) also harbor non human-pathogen bacteria belonging to α-Proteobacteria and γ-Proteobacteria, that might be mutualistic endosymbionts. These microorganisms, usually localized in the Malpighian tubules and/or ovaries, have been identified as Rickettsiales (α-Proteobacteria) or *Francisella/Coxiella*-like endosymbionts (γ-Proteobacteria). Recently, another symbiont of *Ixodes ricinus*, belonging to the γ group, has been identified and isolated in a population from Slovakian forests: *Diplorickettsia massiliensis.*

1.5 An extraordinary endosymbiont of ticks: *Candidatus* Midichloria mitochondrii

Candidatus Midichloria mitochondrii is an intracellular bacterium representing an early divergent lineage within the order Rickettsiales (α-Proteobacteria). These new-type of bacteria were first detected in ovarian cells of the tick *I. ricinus*, and provisionally named IricES1 (*Ixodes ricinus* endosymbiont one). *Candidatus* Midichloria mitochondrii is described as a Gram-negative non spore forming bacterium, with a rippled outer layer, a cell wall, and an inner cell layer. It presents a bacillar shape and an average size of 0.5 μm in diameter and 1.2 μm in length (Lewis,1979, Zhu et al., 1992, Sacchi et al., 2004). *Candidatus* Midichloria mitochondrii is able to enter into mitochondria and replicate in the intra-mitochondrial space, leading to a reduction of the mitochondrial matrix until the organelle achieve the shape of an empty sac full of bacteria. When mitochondria harbour *Candidatus* Midichloria mitochondrii, they may appear degenerated, enlarged and swollen with atypical structural features. The description of bacteria able to invade organelles like mitochondria, originating from bacteria approximately 1.5 billion years ago, has aroused interesting evolutionary questions on the nature of this relationship.

The genome of *Candidatus* Midichloria mitochondrii consists of a single 1,183,732 bp circular chromosome with a G+C content of 36.6%, genome structure and content is similar to that encountered in other members of the Rickettsiales. More precisely, *Candidatus* Midichloria

mitochondrii possesses a relative scarcity of genes encoding amino acid and nucleotide biosynthesis pathways, compared with free-living α-proteobacterial relatives (Sassera et al., 2011). Although genome shrinkage in *Candidatus* Midichloria mitochondrii has led to diminished biosynthetic capabilities, like in many other intracellular bacteria, it maintained genes for the production of several cofactors, including coenzyme A, biotin, lipoic acid, tetrahydrofolate, panthotenate, heme group, and ubiquinone, that might be supplied to host cells.

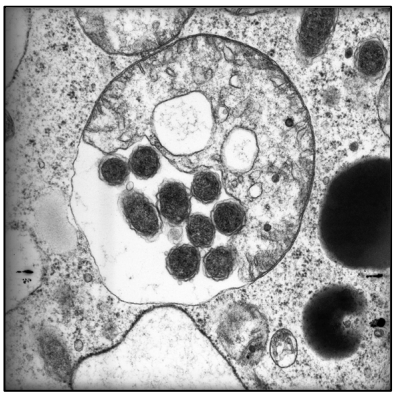


Fig 1.3 – Mitochondrion of a cell of *Ixodes ricinus* colonized by nine *Midichloria mitochondrii* bacteria. The microorganisms are located between the two mithocondrial membranes and apparently are reducing the mithocondrial matrix

Candidatus M. mitochondrii have a functional Krebs cycle, gluconeogenesis pathway, pyruvate dehydrogenase complex and almost all enzymes required for glycolysis. *Candidatus* Midichloria mitochondrii is able to synthesize ATP, and the presence of a gene coding for an ATP/ADP translocase indicates that it may also be able to import/export ATP from/to the host (Sassera et al., 2011). In the following chapters, *Candidatus* Midichloria mitochondrii will be addressed simply as *Midichloria mitochondri.*

Chapter 2

Midichloria mitochondrii in ticks and other Metazoa

2.1 Mito-bacteria in the mithocondria of *Ixodes ricinus*

M. mitochondrii has been primary observed and characterized within various cell types (luminal cells, funicular cells and oocytes) of the ovary of the hard tick *Ixodes ricinus* (Ixodidae). In the cells of the reproductive tissues of females, *M. mitochondrii* was described free in the cytoplasm or included in host-derived structures. In addition, in luminal cells and oocytes, the bacterium has also been observed within the mitochondria, in the periplasmic space between the two membranes of these organelles (Sacchi et al., 2004). At present, *M. mitochondrii* can be considered the first mito-bacteria described. Comparing different TEM images is possible to infer that the bacteria reduce the inner part of the mitochondria and multiply therein consuming intra mitochondrial matrix. *M. mitochondrii* appears to be ubiquitous in females of *I. ricinus* across this species geographical distribution, while lower prevalence is observed in males (44%), where probably the amount of bacteria decline during development to reach undetectable levels (Lo et al., 2006). Different numbers of bacteria have been observed within the mitochondria, from a single bacterium to over 20. Despite the high proportion of mitochondria consumed by the bacterium, indeed not all mitochondria are parasitized; the eggs of the tick develop normally. It is still unclear the role that these bacteria might play in the biology of the tick, but probably *M. mitochondrii* could be regarded as a facultative mutualist. Indeed, *M. mitochondrii* seems to share characteristics of a P-endosymbiont, vertically transmitted to offspring, with beneficial effects on the fitness of its host. However, laboratory data indicate that the number of bacteria in controlled reared tick colonies decrease without visible effects on the biology of the hosts, thus suggesting a facultative role. *M. mitochondrii* could be more important in natural condition where ticks might not be able to make the blood meal for long time and become facultative or even get lost in controlled laboratory conditions where the ticks are nourished regularly. The bacteria might synthesize important metabolites for its host necessary in stressful situation or under particular environmental condition or they simply complement the metabolic capacity of hosts living on a nutrient deficient diet as showed in other insect endosymbionts (Moya et al., 2009). Besides being vertically transmitted to the eggs and larval progeny of the females of *I. ricinus,* this micro-organism is maintained through trans-stadial transmission during the molting to the next instar. In addition, the amount of *M. mitochondrii* blooms after the blood meal of any trans-stadial stage except in the last moult in adult males, indicating a possible role for this bacteria in some host molting processes (Sassera et al., 2008).

The available evidence suggest that the symbiont does not cause sex ratio distortion (for example via male-killing, parthenogenesis, or feminization of male embryos), since it appears transferred equally to both male and female larvae, while some kind of specialization toward females could be established during the nymphal stage. The lower prevalence in adult males could be interpreted as a specialization of *M. mitochondrii* for females and vertical transmission while males represent a dead-end for bacteria that can not be further transmitted. In *I. ricinus*, adult males do not need blood meal to complete sexual development and mate while female are obliged to have a blood meal for completing oogenesis and in this moment we have registered the higher prevalence of *M. midichloria*. The bacteria might supply its host with important metabolizes required in this particular step in the reproductive biology of adult females.

At the moment there is no definitive evidence of horizontal transmission and infectivity of these bacteria for vertebrate hosts during the blood meal of the ticks, even if strongly suspected (see below). So far, the intra-mithocondrial localization of *M. mitochondrii* has been confirmed only in another tick species: *Rhipicephalus bursa* (Epis et al., 2008). It has also been detected in several species belonging to different genera both in Prostriata and Metastriata groups in the hard tick family Ixodidae (Epis et al., 2008), but currently there are no further data to support this very peculiar intramitochondrial localization.

However, the presence of relatives of *M. mitochondrii* in other ticks distantly related to *I. ricinus*, like the uncharacterized *Midichloria sp*. in *Haemaphisalis wellingoni*, may suggest a putative way of horizontal transmission for these bacteria. *M. mitochondrii* is a symbiont present in many tick species and its wide diffusion strongly suggests that this microorganism might also play an important role in the biology of other tick species besides female of *I. ricinus*.

Phylogenetic analyses on *M. mitochondrii* do not indicate a pattern of co-evolution with its main host species, as encountered in the great majority of endosymbionts. In detail, phylogenies of tick hosts and of their uncharacterized *Midichloria* sp. (detected only in some tick species) do not overlap as frequently happen in arthropod and endosymbionts that present pattern of co-evolution (Lo et al., 2003).

In most cases there are only PCR data to confirm the presence of these bacteria in other tick species but ultra structural details are also available at least for two species.

In *Ixodes holocyclus*, a common Australian east-coast tick, *M. mitochondrii* seems not to be present inside the mitochondria of the reproductive tissue of females and also present the same prevalence (100%) in both adult males and females (Beninati et al., 2009). Besides, in *I. holocyclus* were detected two different strains of *Midichloria* presenting 2.5% nucleotide differences at the level of 16S rDNA.

Considering the case of *I. holocyclus* seems that *M. mitochondrii* might have the capacity to invade mitochondria only in few species of ticks (certanly *I. ricinus* and *R. bursa*) but could remain in the cytoplasm in some other species like bacteria of the genus *Rickettsia*. At the moment, there is no evidence to confirm that these bacteria survive inside mitochondria of some

other animal cells, as it is difficult to understand the harmful role on the cells that harbor potentially dangerous microorganisms in mitochondria.

In conclusion, *M. mitochondrii* is certainly an important "component" of the tick microbial community, possibly interacting with other pathogenic and non-pathogenic bacteria and protozoa present in the host and transmitted to vertebrates and might also interfere in different way in the transmission of these microorganisms.

2.2 *Midichloria*-like organisms inside eykaryotic cells

The presence of various 16S rDNA gene sequences with high similarity to that of *M. mitochondrii* in several ticks species and other Metazoa, including in environmental microbial mats suggests the existence of a family of *Midichloria*-like organisms (MLOs). The detection of DNA of MLOs in other hematophagous arthropods could suggest a possibility of transmission and "circulation" of these bacteria between vectors and vertebrate hosts. In the literature, there are few cases of description of microorganisms inside mitochondria of eukaryotic cells, but in the majority of these cases the molecular data are lacking.

At this point, I would like to specify that *M. mitochondrii* sensu stricto is the type species, the one described in *I. ricinus*. The information so far acquired indicates a certain degree of molecular variation of these bacteria in hard ticks (i.e., *M. mitochondrii* from different tick species can show up to about 3% nucleotide differences at the level of the 16S rDNA gene). However, within the same tick species, there is generally limited or no variation in *M. mitochondrii* 16S rDNA genes, with the exception of *I. holocyclus* (see above). Throughout this chapter and in the following ones, I have chosen the option of referring to all of the tick bacteria that cluster within the same group of *M. mitochondrii* sensu stricto with less than 3% nucleotide substitutions at the 16S rDNA level simply as *M. mitochondrii*; the acronym MLO is used to refer to those bacteria that are phylogenetically related with *M. mitochondrii* and show a nucleotide difference on 16S rDNA up to 14% (Fig. 2.1).

As previously discussed, the phylogeny of *M. mitochondrii* does not match with that of the host ticks. This suggests that this bacterium can be transmitted horizontally even between ticks belonging to different species. For example, the distantly related tick species *Riphicephalus turanicus* and *Hyalomma truncatum* harbor *M. mitochondrii* with identical 16S rDNA sequences (Epis et al., 2008). How could *M. mitochondrii* move horizontally between different ticks? A possible hypothesis is that this horizontal transmission might occur through a passage in a vertebrate host.

Is there any evidence for the capacity of *M. mitochondrii* to infect vertebrate hosts? Bacteria showing high sequence similarity with *M. mitochondrii* have been detected by PCR in the blood of roe deer, in the context of a screening for pathogens vectored by ticks (Skarphédinsson et al., 2005). Of course, this bacterial presence revealed by PCR does not provide any evidence

for the capacity of *M. mitochondrii* to infect vertebrates. It is possible that PCR revealed bacteria, or even just bacterial DNA, as simply inoculated by ticks.

There is, however, circumstantial evidence that MLOs could cause pathological alterations in humans. In 2004 a novel microorganism, called unofficially 'Montezuma', was detected by PCR in ticks collected in Far East Russia (*Ixodes persulcatus* and *Haemaphysalis concinnae*) and in samples from human patients presenting acute febrile symptoms (Mediannikov et al. 2004). Based on the 16S rDNA sequences deposited in the databases, Montezuma is a relative of *M. mitochondrii* (10% nucleotide divergence; Fig. 2.1). Thus, based on available information, Montezuma can be regarded as a bacterium belonging to the same clade of *M. mitochondrii*, even though probably too distant to be assigned to the same species.

Beside the possible role of the MLO Montezuma in a human disease, the strawberry disease (SD) of the rainbow trout *Oncorhynchus mykiss* has recently been proposed as being caused by a MLO (Lloyd et al. 2008). SD is a fish skin disorder of unknown etiology, characterized by bright red inflammatory lesions. Analysis using conserved bacterial 16S rDNA primers consistently revealed the presence of a MLO in the skin lesions of fish specimens affected by SD. These bacteria have not been isolated and there is no clear evidence that this microorganism is actually the etiologic agent of SD. These bacteria display 5% nucleotide divergence to the 16S rDNA of *M. mitochondrii* and are thus the most closely related lineage to the cluster of *M. mitochondrii* complex isolated from ticks (Fig. 2.1). The studies performed on SD have provided significant evidence that MLOs can infect vertebrates.

M. mitochondrii and MLOs have been detected by PCR analysis also in other ectoparasitic/haematophagous arthropods: in two tabanid flies *Tabanus bovinus* and *T. tergestinus* (Hornok et al., 2008), in the bed bug *Cimex lectularius* (Richard et al., 2009), in mites of the species *Spelaeorhynchus praecursor* (Acari: Dermanyssoidea) infecting bats (Reeves et al., 2006) and in *Xenopsylla cheopis* (Siphonaptera: Pulicidae), a flea that infests rats (Erickson et al., 2009).

Literature and database searches revealed that there are other 16S rDNA sequences, from different sources, that could be referred to *M. mitochondrii* or to MLOs (Fig. 2.1). Among these MLOs, the best characterized are those harboured by amoebae of the genus *Acanthamoeba* (Fritsche et al., 1999). In addition, sequences from MLOs have been retrieved from a ciliate protozoan, other amoebae species, cnidarians, marine spongiae and environmental microbial mats. In the case of the fresh water cnidarian *Hydra oligactis*, in addition to molecular sequence information (87% identity in 16S rDNA with *M. mitochondrii*), the candidate MLO was observed in electron microscopy. It appears as a rod-shaped bacterium located in the cytoplasm of ectodermal epithelial cells of the host (Fraune and Bosch, 2007).

In summary, there is evidence that *M. mitochondrii* and MLOs are quite widespread symbionts, as evidenced by the variety of hosts in which their DNA was detected. Is the capacity of invading the mitochondria a peculiarity of *M. mitochondrii* of ticks, or is the intramitochondrial

location a common feature of MLOs? It must be emphasized that bacteria with an intramitochondrial location have been observed in two different species of ciliates: *Halteria geleiana* (Yamataka and Hayashi 1970) and *Urotricha ovata* (de Puytorac and Grain 1972). In addition, bacteria strictly associated with mitochondria have been observed in other ciliate species: *Spirostomum* sp. (Fokin et al. 2005) and *Cyclidium* sp. (Beams and Kessel 1973). However, these bacteria have not yet been molecularly characterized. It is thus not known whether these bacteria are phylogenetically related to *M. mitochondrii*. Considering that the 16S rDNA sequence of an MLO (86% identity) has been obtained from the ciliate *Diophrys* sp. (Ciliophora: Hypotrichia) we believe that the role of amoeba/ciliate-like organisms as suitable hosts for MLOs must not be overlooked. Since several MLO 16S rDNA sequences have been obtained both from marine and fresh water metazonans (Fig. 2.1), we could hypothesize that aquatic protozoa might have played an important role in ancient MLOs evolutive success, constituting a sort of reservoir from which MLOs could have reached its current hosts.

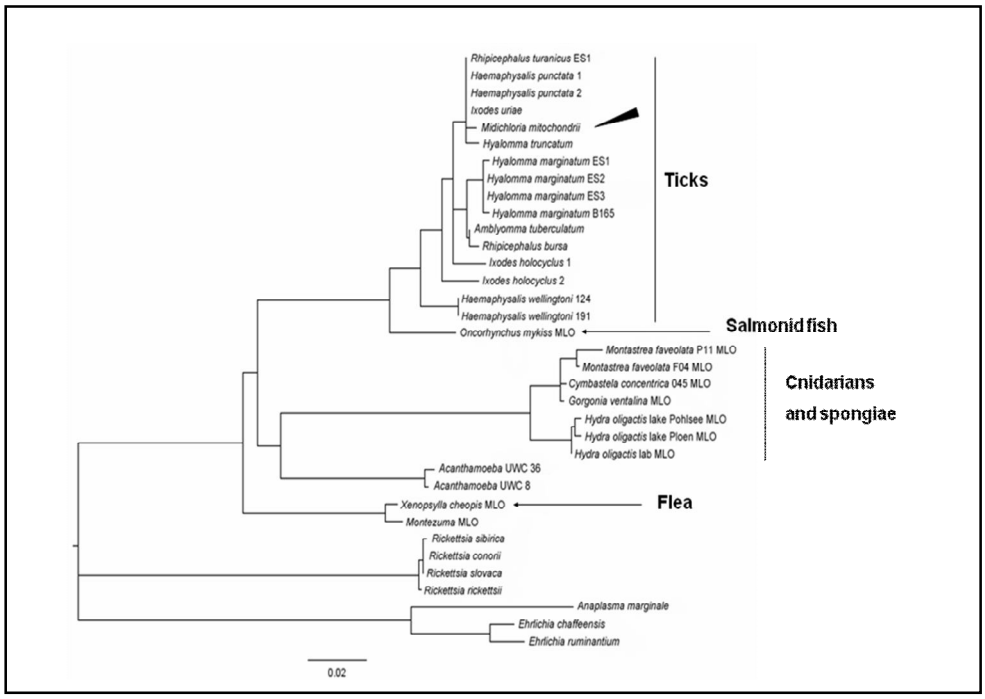


Fig 2.1 – Neighbor-joining tree (Kimura correction) based on 16S rDNA sequences, showing phylogenetic relationship between *Midichloria mitochondrii* symbionts of ticks and *Midichloria*-like organisms (MLOs). Arrowhead indicates *Midichloria mitochondrii* of *Ixodes ricinus*. The names at the terminal nodes of the tree are those of the eucaryotic host species, with the exceptions of bacterial species of the genera *Anaplasma*, *Ehrlichia*, *Rickettsia* and 'Montezuma' that is the unofficial name of a MLO sequence retrieved from ticks and humans. Bacteria of the genera *Anaplasma*, *Ehrlichia* and *Rickettsia* were used as outgroups. The bar indicates the number of substitutions per site.

2.3 Origin of symbiosis: an ancient scenario of endless struggle and cooperation

The concept of symbiosis can be expressed as a form of relationship between individuals of different species living in close association (De Bary, 1879), the nature of this inter-connection range from commensalism to parasitism. In particular, considering intracellular symbiosis between eukaryotic cells and their endocytobionts, the network of connection can reach a very complicated pattern, until the reciprocal exchange in metabolic products between the two actors can become fundamental for the survival of both organisms thus leading to the evolution of interconnected metabolical pathways. In some cases the symbiosis could become obligate and the host may depend upon the symbionts and vice versa. For instance, if the *Wolbachia* symbionts of filarial parasites are removed by antibiotic treatment the host cannot complete sexual development and reproduce. Besides, many cases of obligate and facultative symbiosis are known, like the situation encountered in hematophagous insects and acari that strongly interact with their P-endosymbionts and with a series of other pathogenic bacteria vectored to vertebrates.

These symbioses were established millions of years ago, but the most ancient symbiosis dates back to the origin of the eukariotc cells, when the proto-eukariotic cell started interacting with the alpha-proteobacterium than would later be called mitochondrion.

In a primordial environment, where many different species of prokaryotic free living organisms were surrounded by the first proto-eukaryotic cells, the competition for utilizing the best niche available must have been very strong and the internal cytoplasm of other cells should be recognized as a suitable place protected from environmental physical and chemical stress and rich of nutrients. A central evolutionary pillar of early-life biology states that the eukaryotic organelles involved in the processes of photosyntesis and respiration (chloroplasts and mitochondria) are the degenerate descendants of prokaryotic endosymbionts, anciently established within the eukaryotic cytoplasm. Intracellular lifestyle might have allowed some bacteria to gain a competitive advantage compared to other free living microorganisms. Indeed, this life style could either enable evasion of killing mechanisms that are carried out by predatory cells in the environment, such as amoebae, and gain a first-class niche, once solved the problem to evade host immune defences. If a microorganism is able to defeat the host's surface defenses, it must then overcome the host's phagocytic response to succeed in an infection. In contrast, most bacteria that are successful as pathogens interfere to some extent with the phagocytic activity. The intracellular environment of a phagocyte may be protective, sheltering the bacteria during the early stages of infection, in fact, bacterial pathogens have devised several strategies to avoid phagocytic engulfment and killing, using compounds produced by the same infective microorganism aimed at blocking one or more of the steps in phagocytosis, thereby stopping the process. One typical way to survive inside a cell is based on the inhibition of the fusion of the phagocytic lysosomes with the phagosome, this means that the bacteria survive inside of phagosomes because they prevent the discharge of lysosomal contents into

the phagosome ‘vacuole’. This strategy is very common in species of the Genus *Chlamydia*. Another common mechanism utilized by some bacteria is based on escaping from the phagosome. Early escape from the phagosome vacuole is essential for growth and virulence of some intracellular pathogens and this is the strategy employed by the Rickettsiae. These bacteria enter host cells in membrane-bound vacuoles (phagosomes) but are free in the cytoplasm a short time later and a bacterial enzyme, phospholipase A, may be responsible for dissolution of the phagosome membrane.

In general, the intracellular environment guards the bacteria against the activities of extracellular hazardous agents and maybe just the ability to survive host cell inclusion and digestion could be at the basis of the evolutionary success experimented by Eukarya. On the other hand, we could consider the intracellular current pathogens as victims in a struggle for life, hence, after they were included in the eukariotyc cells trough phagocytosis they just tried to survive cellular degradation developing in long evolutionary time some kind of “virulence” (becoming pathogens) towards eukaryotic cells.

In the countless moments of interaction between eukariotic and prokariotic cells that occurred in the biological history of life, some bacteria might have followed a road for higher virulence killing its host, but some other might have followed a path for establishing a long-lasting collaboration. These Proto-Eukariotic cells continuously interacting with microorganisms might have constituted an evolutionary training ground for intracellular bacteria.

At present, bacterial endosymbionts have been recorded in more than 200 ciliate species, in several cases different species are co-living togheter in the same unicellular eukaryotic organism. Ciliates can be considered as an optimal model for studying interaction between prokaryotes and eukaryotic unicellular organisms. Some protozoa like *Spirostomum minus* and *Spirostomum ambigum* (Ciliophora, Protista) can be described as a bacterial microcosm, they show a contemporary co-presence of different species of endocytobionts (up to 7) with reported geographical variation in bacterial fauna in the same species, and diverse cellular location shifting form free in the cytoplasm, contained inside vacuoles, inside cellular organelles and mitochondria till even inside cell nuclei.

In such an actively evolving scenario, the most fascinating question still waiting for an answer is whether or not ancestors of *M. mitochondri* were already able to penetrate inside the still free living ancestor of the future eukaryotic mitochondria or *M. mitochondrii* developed this capacity after mitochondria already established inside eukaryotic cells and started following the process to became the semi-autonomous power houses in the Eukarya. In this second case the picture would be a bit more complicated because *M. mitochondrii* must be able to penetrate the cellular membrane of the cell, survive in the cytosol to lysosome action and penetrate the mitochondria membranes without activation of apoptosis pathway (see below), and possibly it should have maintained this capacity in our days since the mitochondria invading event must be expected as quite recent. Are MLOs still able to invade mitochondria or these bacteria lost this capacity and

just can survive inside mitochondria without abandoning them or at least without abandoning the eukaryotic cell? In fact, they could ended up in the cytoplasm after they conduct to mitochondrial destruction.

2.4 Living inside an eukaryotic cell

In the order Rickettsiales, to which *M. mitochondrii* belongs, all known species share the common feature of multiplying only inside the eukaryotic cell. Different host types have been described for these microorganisms, ranging from vertebrates to nematodes. Several species in the two main families (Rickettsiaceae and Anaplasmataceae) of the order Rickettsiales are associated with arthropods. Bacteria of the genera *Rickettsia* and *Orientia* (Rickettsiaceae), often associated with ticks, are normally encountered free in the cytoplasm of the host cell: they are not surrounded by a host-derived membrane, and their cell wall is directly immersed into the cytosol (Ray et al., 2009). There are also species of *Rickettsia* that have been observed inside the nucleus, again without a surrounding membrane (Ogata et al., 2006). The lack of a host derived membrane in symbionts is atypical, in that intracellular microorganisms are normally surrounded by this kind of membrane; i.e. they are normally located in a vacuole, which is frequently the result of a process of phagocytosis. This is indeed the case for members of the Anaplasmataceae (genera: *Anaplasma*, *Ehrlichia*, *Wolbachia, Neorickettsia*). In the case of *M. mitochondrii*, three different types of locations have been reported: free in the cytosol; in the cytosol surrounded by a membrane and in the intermembrane space of mitochondria (Fig. 2.2).

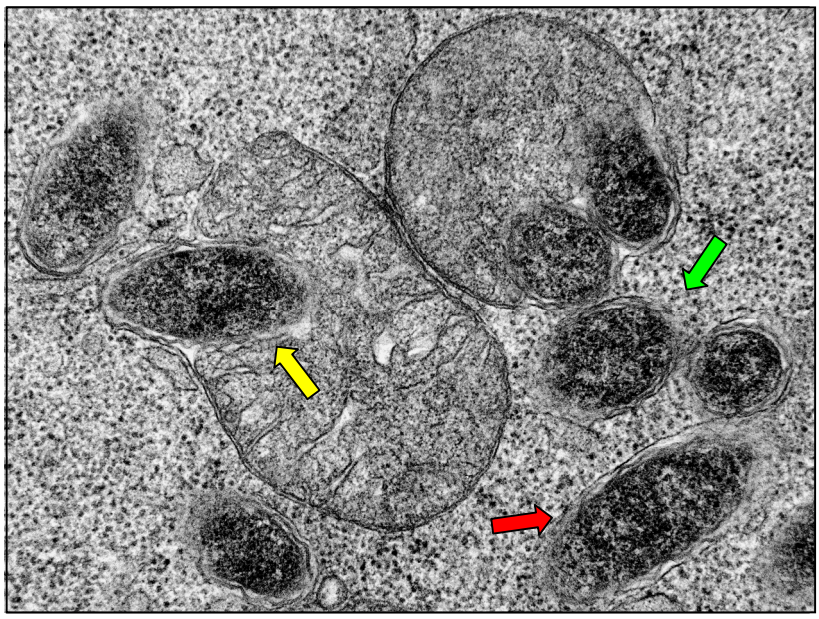


Fig 2.2 – *Midichloria mitochondrii* inside the oocyte of *Ixodes ricinus*. The bacteria are located inside mitochondria (yellow arrow), in the cytosol surrounded by a probably host-derived structure (red arrow) or free in the cytosol (green arrow).

The second location should not necessarily be interpreted as the bacterium being in a vacuole; rather, TEM pictures suggest that the bacteria that are observed within a surrounding membrane are possibly located within a bulge of the mitochondrial membrane (although this interpretation would require further support). In summary, if we exclude the intra-mitochondrial location, *M. mitochondrii* seems to behave in a way similar to the Rickettsiaceae, in agreement with its phylogenetic position.

Intracellular bacteria inside host-derived vacuoles can take advantage of this location, gaining protection in terms of environmental stability, but at the same time they are confined and concentrated in a dangerous area, where they could be under the deleterious action of host lysosomes. Indeed, to avoid this defensive system of the host cell, intracellular bacteria of the genus *Chlamydia*, not phylogenetically related to Rickettsiales, prevent the fusion of the phagosome with the lysosome and thus the formation of a phagolysosome (Eissenberg and Wyrick, 1981). *Rickettsia* has clearly adopted a different strategy, consisting in the early escape from the vacuole soon after cell invasion (Ray et al., 2009).

The strategy adopted by *M. mitochondrii*, based on the invasion and multiplication inside mitochondria, could be interpreted as a third way to escape from the attack of the host cell. While the programmed, partial death of mitochondria (mitoptosis) is known to occur in particular phases of metazoan development (Meier et al., 2000), these organelles are obviously fundamental for the metabolic activity and long-term survival of the cell. We might thus expect that they represent a protected environment, where a bacterium could find a specific niche. Why then are not intramitochondrial bacteria widespread? Mitochondria play an important role in triggering the apoptosis of animal cells, both indirectly (decrease of mitochondrial activity results in an energetic crisis and acidification of the cell), but also in a direct way (mitochondrial pathway of apoptosis). Alteration of the mitochondrial membranes leads to the release of cytochrome C, triggering the apoptotic pathway (Garrido et al., 2006). Integrity of the mitochondrial membranes is thus crucial to avoid the death of the host cell. It is possible that intra-mitochondrial parasitism/symbiosis is rare not because of the unsuitability of the organelles for the symbionts, but for the delicate equilibrium between cell-life and apoptosis, where mitochondria play a central role. We might hypothesize either that *M. mitochondrii* is capable of multiplying within mitochondria without triggering apoptosis, or that the apoptotic pathway in ticks is triggered differently compared to other metazoans. We could go as far as to hypothesize that the mitochondrial pathway of apoptosis was selected as a system to eliminate cells parasitized by intra-mitochondrial pathogens.

2.5 *Is Midichloria mitochondrii* a very efficient energy parasite?

The mitochondrial intermembrane space might represent for *M. mitochondrii* not only a protected shelter but also an advantageous niche very rich in ATP, pumped out of the mitochondrial matrix through the action of mitochondrial ADP/ATP translocase. Could *M.*

mitochondrii take advantage of the ATP molecules that flow from the mitochondrial matrix towards the cytoplasm?

Genes coding for ATP membrane exchange proteins have been found in genomes of Rickettsiae and in MLOs of Achantoamoebae (Schmitz-Esser et al., 2004). *M. mitochondrii* bacteria possess genes for an ATP/ADP translocase, which show homology with the transporters that in other bacteria have been shown to exchange bacterial ADP with host-cell produced ATP, thus parasitizing the energy production of the host cell, representing a perfect example of specialization both from the point of view of function and of intracellular location.

Mitochondrial ADP/ATP carrier and the proteins involved in ATP acquisition in bacteria of the order Rickettsiales (including some MLO) and Chlamydiales (Klingenberg, 2008) have different origin and are not considered homologous. Bacterial translocases act as importers of ATP while the mitochondrial version works in the opposite direction, exporting ATP towards the cytoplasm. An additional difference between these two classes of transporters is their origin (Amiri et al., 2003). It is generally assumed that the current mitochondrial ADP/ATP carrier was not present in the free-living ancestor of mitochondria, and it has been proposed that this protein derived from the eukaryotic cell (Embley, 2006), representing an important evolutionary step in the process of nuclear control over mitochondria.

In the end mitochondria are bacteria that followed a path in evolution that brought them to loose completely their independence to serve their eukaryotic host (examples are encountered in other symbionts, i.e. genome shrinkage in *Carsonella ruddi* - Tamames et al., 2007). During evolution mitochondria lost almost all stored genetic information and only a small number of original genes are nowadays found in mitochondrial genomes.

Despite the huge diversity of bacterial types and their capacity to utilize different energy sources, the majority of them contain an ATP synthase in the plasma membrane similar to the one present in mitochondria. In bacteria that use an electron transport chain to produce energy, H^+ ions are pumped out of the cell membrane to establish a proton-motive force across the aformentioned membrane to promote ATP production trough ATP synthase. In different conditions ATP synthase can work in reverse, hydrolizing ATP obtained from glycolysis or other fermentation pathways to pump H^+ ions and generate a proton gradient across the plasma membrane. *M. mitochondrii* could likely use ATP produced by the mitochondrion, also to generate a proton motive force using ATP synthase. In the *M. mitochondrii* genome are present genes for glycolysis (not complete) and Kreb cycle (complete) and this microorganism is thus capable of generating its own ATP; the use of an ADP/ATP translocase to get ATP from its host might anyway be important in some parts of its life cycle. This location is not so astonishing considering that the outer membrane of a mitochondrion is highly permeable to many ions and cellular product of small dimension and does not represent an environment very different from the cytoplasmatic space (but the intermembrane space of the mitochondrion is likely an environment with high concentration of oxygen radicals, considering that the mitochondrion is a

main site of generation of these radicals in the cell). On the contrary, the major working part of the organelle, the matrix and inner membrane are highly specialized. The presence of phospholipid cardiolipin in its lipis bilayer with its four hydrofobic chains fatty acids instead of two assure that the membrane is impermeable to ions. Anyway, we might guess that the concentration of hydrogen ions must be higher compared to the rest of the cellular cytoplasm due to the activity of the electron transport chain that generates the proton motive force. The advantageous position of *M. mitochondrii*, in an environment rich of hydrogenous ions, could allow the bacteria to exploit the proton motive force created by the mitochondria to generate its own ATP with a low cost.

In several species of *Rickettsia* belonging both to spotted fever group (*R. rickettsii*, *R. conorii*, *R. montanensis*, *R. sibirica* and *R. felis*) and typhus group (*R. prowazewkii* and *R. typhi*) a series of homologues proteins with putative ADP/ATP translocase activities has been identified. This membrane pump protein has been shown to work moving ATP in the intracellular cytoplasm expelling ADP.

In particular, *Rickettsia prowazekii* the etiological agent of epidemic typhus in humans, is an intracellular bacterium that grows inside eukaryotic cells, free in the cytoplasm, not enclosed in host-derived vacuoles. This species represent an ideal model to make important considerations on intracellular living. *R. prowazekii*, in such a nutrient-rich niche, has evolved several unique transport system specific for large and charged molecules including ATP trough ADP/ATP translocase as a means to acquire high energy compounds to cope with its own energetical needs and all these transport abilities might also have contributed to the shrinkage of the genome. All the previous consideration might be valid also in the case of *M. mitochondrii*. In this microorganism is present a protein homologous to the rickettsial transport protein, actively working to exchange internal bacterial ADP with host cell produced ATP as a source of energy.

2.6 Mitochondrial pathogens

Mitochondria are prokaryotic-like partially autonomous intracellular organelles that produce energy within eukaryotic cells. Mitochondria are extremely dynamic organelles that migrate, divide and fuse inside eukaryotic cells. Continuous cycles of mitochondrial division and fusion guarantee mixing of metabolites and mitochondrial DNA (mtDNA) (Knott, 2008). This process might also guarantee the repair of mithocondria damaged by physical or chemical stresses.

All these dynamic processes modify shape, number and bioenergetic functionality of mitochondria and also allow the cell to face changing of energy demand and environmental condition. There is mounting evidence that mitochondrial dysfunction has deleterious consequences on the functionality of cells, tissues and organs. The origin of mitochondria from symbiotic bacteria raises the possibility that mitochondria themselves, like free-living bacteria, have their own pathogens, like predatory/parasitic bacteria and phages (Bongaerts and van den Heuvel, 2008). It is thus worth considering whether pathogens of mitochondria do exist, and

whether these pathogens might cause any disease in eukaryotes.The discovery of *M. mitochondrii* clearly demonstrates that microorganisms capable of living inside mitochondria do exist. Are there other examples? Is it possible that MLO in organisms other then ticks cause pathologies? Or, is there any MLO-tick interaction that could represent a form of parasitism/symbiosis? There is limited knowledge of pathogenic microorganisms for mitochondria. Since mitochondria still resemble in several aspects bacteria it is not difficult to infer the presence of mitochondrial pathogens capable of attaching and damaging mitochondria with mechanisms analogous to those involved in bacterial predation (Bongaerts microbial mito pathogens 2007). *Bdellovibrio bacteriovorus* is a small flagellate bacterium with the ability to invade and consume Gram-negative bacteria, entering between the inner and outer bacterial layers, in the periplasmic space. This behaviour has already been associated with the way in which *M. mitochondrii* attacks mitochondria and could be similar to the mechanism utilized by intramitochondrial bacteria in ciliates and other animal groups. Mitochondria are organelles with a double membrane system, also evolutively derived from Gram-negative *Midichloria*-like bacteria, hence this similarity is not to be overlooked. The previously mentioned review article by Bongaerts and van den Heuvel (2008) proposed the word 'mitopathogens' to indicate infectious agents capable of determining alterations of mitochondria. In addition to *M. mitochondrii*, we can mention the mitochondrial viruses of the genus *Mitovirus* (Family Narnaviridae), for example the one infecting the mould *Ophiostoma novo-ulmi* (a pathogen of elm trees). Both a bacterium (*M. mitochondrii*) and a group of viruses (*Mitovirus*) have thus been shown to invade mitochondria. Whether *M. mitochondrii* is to be regarded as a pathogen, a commensal, or a beneficial symbiont is still unclear, and certainly this bacterium is not pathogenic for *I. ricinus*. However, from the point of view of the single mitochondrion that is consumed by *M. mitochondrii*, this bacterium is certainly a mitopathogen. Mitochondria, in mammalian cells, play an important role in programmed cell death, not only because decrease in these organelle activities result in an energetic crisis and acidification of cellular internal medium, but also considering that damages in mitochondrial membranes altering organelle permeability could activate the kinase cascade and apoptotic mitochondrial pathways trough citocrome C releasing. Mitochondrial specific pathogens could start processes leading to cell death.

Chapter 3

Genetic variability of *Ixodes ricinus* and diffusion of *Borrelia burgdorferi* sensu latu in Europe and North Africa

3.1 Introduction

3.1.1 Genetic variability of *Ixodes ricinus*

The tick *Ixodes ricinus* is recognized as the primary European vector of disease-causing bacteria in humans (Carpi et al., 2011). This study on genetic structure of population of *Ixodes ricinus* aimed to determine the genetic variability and intraspecific relationship between different European and North African populations of this species. Previous investigations on the population genetics of *I. ricinus* were focused only on few European populations (McClain et al., 2001 and Paulauskas et al., 2006). The first study on the genetic variability of this tick was carried out on five populations of western Switzerland, and it was based on 18 allozime loci that showed low variability and the lack of a significant difference between the localities (Delaye et al., 1997). Three years later, another work, analyzing the sequence of the D3 region of 28s rDNA, also confirmed the absence of substantial genetic differentiation within the same population and between populations (McClain et al., 2001). In both works, as outlined by the authors, a strong limit is represented by the choice of unsuitable markers (allozimes and sequence of D3 region) presenting low variability. Later, using RAPDs, another investigation showed high level of genetic diversity between 6 populations of Norway and Latvia (Lituania) and low level inside the same populations (Paulauskas et al., 2006).

The results had been justified by the presence of migratory birds that used to aggregate in colonies in costal area, favoring differentiation in their populations of ectoparisites.

The genetic variability of *I. ricinus* on regional scale had been investigated trough microsatellites (5 loci) on samples from 8 different areas in Switzerland. The result obtained, in accordance with the other studies, evidenced the low level of genetic differentiation even between populations separated by geographic barriers, like the Alps. Anyway a comparison between these ticks and specimens collected in North Africa permitted to hypothesize higher level of differentiation between the two continents (DeMeeûs et al., 2002).

Investigations on the intraspecific variation of *I. ricinus* on wide geographical scale brought interesting results. Populations collected in Switzerland, Italy, Austria, Finland, Denmark and Sweden were studied through the analysis of 5 mitochondrial genes (COI, COII, cytb, 12S, CR), but results obtained did not evidenced any correlation between the identified aplotypes and their geographical origin, suggesting that European populations of this species lack phyleogeografic

structure (Casati et al., 2008). Recently, the absence of geographic association has been confirmed by another study of genetic diversity at local, regional, Euroasiatic and western Paleartic level, using both nuclear and mitochondrial genes (Noureddine et al., 2010). In accordance with precedent suggestions emerge a genetic discontinuity between European and African populations.

The absence of genetic differentiation in European populations of *I. ricinus* is explained with two different hypotheses. The first hypothesis is linked to the role played by hosts. Several authors suggested that wide areal of distribution and capacity to cover long distances of some kind of hosts (birds and large mammals) might be the responsible element for the lack of variability observed at present (Delaye et al., 1997; McClain et al., 2001; Casati et al., 2008; Noureddine et al., 2010). The second possibility sustains that the low genetic variability observed, might be the consequence of a population post glacial expansion from Southern shelters towards Northern Europe (McClain et al., 2001; Casati et al., 2008; Noureddine et al., 2010). Both these two plausible hypotheses remain to be verified. Nowadays, the level of association between *I. ricinus* and the vertebrate hosts is still unclear. This parasite is considered a generalist, able to feed on a great variety of animals (Gern & Humair, 2002); but a sort of local adaptation or specialization for some species of vertebrates has been proposed (Kempf et al., 2010). Anyway, the parasite-host association in the dynamic of dispersion of the species is difficult to assess and the influence of this process on the level of genetic diversity of population is even less clear.

In the same way, the second conceivable hypothesis, the existence of shelter areas of post glacial expansion, though proved for other animal species (Hewitt, 2004), remains to be verified for *I. ricinus*. The genetic variability encountered between European and North African populations, also difficult to explain, might derive from demographical and ecological factors. The action of genetic drift and difference in seasonal activity, beside the geographical separation of the two continents, might have contributed to insurgence of such diversity (Noureddine et al., 2010). The role of migratory birds in large-scale transport of individuals and mixing of different populations remain to be clarified (DeMeêus et al., 2002). The level of genetic divergence observed in these two different populations might be due to a speciation event occurring in the two continents (Noureddine et al., 2010).

Few attempts have been made to determine intraspecific taxonomic markers in ticks. However, a study on cuticular hydrocarbons, which can be useful markers for insect taxonomy, showed the existence of at least 10 distinct *I. ricinus* groups and a geographical pattern in their distribution (Estrada-Peña et al., 1996).

Variations in susceptibility of different tick populations to tick-borne pathogens may also occur. As demonstrated in a study on the susceptibility of *I. ricinus* larvae derived from females collected in Spain, Ireland and Germany to *B. afzelii* where it was showed that Spanish ticks were more susceptible (though *B. afzelii* does not occur in Spain) than either German or Irish

populations (Estrada-Peña et al., 1998). Surprisingly, the German ticks were the least susceptible to this German *B. afzelii* strain, which was from the same region. Geographical differences in the epidemiology of tick-borne diseases are often explained in terms of pathogen characters, but this kind of study suggests that more attention should be given to variations in vector genetic variability and competence.

The present study was focused on the genetic variability of populations of *I. ricinus* in the complete European and North African geographical areal of distribution of the species, through the analysis of variation in the genetic sequences of nuclear and mithocondrial markers. Principal objectives of our study are: I) to evaluate the presence of group of populations differentiated from a genetic point of view II) to study the pattern of connectivity between populations. So far, several factors still remain unclear and complicate the comprehension of the real evolutionary history of *I. ricinus*.

3.1.2 *Borrelia burgdorferi* sensu latu

The genus *Borrelia* is one of the four genera (*Troponema*, *Leptospira, Borrelia* and *Brachyspira*), belonging to the phylum Spirochaetes, that comprehend human pathogens. *Borrelia* species are characterized by a parasitic life style involving both an arthropod vector and a vertebrate hosts. The complex of *B. burgdorferi* sensu latu contains pathogens causing Lyme disease in humans. When this bacterium was described for the first time (Burgdorfer, 1982), *B. burgdorferi* was hypothesized to be a single species. Effectively the first 12 isolates (Johnson et al., 1984) belonged to a single species lately named *Borrelia burgdorferi* sensu stricto (Baranton et al., 1992). Nowadays, *B. burdorgferi* sl complex comprises 13 genopsecies: *Borrelia burgdorferi* s.s, *B. garinii*, *B. afzelii*, *B. spielmanii*, *B. bissettii*, *B. valaisiana*, *B. lusitaniae*, *B. japonica*, *B. tanukii*, *B. turdi*, *B. sinica*, *B. andersonii*, *B. californiensis*, and other genospecies recently isolated. These genospecies present variable degree of pathogenicity, and also differ for ecological characters, areal of distribution, host preference, vector competence and clinical manifestations. *B. burgdorferi* s.l. complex is widely distributed in Northern Hemisphere. *B. garinii* and *B.afzelii* associated with *I. ricinus* and *I. persulcatus* and an ample range of reservoir hosts, respectively birds and rodents, show the widest area of expansion (Asia and Europe) (Fig.3.1). *Borrelia burgdorferi* s.s., present the higher genetic diversity in California, where it probably evolved locally in association with *I. pacificus*, later the adaptation to *I. scapularis* might have permitted to reach the east cost of USA, and successively, after adaptation to *I. ricinus*/*I. persulcatus*, moved to Taiwan (Baranton & De Martino, 2009) to colonize Asia and Europe. Other species of *Borrelia* present a more limited range. *B. lusitanie* is mainly present in Portugal, North Africa and few European states (Gern & Humair, 2002; Rauter & Hartung, 2005). *B. andersonii* is restricted to the eastern part of USA.

The tick vector acquires the spirochetes feeding on infected hosts, and maintains the infection during trans-stadial molting. In some species, like *I. ricinus*, tras-ovarian transmission might also

occur, but at low degree (Gern & Humair, 2002). The persistence of *Borrelia* infection involves the presence of competent reservoir hosts. In Europe, only few of the over 300 possible host species for *I. ricinus* had been identified as reservoir hosts for *B. burgdorferi* s.l. spirochetes. Rodents like *Apodemus flavicollis*, *A. sylvaticus*, *A. agrarius* and *Clethrionomys glareolus* seem to be the most important, playing a central role in the ecology of Lyme disease. Besides, only recently the role of birds, as reservoir hosts, for *Borrelia* had been recognized (Gern & Humair, 2002). The importance of avian hosts, must not be underestimated, since they can also transport infected ticks during migration and introduce new *Borrelia* species where they normally were not present, like it has been demonstrated for *B. lusitaniae* (Poupon et al., 2006). Even if ungulates and other mammals, probably do not act as reservoir hosts, they can contribute indirectly to amplify transmission. Heavy tick population density is related to the abundance of ungulates (e.g. roe deer and red deer), and also, different ticks feeding contemporary on the same hosts (co-feeding) can become infected with the borreliae. In several cases, a specific association between a *Borrelia* species and vertebrate hosts has been recorded; it has been demonstrated for *B. azfelii* and the genus *Apodemus*, *B. garinii* and birds (Gern & Humair, 2002) and finally between *B. lusitaniae* and lizards (Dsouli et al., 2006; Richter & Matuschka, 2006; Amore et al., 2007).

Humans can contract *Borrelia* spp. infection after a tick bite. Spirochetes might be transmitted during blood feeding, and more time the tick remains attached, higher is the risk of bacteria transmission. Hence the efficiency of transmission increases with the duration of the blood meal (Kahl et al., 1998; Crippa et al., 2002). Before feeding the spirochetes are normally located in the intestines of the ticks. Spirochetes transmission do not occur trough regurgitation of intestinal contents, but trough infected saliva injected while feeding. More recent study also demonstrated that spirochetes might be present in salivary glands before the tick start the feeding process (Leuba–Garcia et al., 1998). Anyway the retard observed in transmission has been attributed to the spirochetes migration previously described. Some species present lower latency time and hence higher rate of infection (Crippa et al., 2002). The migration phase inside the vector is accompanied by the expression of specific proteins. In the model *I. ricinus*, before the tick start feeding, *Borrelia burgdorferi* expresses *Osp*A (outer surface protein A), but its concentration decrease while feeding. In this phase *Osp*A synthesis is inhibited and a new protein, called *Osp*C (outer surface protein C), is synthesized. This factor, demonstrates the complex interaction between *Borrelia* and the vector, but other aspects involved in the pathogen transmission need to be clarified to promote action to reduce the risk of Lyme disease.

Lyme disease, or Lyme borreliosis, represents the human pathology, transmitted by ixodid vectors, more frequent at world level, even if, it is present only in the Northern Hemisphere (Hubálek, 2009). In fact, the distribution of this disease corresponds to the range of distribution of its principal vector, ticks of the *I. ricinus* complex, and for this reason the incidence is higher in areas favourable for tick abundance. The incidence rate varies with latitude and altitude that

influence the environmental parameters, like temperature and humidity, of primary importance for ticks. Lyme disease is a multi-system pathology that affects humans independently from sex and age, although some working categories might be more exposed to risk (Hubálek, 2009). The diverse symptoms rarely occur at the same time. The skin is the body part more frequently affected, with different lesions involving cutaneous tissues (Strle & Stanek, 2009). Between these lesions, the most frequent is called *erythema migrans*. This typical red spot appears in the site of tick bite within days or weeks, where the spirochetes had been inoculated, and as the time goes by, it expands to assume an oval shape. But epidermis and skin are not the only tissue damaged in *B. burgdorferi* s.l. infections, in fact, nervous and circulatory systems and junctions are often seriously injured. Lyme disease can provoke meningitis, paralysis, insomnia, tachycardia and circulatory problems, finally at junction level arthritis and atralgia are common manifestations (Strle & Stanek, 2009).

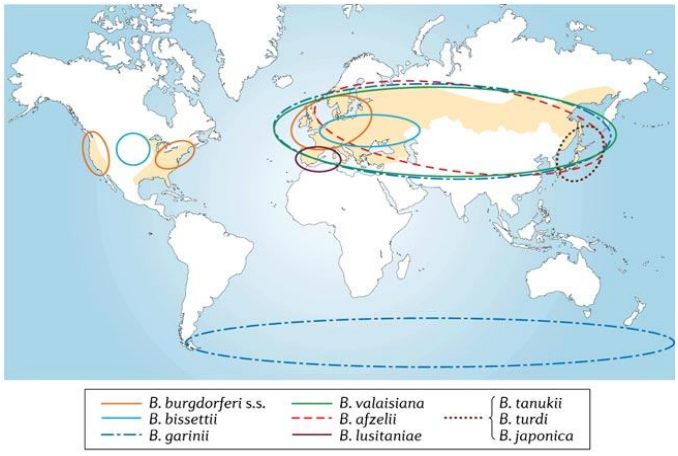


Fig 3.1 - Beige-shaded areas indicate geographical distribution of clinical cases of LD. The highest species richness is recorded in Eurasia. In the USA, *B. burgdorferi* s.s. is expanding in population size and geographical range, causing LD epidemics. *B. afzelii* seems to be less abundant in the British Isles compared with continental Eurasia. The prevalence of *B. burgdorferi* s.s. phases out towards eastern Europe. *B. garinii* and *B. valaisiana* are found across terrestrial Eurasia. *B. garinii* is also maintained by seabird species and *I. uriae* ticks in pelagic transmission cycles in both hemispheres. *B. bissettii* and *B. lusitaniae* have occasionally been found in locations beyond their core range. Other *Borrelia* species are omitted from the figure, due to little information on distribution. (Kurtenbach et al , 2006).

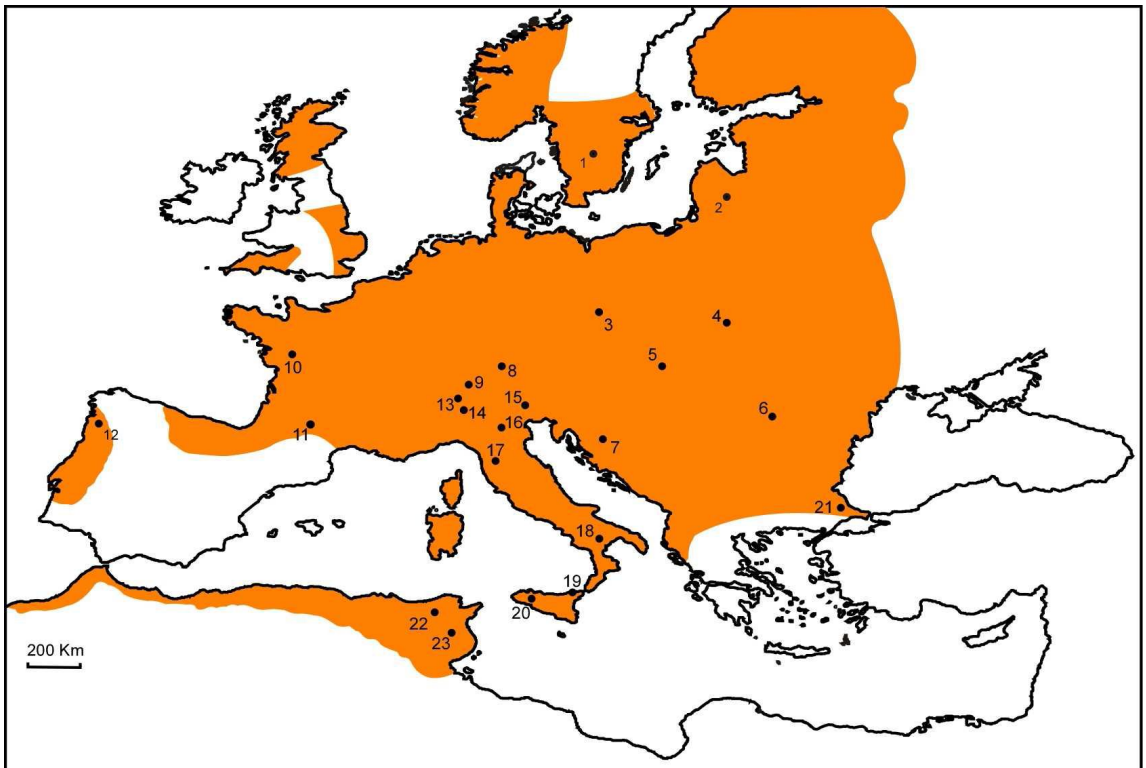
3.2 Materials and methods

3.2.1 Tick sampling

In this work, we analyzed a total of 145 individuals (50 males and 95 females) of *Ixodes ricinus*, collected by dragging (Fig. 3.2), from 23 different localities within its range of geographic distribution in Europe and North Africa (Fig. 3.3). The specimens has been collected on vegetations using dragging method by expert tick hunters (Fig 3.2). The collected individuals were immediately transferred in a single 1.5 ml plastic vial with 1 ml of ethanol 90% and transported in laboratory facility to be stored at 4°C.

The majority of the ticks used in this study where collected in summer/autumn 2009 in representative areas in continental Europe. Unfortunately, ticks were not collected in the complete geographical area of distribution of the species and some interesting representative zones (England and Norway) remain out of this study or are not well represented (Northern Europe). In the costal areas of Northern Africa, *Ixodes ricinus* ticks are active throughout the year, especially in winter, and for our research purpose we organized the sampling campaign in November 2009. In the next page, all the tick sampling sites are reported in a map (Fig 3.3).

Fig 3.2 – Tick hunter at work for collection of *Ixodes ricinus* and other tick species by dragging on vegetation in rural area, Le kef, Tunisia. In Northern Africa *Ixodes ricinus* is also active in wintry season.



Locality	Latitude	Longitude	Locality	Latitude	Longitude
1. Uppsala, Svezia	59°51'N	17°38'E	13. Domodossola, Italia	46°07'N	08°17'E
2. Kaunas, Lituania	54°33'N	23°53'E	14. Varese, Italia	48°49'N	08°49'E
3. Pribice, Rep. Ceca	48°57'N	16°33'E	15. Trento, Italia	46°04'N	11°07'E
4. Kosice, Rep. Slovacca	48°43'N	21°15'E	16. Parma, Italia	44°44'N	10°12'E
5. Budapest, Ungheria	47°30'N	19°02'E	17. Pistoia, Italia	43°56'N	10°53'E
6. Cluj-Napoca, Romania	46°46'N	23°26'E	18. Potenza, Italia	40°38'N	15°48'E
7. Zagabria, Croazia	45°49'N	15°58'E	19. Barcellona, Italia	38°09'N	15°12'E
8. Monaco, Germania	48°08'N	11°34'E	20. Corleone, Italia	37°48'N	13°18'E
9. Zurigo, Svizzera	47°22'N	08°32'E	21. Silvri, Turchia	39°06'N	29°33'E
10. Villecartier, Francia	48°44'N	00°43'E	22. Airdraham, Tunisia	36°51'N	08°46'E
11. Gardouch, Francia	43°23'N	01°41'E	23. Eljouza, Tunisia	36°04'N	09°36'E
12. Galizia, Spagna	42°46'N	07°31'E			

Fig. 3.3 – Sampling areas of *Ixodes ricinus* ticks collected by dragging in Europe and North Africa with the aim to cover the entire areal of distribution of the tick species. Geographic coordinates of the sampling points are also reported.

3.2.2 Laboratory procedures

DNA extraction

DNA was extracted from ticks using the commercial Illustra™ tissue & cell genomic Prep Spin GE® Healthcare kit (Little Chalfont, Buckinghamshire, UK) following the manufacturer instructions. Before DNA extraction each tick has been rinsed in sterile water in order to rehydrate the tissues, then places in a sterile 1.5 ml microtube, sliced with a blade and homogenized by using a sterile micropestle.

3.2.3 Amplification of nuclear loci, molecular cloning and sequencing

We performed PCR amplification of part of DNA regions coding for two different nuclear genes called Defensin and TROSPA. The immune system of ticks is stimulated to produce many pharmacologically active molecules during feeding and especially during pathogen invasion. The family of cationic peptides - defensins - represents a specific group of antimicrobial compounds with six conserved cysteine residues in a molecule. Defensins have been identified from insects, scorpions, mussels, ticks and other arthropods. The primary mechanism of action of tick defensin is bacterial cytoplasmic membrane lysis; they are particularly active toward Gram-positive bacteria (Nakajima et al., 2003). The tick receptor TROSPA (tick receptor for *Osp*A) has been shown to be required for spirochetal colonization of *Ixodes* sp. vectors (Pal et al., 2004). In particular, *B. burgdorferi* outer surface protein A, which is abundantly expressed on spirochetes within the arthropod and essential for pathogen adherence to the vector, specifically bounds to TROSPA. TROSPA mRNA levels in ticks increase following spirochete infestation and decreased in response to engorgement, events that are temporally linked to *B. burgdorferi* entry into and egress from the vector. The blockade of TROSPA by TROSPA antisera or by the repression of TROSPA expression via RNA interference reduced *B. burgdorferi* adherence to the tick gut in vivo, thereby preventing efficient colonization of the vector and subsequently reducing pathogen transmission to the mammalian host (Hovious et al., 2007).

Region coding for Defensin was amplified using a previously published pair of primer Def_12_for ATGAAGGTCCTTGCCGTCTC / Def_12_rev CAGCGATGTAGTGCCCATGT (Noureddine et al., 2010). Part of the region coding for TROSPA has been amplified using primer Tro_V_for GCTACGGACACGGTGGTT / Tro_V_rev TGGTTTCCCTTTGAGATG created on the base of the sequences of *I. ricinus* deposited in GeneBank. For both gene fragments the PCR reaction was performed in a finale volume of 25 µl, containing 50 ng of DNA template, 10X buffer , 2.5 mM $MgCl_2$, 0,2 mM dNTPs, 0,2 µM of each primer 0,2 unit of Taq polimerase (Promega). The thermal profile was the following: 94° C for 15 min, 40 cycles at 94° C for 45 sec, 30 sec annealing at different temperatures for Defensin (59°C); and for *TROSPA* (55°C), 45 sec at 72°C and a final step of 72°C for 15 min.

The amplified products had been sequenced using ABI PRISM 3700 DNA sequencer of Macrogen Inc. (www.macrogen.com). All the individuals had been sequenced using both forward and reverse primers. Sequences were corrected and allineated using respectively softwares CHROMAS 2.33 (Technelysium Pty Ltd, Australia) and CLUSTALX 2.0 (Thompson et al., 1997). In the sequences, the presence of heterozygotic nucleotidic positions had been identified considering double peaks in the electroferograms (Brumfield et al., 2003).
In order to obtain the sequences of the different alleles we effected the molecular cloning *in vivo* of the products of amplification. Such technique is summarized in the following phases:

- Insertion of amplified fragments in the vector pGEM-T
- Transformation of competent cells
- Screening for positive colonies
- Extraction and purification of plasmids
- Sequencing of the inserts

Insertion of amplified fragments in the vector pGEM-T

Fragments obtained in the previous amplification had been introduced in the vector pGEM-T (Promega) that allows molecular cloning of PCR products. This vector is prepared from other pGEM plasmids, after cutting with restriction enzyme, such as Eco RV and after adding of Tymidine at the two 3' ending. These protrusions at 3' avoid plasmid re-circularization and improve efficiency of ligation. Plasmid pGEM-T (3Kb) contains:

- Initiating site of transcription of RNA polymerase T7
- Initiating site of transcription of RNA polymerase Sp6
- Promotor of RNA polymerase T7
- Promotor of RNA polymerase Sp6
- A region of multiple cloning
- The starting codon of *lac*Z
- Sequences of *lac* operone
- Operator *lac*
- Codifying region for β lattamase

Ligation of purified PCR products with the vector was performed in a finale volume of 10µl with the following ligation mix: 2X Rapid ligation buffer Promega (60mM Tris-HCl pH7.8, 20mM $MgCl_2$, 20mM DTT, 2mM ATP 10% polietilenglycole); 50 ng of plasmid pGEM-T (Promega); 6.6 ng of purified PCR product;1µl of T4 DNA ligase (3u/µl). The reaction mix remained incubated at 4°C for four days.

Transformation of competent cells

After insertion of a PCR fragment in the cloning plasmid vector, the new construct was inserted in a strain of competent cells. We used *Escherichia coli* JM109 cells (Promega) transformed trough heat shock. The heat shock causes the opening of pores in bacterial wall structure that allows the penetration of plasmid vector in the cell. In 1.5 ml plastic vials we placed 2 µl of each ligation reaction and 50 µl of competent cells, previously stored at -70°C and incubated in ice for 20 minutes. Cells had been heat shocked at 42°C for 50 sec and again placed in ice. At this stage, we added 950 µl of SOC medium (20 g of Bacto-Tryptone, 0.5 g of Bacto-yeast extract, 1ml NaCl 1M, 0.25ml KCl 1M, 1ml Mg^{2+} 2M, 1ml of sterilized glucose 2M) in each tube containing the competent cells and the ligation reaction. Cell transformation takes place at 37°C for 1 hour and 30 minutes. The cells were plastered on a solid LB growth medium containing ampicillin/IPTG/X-gal and incubated overnight at 37°C. The preparation of 1l of LB medium require 10g of meat peptone, 5 g of yeast extract, 10 g NaCl at 7 pH, 15 g of agar. After autoclavation for 20 minutes at 2 atm, the medium was cooled and when it reached a temperature inferior of 40°C ampicilline 1% was added (10 mg/ml). This medium had been let to solidify in Petri dish, first at room temperature, and later in a fridge at 4°C for 1h. In each Petri dish was added IPTG 0.1M and X-gal 50 mg/ml (100mg X-gal in 2 ml of NN dimetilformammide). Only the cells containing the plasmid, with the gene that provides resistance to ampicilline, are able to growth on this medium.

Screening for positive colonies

The colonies of cells on the Petri dishes can be white or blue. This white-blue coloration allows to distinguish positive colonies containing both the plasmid and the DNA insert. Indeed, in the medium is present IPTG (isopropiltio β galattoside) and X-gal. The first substance is an inductor of the gene codifying for β-galattosidasi (enzyme that hydrolyze lattose) of *E. coli*. X-gal is an incolour substance, that when hydrolyzed produce a molecule of blue colour. The blue coloration is due to the formation of cromogen compound generated by scission of the x-gal substrate for the action of β-galattosidase codified by *lac*Z gene induced by IPTG.

The transformed colonies does not present blue coloration since the DNA insert break the *lac*Z gene, and even if the inductor IPTG is present, the β-galattosidase enzyme is not transcripted. Only the white colonies were removed and transferred in microplate with LB medium at 4°C.

Extraction and purification of plasmids

An aliquot (20µl) of the bacterial colonies was transferred in Falcon collection tubes (10 ml) with 3 ml of LB medium and incubated at 37°C overnight. The plasmid purification had been performed using commercial kit Wizard Plus SV Minipreps (Promega) following manufacturer instructions.

Sequencing of vector-inserted DNA

The purified plasmids were prepared for automatic sequencing using ABI Prism Automated Sequencer, Perkin Elmer at Macrogen Inc. For each samples had been sent 20 ng of plasmidic DNA and 5 pmol of oligonucleotids Sp6 (5'-ATTTAGGTGACACTATAG-3') and T7 (5'-AATACGACTCACTATAG-3').

3.2.4 Amplification of mitochondrial DNA and sequencing

Two partial regions of mitochondrial DNA (mtDNA), respectively coding for cytochrome c oxidase subunit I (COI) and cytochrome c oxidase subunit II (COII) were PCR amplified in this study. The COI coding region has been amplified using primer pair:

z-coI-for (5'-TTTTAATTCGAACTGAATTAGGACAA-3')
z_coI_rev (5'-TCATCAATAAATCCTAAAAATCCAA-3')

The primers were designed on the base of sequences of *I. ricinus* deposited in Gene Bank, while the COII region has been amplified using the previously published primer pair (Casati et al., 2007):

C1-J-2797 (5'-CCACGACGATACTCAGATTATC-3')
TK-N-3785 (5'-TTTAAGAGACCATTGCTTA-3')

For both gene fragments the PCR reaction was performed in a finale volume of 25 µl, containing 50 ng of DNA template, 10X buffer , 2.5 mM $MgCl_2$, 0,2 mM dNTPs, 0,2 µM of each primer 0,2 unit of Taq polimerase (Promega). The thermal profile was the following: 94° C for 15 min, 40 cycles at 94° C for 45 sec, 30 sec annealing at different temperatures for COI (57°C); and for COII (52°C) elongation of 45 sec at 72°C and a final step of 72°C for 15 min. The amplified products were sequenced using ABI PRISM 3700 DNA sequencer of Macrogen Inc. (www.macrogen.com). All the individuals had been sequenced using both forward and reverse primers. Sequences were corrected and allineated using respectively software CHROMAS 2.33 (Technelysium Pty Ltd, Australia) and CLUSTALX 2.0 (Thompson et al., 1997).

3.2.5 PCR detection and identification of *Borrelia* species

A PCR screening for *B. burgdorferi* s.l. bacteria was performed on the DNA of all the collected ticks using primers BBLD5' and BBLD3' targeting the 16S rDNA (Marconi and Garon, 1992). Positive samples were further examined using a nested PCR targeting the ITS2 region of *Borrelia* spirochetes, using primers 23S3 and 23Sa (Chu et al., 2008). PCR reactions were performed in final volumes of 20µl, following the published protocols. Sequencing of amplified fragments from the 16S rDNA confirmed the detection of spirochetes belonging *B. burgdorferi*

s.l., but did not allow determining the genospecies, and for this purpose we sequenced ITS2 region.

3.2.6 Data Analysis

We evaluated the nucleotidic and haplotypic polimorphisms of mtDNA genes COI and COII and of nuclear genes Defensin and TROSPA using software MEGA 4.0 (Tamura et al., 2007). For mitochondrial genes we performed all the analysis using concatenated sequences, while for nuclear genes the analysis were conducted on single loci. Haplotipic diversity ($\hat{H}$) and nucleotidic diversity (π) defined by Nei (1987), had been estimated using the software ARLEQUIN 3.1 (Excoffier et al., 2005). $\hat{H}$ is defined as the probability that two haplotypes randomly chosen in the sample set are different. It is estimated using the algorithm:

$$\hat{H} = \frac{n}{n-1}(1 - \sum_{i=1}^{k} p_i^2)$$

where n is the number of gene copies present in the sample set, k is the number of haplotypes, and pi is the frequency of i-esimo haplotypes (Nei, 1987).

Π is defined as the probability that two homologous sites, randomly chosen are different. It is the equivalent of $\hat{H}$, but at nucleotidic level. This parameter is expressed by the following formula:

$$\hat{\pi}_n = \frac{\sum_{i=1}^{k} \sum_{j<i} p_i p_j \hat{d}_{ij}}{L}$$

where p_i is the frequency of haplotype i, p_j is the frequency of haplotype j, d_{ij} is an estimation of the number of mutation that happened starting from the divergence of i and j haplotypes, and k is the number of haplotypes (Nei, 1987).

The genealogical relationship between different haplotypes was analyzed trough the construction of a phylogenetic network, following a described method (Templeton et al., 1992). The network represents the most appropriate method to show the variation at intra specific level (Posada & Crandall, 2001). Due to the relationship linking the individuals of the same species, it is not possible to use a phyilogenetic tree representation. More in details, the relationships between individuals belonging to the same species are not gerarchical, as encountered between individuals of different species. In fact, while phylogenetic processes (i.e. between different species), bring to the appearance of two different species derived from an ancestral one, on the contrary, the tokogenetic processes (i.e. within the same species) cause a mixing of parental genes to generate newborns. This means that methods traditionally used to estimate the variation between different species, like phylogenetic tree, are not feasible to be applied at population level, where fundamental basic assumption are not maintained. Another important aspect is linked to the divergence time. Co-specific individuals diverge later, compared to individuals of different species, consequently the characters for phylogenetic analysis decrease

inside the same species, reducing the statistical power of traditional methods. Frequently, in natural population, the derived genes co-exist with the ancestral ones and event of recombination can produce a complex network of interconnections. For these reasons the network representation is more suitable for showing the genetic variation within a species. The network shows the ancestral haplotypes and all the other recent haplotypes that originated form the ancestral one, evidencing possible recombination events, hybridation between lineages and homoplasy. On the contrary, the phylogenetic tree is able to show only terminal nodes and not the ancestral haplotypes that remain at the basis of a specific cluster with a branch length equal to zero. And also it is not able to show eventual interconnection between individuals due to recombination events (Posada & Crandall, 2001).

In the present work, we built the network using the software TCS 1.21 (Clement et al., 2000), based on an algorithm of statistical parsimony. This algorithm estimates the maximal number of differences between haplotypes due to single substitutions with a statistical confidence of 95% (limit of parsimony). Hence, the haplotypes differing for one position are connected, then the ones differing for two positions, and so on, until the limit of parsimony is reached. This algorithm, more than empathizing the differences between haplotypes, empathizes what they share giving a statistical evaluation of deviation from parsimony (Posada & Crandall, 2001). The genetic structure of the population had been studied applying the Spatial Analysis of Molecular Variance, using the software SAMOVA1.0 (Dupanloup et al., 2002). This approach allows defining group of populations that are geographically homogenous and well differentiated, on the basis of genetic data, without knowing *a priori* physical and ecological characters. This method is based on the algorithm of 'Simulated Annealing' (SA), procedure that allow to find the composition of K group in which the value of *FCT*, or the proportion of the total genetic variation due to differences between groups of population, is maximized. Initially, it is built a set of poligoni of Voronoi starting from geographic locations of the *n* sampling points. In this way, it is obtained a partition of the space in *n* polygons that correspond to the populations, separated by genetic barriers represented by the sides of polygons. Hence, it is arbitrary established the value of K, meaning the number of groups to distribute the *n* populations, and it is calculated the value of the index *FCT*. At this point the SA phase starts; one side of the barriers is randomly selected, as the two populations at the two side of the barrier and one of the two populations will be included in the other, delineating a new configuration. The new value of the index *FCT*, associated to this subdivision is calculated with a probability p:

$$p = \begin{cases} 1 & \text{if} \quad F_{CT}{}^{*} \geq F_{CT} \\ e^{(F_{CT}{}^{*} - F_{CT})S^{A}} & \text{if} \quad F_{CT}{}^{*} < F_{CT} \end{cases}$$

where S is the number of simulated steps in the process of annealing and A is a constant. These steps are repeated 10.000 times. Besides, to avoid that the final configuration of K is not

biased by the initial configuration, the SA process is repeated 100 times, starting every time from a different partition of *n* samples in K groups. The configuration with associated the higher value of the index *FCT*, after 100 simulations will be chosen as the best grouping of the populations (Dupanloup et al., 2002). Our analysis has been conducted testing K values from 2 to 22 and, to control the coherence, we repeated every analysis 5 times with 10.000 processes of annealing. Finally we tested the eventual existence of correlation between genetic distance and geographic distance by means of Mantel test. The genetic distance between populations had been estimated using the index of pair difference *FST*, according to described method (Weir & Cockerham 1984). The indexes of genetic difference had been transformed as *FST*/1-*FST* and for geographic distance was used the natural logarithm of geographic distance expressed in Km. The analysis had been run using the software Genepop 4.1 (Raymond M. & Rousset F., 1995).

Analysis of demographic changes in populations

In order to study signs of possible demographic changes in populations we analyzed the distribution of nucleotidic pair wise differences between haplotypes (mismatch distribution) using ARLEQUIN 3.1 (Excoffier et al., 2005). This distribution is generally multimodal in populations that stand in condition of equilibrium, on the contrary, assumes a unimodal and Gaussian aspect in population that suffered recent event of expansion (Rogers & Harpending, 1992). In this second case, the probability to observe S difference between two non-recombinant haplotypes randomly chosen is equal to:

$$F_S(\tau,\theta_0,\theta_1) = F_S(\theta_1) + exp(-\tau\frac{\theta_1+1}{\theta_1})\sum_{j=0}^{S}\frac{\tau^j}{j!}\left[F_{S-j}(\theta_0) - F_{S-j}(\theta_1)\right],$$

where:

$$F_S(\theta) = \frac{\theta^S}{(\theta+1)^{S+1}}$$

is the probability to observe *S* difference between two randomly chosen haplotypes in a stationary population; $\theta_0 = 2\mu N_0$, $\theta_1 = 2\mu N_1$, $\tau = 2\mu t$ and μ is the rate of mutation of the entire haplotypes. In order to analyze the existence of significant differences between mismatch distribution observed and expected in case of demographic expansion was used the sum of square deviations (SSD). Besides, the measure of unimodality of distribution was calculated the index of raggedness *r* and its significance was tested through 1000 replicates. This index is defined by Harpending (1994) as:

$$r = \sum_{i=1}^{S+1}\{x_i - x_{i-1}\}^2$$

where: *d* is the maximum number of differences observed between haplotypes and X are the relative frequencies observed of the mismatch classes. The index assumes higher values in case of multimodal distribution, when population is stationary, compared to cases of unimodal or Gaussian distribution. To determine signs of eventual demographic changes had been used the Fu's index Fs (1997). This test is based on the probability to observe k alleles in a sample of *n* sequences, given a value θ, assumed as mean value of pair wise differences. This test is particularly sensible to events of population expansion that are usually characterized by negative *Fs* index values. In fact, great negative values of parameter *Fs* indicate en excess of single mutation (singleton), as expected in population that experienced recent demographic expansion. This index had been calculated in ARLEQUIN 3.1 (Excoffier et al., 2005) and significant was tested with 1000 replicates.

3.3 Results

3.3.1 Results of the analysis on mitochondrial and nuclear loci

In the analysis of mitochondrial loci, the final alignment included 205 sequences for a total length of 1486 bp (685 from the COI fragment and 801 from COII fragment). Considering the concatenated fragments we defined 100 different haplotypes defined by 139 nucleotidic substitutions. For the entire set of mitochondrial data the estimations of haplotypic and nucleotidic diversity are respectively 0.867 (± 0.001) e 0.0037 (±0.0017). The estimates of haplotypic and nucleotidic diversity for each locality are reported in Table 3.1.

In the analysis of nuclear loci, the final alignment included 136 sequences with a length of 288 bp for Defensin and 70 sequences with a length of 479 bp for TROSPA. A total of 49 and 42 haplotypes had been discovered respectively for the loci Defensin and TROSPA. The estimations of haplotypic and nucleotidic diversity are respectively 0.876 (± 0.001) e 0.0027 (±0.0017) for Defensin and 0.823 (± 0.001) e 0.0047 (± 0.0022) for TROSPA. The estimates of haplotypic and nucleotidic diversity for each sample for the two genes are reported in Table 3.2. The network of parsimony showing phylogenetic relationships between haplotypes encountered at mitochondrial loci and between haplotyes encountered in Defensin and TROSPA nuclear loci are shown respectively in (Fig. 3.4 and 3.5). According to mitochondrial marker, all the 100 haplotypes are clustered in a single network forming two principal groups (1.12% of mean divergence) and show a clear geographic pattern. Group I is constituted by all the individuals collected on the European continent while group II comprises only individuals collected in Tunisia, North Africa (Table 3.1 – Fig. 3.4). No sub-group could be noticed inside the two groups. For the nuclear locus Defensin, all the 49 haplotypes recorded are joined in a unique network with two principal groups of haplotypes (0.93% of mean divergence). Likewise for mitochondrial marker, the group I comprises individuals collected in Europe, while in group II are gathered individuals collected in Tunisia, north Africa (Table 3.2, Fig. 3.5A). The haplotypes

D46 e D47 represent an exception, since they were found in Tunisia even if they belong to European haplogroup. On the other hand, the two haplotypes D19 e D42, belonging to Tunisian haplogroup were found in Europe (D19 in Switzerland and D42 in Sicily). No sub-groups were individuated inside these two groups.

For the nuclear locus TROSPA, the 42 haplotypes found are jointed in two networks (2.1% of mean divergence). Group I comprises European individuals and group II individuals collected in Tunisia (Table 3.2, FIG. 3.5B). Haplotypes T8, T28 and T29, even tough belonging to Tunisian haplogroup are found in Europe (T8 in Czech Republic, T28 in Slovak Republic and T29 in Domodossola, Italy). No sub-group had been evidenced inside the two groups. The spatial analysis of molecular variance (SAMOVA) established K=2 as better option of grouping of populations and as best explanation of data both for mitochondrial marker and for both nuclear loci studied (Fig 3.6). This patter of grouping separated European populations from Tunisian ones (Fig. 3.6). Considering only European population the index of genetic differentiation *FST* for the entire set of data is 0.23 (± 0,12) ($P < 0.05$). Mantel test indicate the lack of correlation between genetic and geographic distances ($R2 = 0.048$ $P > 0.05$) (Fig. 3.7).

Code	Locality (Country)	N. individuals	Haplotypes	$\hat{H}$	π
1.	Uppsala, Svezia	11	h1; h2(4); h3; h4; h5; h6; h7; h9	0.809 (0.091)	0.0054 (0.0030)
2.	Kaunas, Lituania	3	h10; h11; h12	1.000 (0.127)	0.0080 (0.0051)
3.	Pribice, Rep. Ceca	12	h2(6); h13; h14; h15; h16; h17; h18	0.808 (0.113)	0.0025 (0.0015)
4.	Kosice, Rep. Slovacca	7	h2(5); h14(2)	0.476 (0.171)	0.0010 (0.0008)
5.	Budapest, Ungheria	7	h2(2); h11; h19; h20; h21; h22	0.952 (0.096)	0.0014 (0.0010)
6.	Cluj-Napoca, Romania	9	h2(2); h23; h24; h25(2); h26; h27; h28	0.944 (0.070)	0.0027 (0.0017)
7.	Zagabria, Croazia	4	h29; h30; h31; h32	1.000 (0.177)	0.0048 (0.0034)
8.	Monaco, Germania	4	h2(3); h33	0.700 (0.218)	0.0038 (0.0025)
9.	Zurigo, Svizzera	13	h2(6); h22; h34; h35; h36; h37; h38; h39	0.835 (0.101)	0.0024 (0.0015)
10.	Villecartier, Francia	7	h2(2); h14; h40; h41; h42; h43	0.956 (0.059)	0.0060 (0.0034)
11.	Gardouch, Francia	5	h2(2); h14; h40; h44	0.929 (0.084)	0.0057 (0.0034)
12.	Galizia, Spagna	12	h2; h14(4); h45(2); h46; h47; h48; h49; h50	0.905 (0.054)	0.0056 (0.0031)
13.	Domodossola, Italia	15	h2(5); h14; h40; h51; h52; h53; h54; h55; h56; h57; h58	0.917 (0.064)	0.0027 (0.0016)
14.	Varese, Italia	15	h2(8); h14; h22; h37; h45; h59; h60; h61	0.767 (0.113)	0.0021 (0.0013)
15.	Trento, Italia	7	h2(3); h17; h45; h62; h63	0.893 (0.111)	0.0034 (0.0021)
16.	Parma, Italia	10	h2(2); h14(2); h32; h44; h64; h65; h66; h67;	0.970 (0.044)	0.0051 (0.0030)
17.	Pistoia, Italia	12	h2(8); h14; h68; h69; h70	0.641 (0.150)	0.0027 (0.0016)
18.	Potenza, Italia	10	h2(5); h14; h71; h72; h73; h74	0.778 (0.137)	0.0025 (0.0016)
19.	Barcellona, Italia	5	h2(4); h75	0.400 (0.273)	0.0003 (0.0003)
20.	Corleone, Italia	6	h2(4); h76; h77	0.600 (0.215)	0.0007 (0.0006)
21.	Silvri, Turchia	7	h2; h78; h79; h80; h81; h82; h83	1.000 (0.076)	0.0026 (0.0017)
22.	Airdraham, Tunisia	13	h84; h85; h86(3); h87; h88; h89; h90; h91; h92; h93; h94	0.962 (0.050)	0.0027 (0.0012)
23.	Eljouza, Tunisia	11	h86(5); h95; h96; h97; h98; h99; h100	0.818 (0.119)	0.0016 (0.0011)

Table 3.1 - Genetic diversity on mitochondrial markers. In the column of the haplotypes is indicated the number of times that each single haplotype was found (in brackets). The values of haplotypic diversity ($\hat{H}$) and nucleotidic diversity (π) are indicated as standard deviation (in brackets).

Codice	Località (Paese)	TROSPA			Defensina		
		Aplotipi	$\hat{H}$	π	Aplotipi	$\hat{H}$	π
1.	Uppsala, Svezia				D1(2); D2(3); D3	0.733 (0.155)	0.0114 (0.0074)
2.	Kaunas, Lituania				D2; D4 (3); D5(2); D6; D7	0.933 (0.121)	0.0071 (0.0053)
3.	Pribice, Rep. Ceca	T1(2); T2; T3; T4; T5	0.933 (0.121)	0.0130 (0.0083)	D4; D8	-	-
4.	Kosice, Rep. Slovacca	T6(2); T7; T8; T9; T10	0.900 (0.161)	0.0296 (0.0019)	D9; D10	-	-
5.	Budapest, Ungheria	T6; T11	-	-	D4; D7; D11; D12; D13; D14	1.000 (0.096)	0.0118 (0.0081)
6.	Cluj-Napoca, Romania	T4; T12	-	-	D1; D4(5); D15; D16	0.944 (0.070)	0.0051 (0.0039)
7.	Zagabria, Croazia				D4(2)	-	-
8.	Monaco, Germania				D3; D4(3); D17; D18	0.800 (0.172)	0.0046 (0.0038)
9.	Zurigo, Svizzera	T6(2); T13; T14; T15; T16	0.933 (0.121)	0.0087 (0.0058)	D1; D4(5); D11; D19	0.524 (0.208)	0.0089 (0.0062)
10.	Villecartier, Francia	T6(2); T14; T17; T18; T19; T20(2)	0.929 (0.084)	0.0085 (0.0054)	D2; D4(3); D6; D20; D21; D22	0.800 (0.172)	0.0057 (0.0045)
11.	Gardouch, Francia	T4; T6(3); T14; T21; T22(2); T23; T24	0.733 (0.155)	0.0082 (0.0055)	D2(2); D6; D22; D23	1.000 (0.177)	0.0104 (0.0081)
12.	Galizia, Spagna	T4; T25; T26; T27	1.000 (0.177)	0.0052 (0.0042)	D1; D4(4); D11; D24; D25	0.833 (0.222)	0.0121 (0.0092)
13.	Domodossola, Italia	T28; T29	0.934 (0.097)	0.0083 (0.0051)	D4(2); D6(2); D23; D26; D27	0.867 (0.129)	0.0072 (0.0054)
14.	Varese, Italia				D2; D4(2); D7; D28; D29	0.933 (0.122)	0.0088 (0.0063)
15.	Trento, Italia				D2; D4(3); D6; D7	0.800 (0.172)	0.0035 (0.0031)
16.	Parma, Italia				D4(4); D6; D23; D26; D30	0.786 (0.151)	0.0061 (0.0045)
17.	Pistoia, Italia				D4(2); D6; D23; D31; D32; D33; D34	0.964 (0.077)	0.0058 (0.0043)
18.	Potenza, Italia	T4; T6(2); T17; T22; T30(2); T31	0.929 (0.084)	0.0095 (0.0059)	D2; D4(4); D35(2); D36(2); D37	0.778 (0.110)	0.0042 (0.0034)
19.	Barcellona, Italia	T17; T32	-	-	D23(2); D35(2)	0.667 (0.204)	0.0046 (0.0042)
20.	Corleone, Italia				D4(2); D31; D38; D39; D40; D41; D42	0.964 (0.077)	0.0155 (0.0097)
21.	Silvri_Turchia	T4; T6(2); T30	0.833 (0.222)	0.0094 (0.0069)	D2; D4; D32; D43	1.000 (0.177)	0.0046 (0.0042)
22.	Airdraham, Tunisia	T35; T36	-	-	D44; D45; D46; D47;	1.000 (0.176)	0.0021 (0.0154)
23.	Eljouza, Tunisia	T33;T34; T37; T38; T39; T40; T41; T42	1.000 (0.500)	0.0084 (0.0093)	D48; D49	-	-

Table 3.2 - Genetic diversity at nuclear loci TROSPA and Defensin. For each locus are indicated alleles found in each locality (In haplotype column the number of times of the alleles is reported in brackets) the estimated value of haplotypic diversity $\hat{H}$, and nucleotidic diversity π (in brackets it is reported standard deviation).

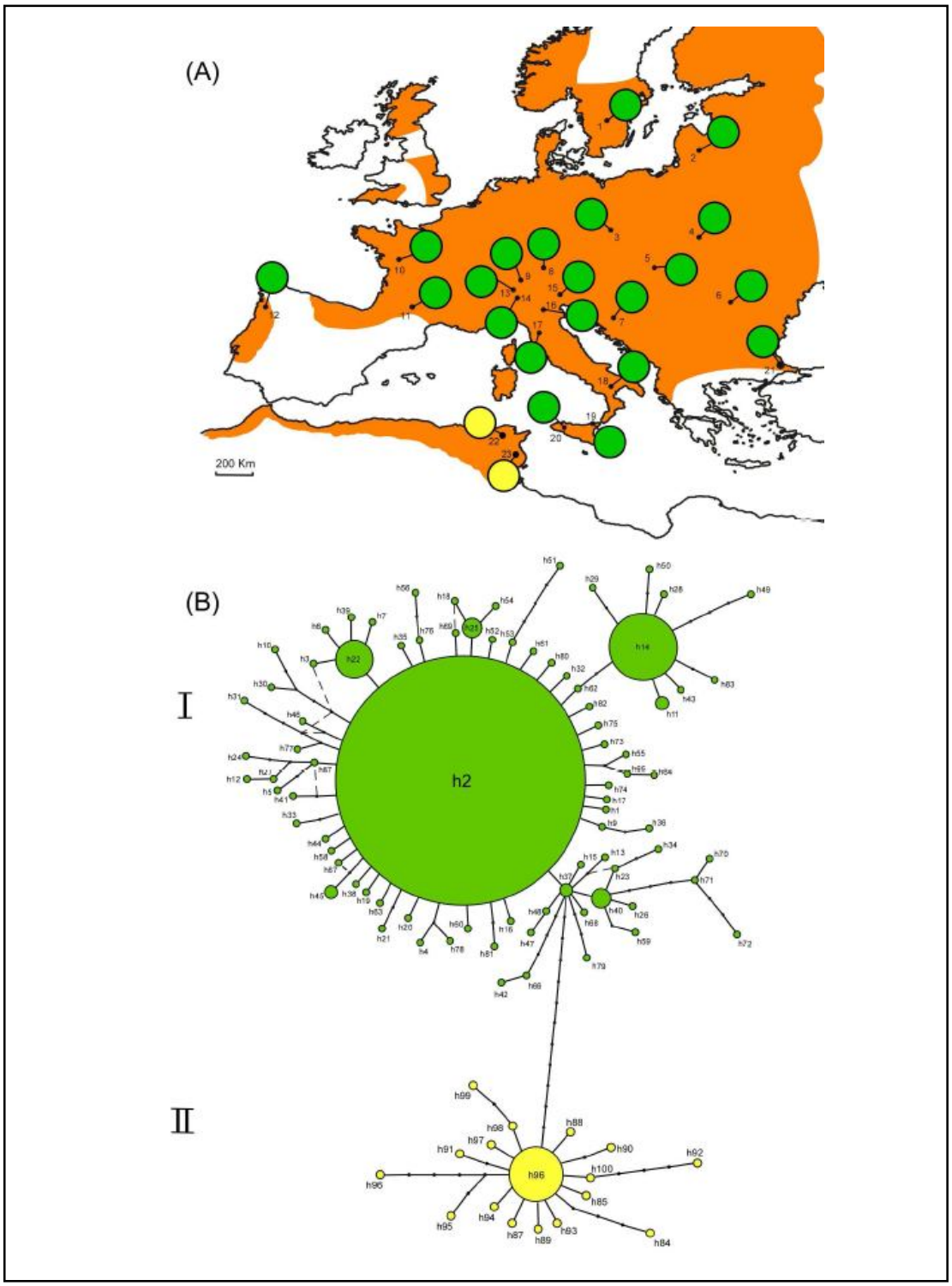


Fig. 3.4 - Geographic distribution and genetic relationship between encountered mitochondrial haplotypes. (A) Geographic distribution of mitochondrial haplogroup I and II. (B) Parsimony network showing genetic relationship between haplotypes. The dimension of circles is proportional to frequency of haplotypes.

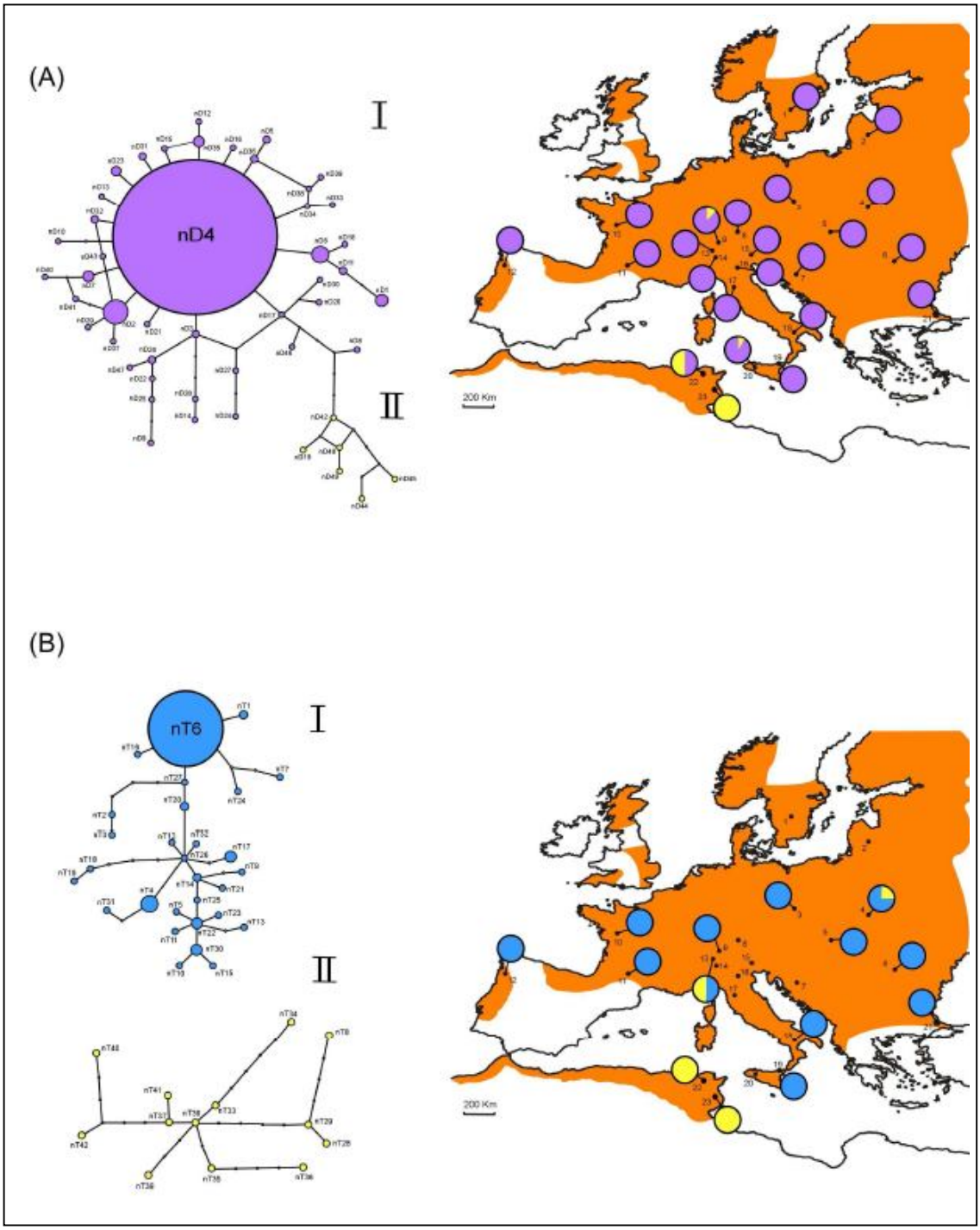


Fig. 3.5 - Geographic distribution and phylogenetic relationship between haplotypes encountered at nuclear loci coding for Defensin (A) and TROSPA (B). In the right panel is shown the geographic distribution of haplogroup I and II for each gene.20

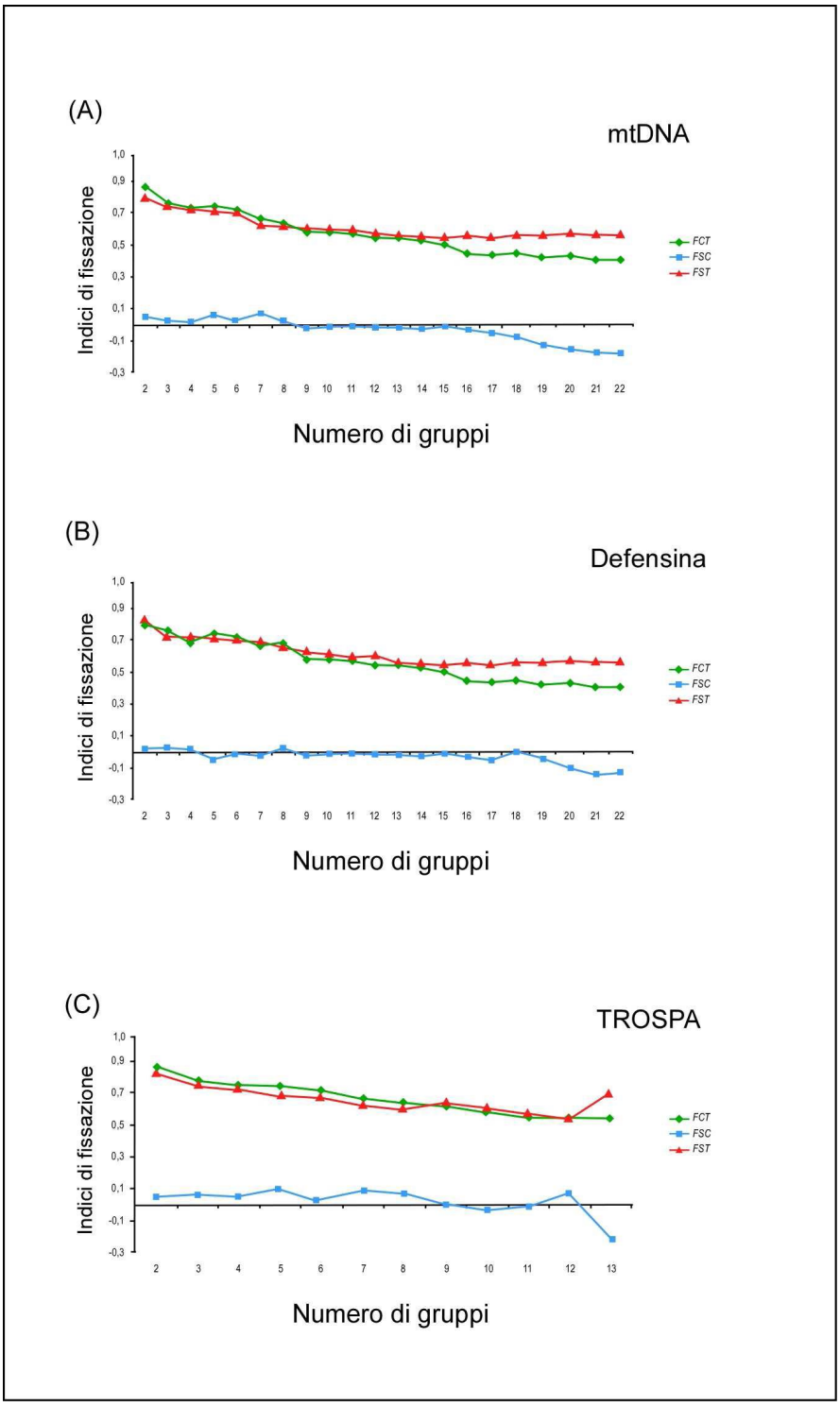


Fig 3.6 – Spatial Analysis of Molecular Variance (SAMOVA) for the mitochondrial marker (A) for the nuclear loci encoding Defensin (B) and TROSPA (C).

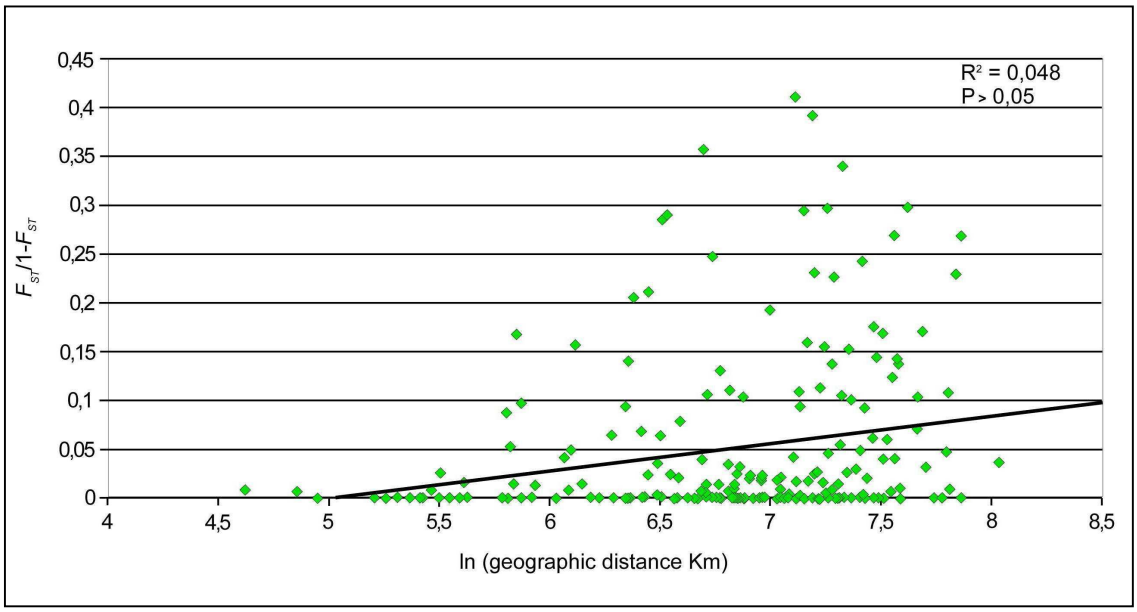


Fig. 3.7 - Mantel Test showing the relation between geographic and genetic distances for European populations of *Ixodes ricinus*. In ordered pairwise genetic distances FST / 1 - FST for the mitochondrial genes COI and COII, in the x-axis the value is expressed as ln (geographic distance in kilometers). The P value represents the estimated correlations from Mantel test (10,000 permutations).

3.3.2 Analysis of demographic changes in the populations

This part of the study was focalized on European populations. Since the genetic analysis on the haplotypes revealed the absence of significant genetic structure inside the populations of continental Europe, analysis of historic demography was run both for mitochondrial marker and for nuclear loci on the entire set of data of European samples (group I in Fig. 3.4 and Fig. 3.5 A-B). For mitochondrial marker the distribution of nucleotidic differences between couple of haplotypes (mismatch distribution) appears unimodal and Gaussian (Fig. 3.8A) and no significant difference was observed between mismatch distribution compared with the expected distribution in a model of demographic expansion (SSD = 0.0008, P = 1.00). The raggedness index *r* resulted low (r = 0.00075, P = 1.00), as expected in case of expansion. The estimates of parameters of expansion for mismatch distribution resulted θ_0 = 0.893 and θ_1 = 5.376 e τ = 0.52 (low bound 0.109, up bound 2.802). The time of expansion can be estimated trough the parameter τ ($\tau = 2\mu t$; where μ = is the rate of mutation per site per million of year and t is the generation time of the species) form mismatch distribution of mtDNA (Rogers and Harpending 1992). Assuming $\mu = 1 \times 10^{-8}$ and one generation per year, the expansion occurred 20.800 years ago, with a time window of 4.300 - 84.000 years. The occurrence of a past demographic expansion is suggested from statistical results on Fu's index Fs, with a value, highly negative (*F*s = – 26,427; *P* < 0,001), demonstrating the presence of an excess number of single mutations, as expected in population that undergone sudden demographic expansion.

For the nuclear gene Defensin, the distribution of nucleotidic difference between couple of haplotypes (mismatch distribution) appears unimodal and Gaussian (Fig. 3.8B) and no significant difference is recorded between observed mismatch distribution and distribution expected with a model of demographic expansion (SSD = 0.002 P = 0.70). The raggedness index *r* resulted low and not significant (r = 0.0315, P = 0.50), as expected in population expansion, as well. The estimates of parameters of expansion for mismatch distribution resulted θ_0 = 1.376 and θ_1 = 999.00 and τ = 0,24 (low bound 0,057, up bound 3,293). Also for this gene, the occurrence of a past demographic expansion is suggested from statistical results on Fu's index Fs, with a value highly negative (Fs = – 13.583; P < 0.001), demonstrating the presence of an excess number of single mutations, as expected in population that undergone sudden demographic expansion.

Also for nuclear gene TROSPA the distribution of nucleotidic differences between couple of haplotypes (mismatch distribution) appears unimodal and Gaussian (Fig. 3.8C) and no significant difference was observed between mismatch distribution compared with the expected distribution in a model of demographic expansion (SSD = 0.0038 P = 0.60). The raggedness index *r* is again low and not significant (*r* = 0.0126, *P* = 0.80). The estimates of parameters of expansion for mismatch distribution resulted θ_0 = 0.011 and θ_1 = 14.785 and τ = 5.184 (low bound 3.143, up bound 8.148). Also for this gene, the occurrence of a past demographic expansion is suggested from statistical results on Fu's index Fs, with a value highly negative (Fs

= – 13.583; P < 0.001), demonstrating the presence of an excess number of single mutations, as expected in population that undergone sudden demographic expansion.

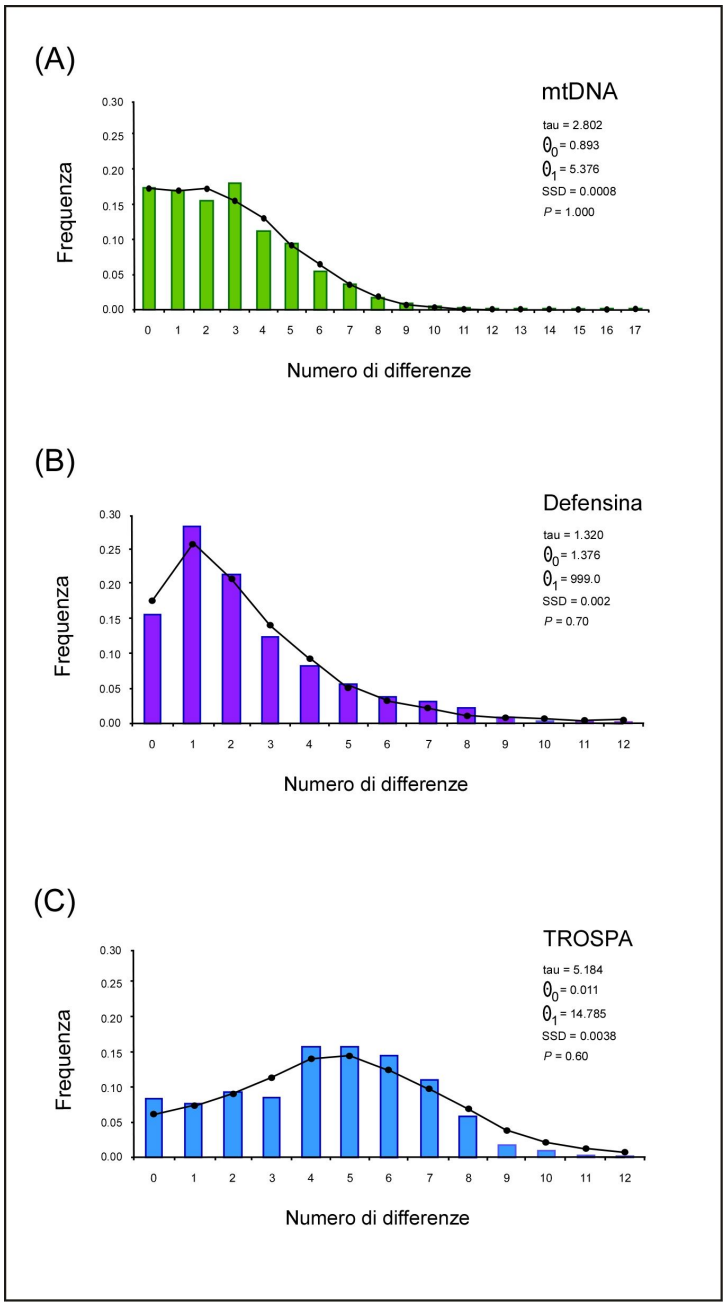


Figure 3.8 Distribution of pairwise nucleotide differences (mismatch distribution) for mitochondrial genes (A), coding for the nuclear locus for defensin (B) and for the nuclear locus coding for trospium (C). With the histogram shows the observed distribution, while the line shows the distribution expected in case of population growth.

3.3.3 Genospecies of *Borrelia burgdorferi* s.l. complex in Europe and North Africa

The Lyme Disease (LD) agent *Borrelia burgdorferi* s.l. naturally persists in a cycle that primarily involves ticks and mammals. We performed a molecular screening for the presence of borreliae causing LD on the collected ticks. Thirty-five ticks on a total of 145 individuals (24%) resulted positive for spirochetes of the *B. burgdorferi* s.l. complex (Table 3.3). In Europe, several *Borrelia* species circulate between different vertebrates apparently without a strong association with specific hosts or locations. Since the number of positive ticks is low (35/145 from 23 locations), our results does not allow to make any association between specific *Ixodes ricinus* genotypes and the positivity for *Borrelia* sp. or the genospecies of *Borrelia*. Nine *Borrelia* sp. positive individuals were associated with *I. ricinus* presenting h2 haplotype; anyway this haplotype is the most frequently reported in European populations of this tick species. Further development of this study, might concentrate on the genetic variability of tick receptors (in primis TROSPA) for borreliae and presence of these bacteria. In this study we show that *Borrelia lusitaniae*, currently thought to the predominant species only in Portugal and North Africa, seems to present an areal of distribution more expanded that expected in continental Europe and it is also frequent in Italy.

Code – Locality (Country)	Species of *Borrelia burgdorferi* sl complex and mitochondrial haplotypes
1 – Uppsala, Sweden	*B. burgdorferi* ss (h2), *B. afzelii* (h1, h4, h6, h10) and *B. garinii* (h3)
2 – Kaunas, Lituania	*Borrelia* spirochetes were not detected
3 – Pribice, Czech Republic	*B. garinii (h20), B. lusitaniae (h6, h19)* and *B. afzelii (h22)*
4 – Kosice, Slovak Republic	*B. burgdorferi* ss (h2)
5 – Budapest, Hungary	*B. afelii (h2, h27)*
6 – Cluj-Napoca, Romania	*Borrelia* spirochetes were not detected
7 – Zagreb, Croatia	*Borrelia* spirochetes were not detected
8 – Monaco, Germany	*B. burdorferi* ss (h2)
9 – Zurich, Switzerland	*Borrelia* spirochetes were not detected
10 – Villecartier, France	*Borrelia* spirochetes were not detected
11 – Gardouch, France	*Borrelia* spirochetes were not detected
12 – Galizia, Spain	*Borrelia* spirochetes were not detected
13 – Domodossola, Italy	*B. garinii* (h43)
14 – Varese, Italy	*B. garinii* (h54)
15 – Trento, Italy	*B. lusitaniae* (h2, h25) *B. afzelii* (h19, h54, h55, h61)
16 – Parma, Italy	*B. garinii* (h2)
17 – Pistoia, Italy	*B. lusitaniae* (h2)
18 – Potenza, Italy	*B.* sp. (h95)
19 – Barcelona, Italy	*Borrelia* spirochetes were not detected
20 – Corleone, Italy	*Borrelia* spirochetes were not detected
21 – Salvri, Turkey	*B. lusitaniae* (h101, h102)
22 – El Jouza, Tunis	*B.* sp. (h108, h114)
23 – Aim Draham, Tunis	*B. lusitaniae* (h108, h122)

Table 3.3 – In the table the locations indicating the sampling areas for ticks are reported. For each locality it is indicated the *Borrelia* species detected. In brackets it is also specified the tick mitochondrial haplotype/s encountered in the spirochetes-positive ticks.

3.4 Discussion

3.4.1 Genetic analysis on *Ixodes ricinus* population

The genetic analysis on both two mitochondrial loci and two nuclear loci (Table 3.1 and 3.2, Fig. 3.4 and 3.5), confirmed the existence of two distinct groups of haplotypes showing a clear geographic pattern. The first group comprises individuals collected mainly in the European continent, while the second group comprises individuals collected mainly in Tunisia, North Africa. The existence of two groups of populations genetically differentiated in the two continents is also supported by the spatial analysis of molecular variance (SAMOVA) congruently for both mtDNA loci and the two nuclear loci (Fig. 3.6).

In previous studies on European and North African populations (DeMeeûs et al., 2002; Noureddine et al., 2010) the lacking of data from intermediate areas does not allowed determining the entity and the nature of the observed discontinuity.

In our study, in addition to a considerable number of individuals from central and Northern Europe, Southern populations were included, sampled in Southern Italy (Sicily). These individuals belong to European group, hence marking the existence of an abrupt and strong genetic discontinuity. Several hypotheses had been proposed to explain this pattern. It could be linked to geographic discontinuity due to the presence of the Mediterranean sea separating the two continents. Anyway the absence of discontinuity in the entire European continent and the possibility for *I. ricinus* to cover large distance feeding on migratory birds, make questionable this hypothesis. Besides, there could be implicated ecological factors linked to *I. ricinus* biology and interactions with its hosts. Phenogical differences might be present between European and North African populations. African ticks are active in winter (Boulkaboul, 2003); on the contrary European ticks present two peaks of activity, one in spring and the other in summer. (Gray, 1991). For this reason when individuals are transported from a geographic area to the other, might not seriously contribute to genetic flux due to different reproductive period. A second hypothesis, not necessary excluding the previous ones, considers the role of interaction between species. *I. ricinus* is a parasites and a vector of other parasites/pathogens, interacting both with its hosts and with the transmitted pathogens, and this fact might be the cause of the genetic difference in the two populations. In Europe, *I. ricinus* parasitizes mammals and birds, while in Tunisia this parasite is common on the lizard *Psammodromus algirus* (Bouattour et al., 2004). Besides, in Europe *I. ricinus* is infected with several species belonging to *Borrelia burgdorferi* s.l. complex (mainly *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s.), while in North Africa *B. lusitaniae* is the prevalent species, suggesting the existence of an association between *I. ricinus*, *B. lusitaniae* e *P. algirus*.

All these selective pressures might have contributed to the insurgence of the observed differences between European and Tunisian populations of *I. ricinus*. Nuclear and mitochondrial loci are concordant in evidencing a genetic discontinuity between the two continents, but the

two markers show different patterns. For mitochondrial DNA, no Tunisian haplotypes were encountered in Europe and vice versa. Nuclear loci show a degree of sharing of haplotypes of the two different haplogroups, probably due to long-distance migration of avian hosts (Table 3.4, Fig. 3.5). Further interesting ecological and evolutionary scenarios, in a speculative way, might be suggested to explain such differences. For example, genetic drift, a strong micro evolutive force, is able to drive the loss of genetic variants in a totally random process. The flux of haplotypes might involve not only nuclear genes, but also mitochondrial genes, but these ones are lost due to genetic drift that on this marker is stronger compared to nuclear loci. An alternative hypothesis proposes the existence of differential migration of males and females. Indeed, females of *I. ricinus* resulted more philopatric compared to males and less incline to dispersion (DeMeeûs et al., 2002). Host preference with different dispersion capacity might explain this pattern, males tend to parasitize more often birds and females are more frequently encountered on large mammals. Another hypothesis might be linked to a selective disadvantage of females in the two geographic regions.

3.4.2 Pattern of genetic diversity inside the European continent

In the European population of *I. ricinus* was not evidenced any phyleogeographic structure. I would suggest that the pattern of low genetic diversity observed at nuclear and mitochondrial loci is a consequence of historical and contemporary factors. Both markers show traces of demographic expansion, in fact, mismatch distribution resulted unimodal and not deviate from the model expected in case of demographic expansion (Fig. 3.8). Besides, the result is confirmed from the values of Fu's Fs index that are negative and significant. Over crossing the actual distribution of the species *I. ricinus* and the distribution of ice in glacial phases in European continent is reasonable suppose that the demographic expansion started from glacial refugia. The value of parameter τ of mismatch distribution of mitochondrial DNA (Rogers & Harpending, 1992), suggests that this expansion happened almost 20.000 years ago. Thus historical event might be linked to deep climatic changes in temperate areas during glacial/interglacial phases in late Pleistocene. During this period, according to the general model of expansion/contraction (Hewitt, 2004), populations of thermophil species, like *I. ricinus*, to defend from ice advancing, moved to lower latitudinal refugia in suitable areas for survival, identified in the Southern Mediterranean peninsulae (Hewitt, 2004). In these refugia, various *I. ricinus* populations survived during glacial ages, and after ice melting and temperature rising, migrated to Northern areas. Previous studies on *I. ricinus* (McClain et al., 2001; Casati et al., 2008; Noureddine et al., 2010), hypothesized that this model, already validated for other species (Hewitt, 2004), is also valid for this parasite. But the results of this study, suggest a different evolutive scenario. In fact, even if the genetic data support the hypothesis of an event of demographic and spatial expansion after glacial ages, on the contrary, the absence of different lineages localized inside the European continent, as the absence of a clinal variation of

haplotypes frequencies do not support the glacial genetic fragmentation. The pattern observed is more adapted to explain a scenario were populations of *I. ricinus* remained inter-connected probably due to continuity offered by its multiple hosts, as suggested for other species with wide distribution in western Paleartic (Porretta et al., 2011). In the case of *I ricinus* is of fundamental importance to consider the ecological characteristics of the species in order to determine the response in front of the Pleistocenic climatic changes. The present analysis consents to evidence an historical component at the origin of actual pattern of distribution of genetic diversity, but also a variety of actual processes that act on populations. The migration on long distances mediated by different *I. ricinus* hosts in different stages of its biological cycle might be implicated in the absence of correlation between genetic and geographic distances, the presence of haplotypes shared by populations distantly located and the lack of areas with major and minor diversity. On the contrary, the presence of specific and private haplotypes in areas in close vicinity suggests a restricted genetic flux on small geographic scale.

4.4.3 Considerations on Lyme borreliosis

The Lyme disease is the most frequent human pathology transmitted by arthropods in Europe (WHO, 2004) with an annual incidence of 65.000 cases, but possibly a number of cases are not diagnosed (Hubálek, 2009). The Lyme disease geographic distribution corresponds to the geographical range of its main vector *I. ricinus*, and diverse evidences suggest a correspondence between the increase in the presence of the vector and the increase in the incidence of the disease (Hubálek, 2009). The control in density of population of *I. ricinus* is a key point in the control of Lyme disease. Groups of populations differentiated by a genetic and ecological point of view can present different vectorial capacity, different association with host/pathogen species and also a different susceptibility to substances used for control. Nowadays, the control of *I. ricinus* is based on the use of acaricides, but the insurgence of resistance towards the majority of commercial products poses at risk the use of the tools actually available and the efficacy of control measures, that normally do not consider criteria linked to biology and ecology of the species. The knowledge of connectivity pattern between vector-host populations is of fundamental importance to define the pattern of diffusion of pathogens, the appropriate geographic scale to effectuate controls, as the risk of diffusion of alleles conferring resistance. The genetic difference at local level of *I ricinus* showed in this study, supports the efficacy of local control measure, but, at the same time, the interconnection between European populations show that this species does not recognize the political boundaries and stress the importance of concerted and coordinated control actions in different EU states.

Chapter 4

Screening for *Midichloria mitochondrii* in blood and tissues of vertebrate hosts

4.1 Introduction

The PCR evidence for the presence of 16S rDNA gene sequences with high similarity to that of *M. mitochondrii* in several ticks species, in a few protozoan species (*Achantamoeba* spp. and ciliates), in environmental microbial mats, in Porifera, Celenterates, in hematophagous insects, and in other Metazoa, including vertebrates and humans, might represent an indirect evidence that *Midichloria*-like organisms (MLOs) circulate between different animals possibly vectored by ticks in vertebrate hosts. Besides, an additional indirect evidence of horizontal transmission is provided by comparisons of the phylogeny of the ticks to that of *Midichloria mitochondrii* bacteria present in diverse tick species; they are not congruent (Fig 4.1). More in details, *Midichloria* endosymbionts were encountered in several species of the Metastriate group, even not closely related. In addition, *Midichloria* bacteria detected in different vector hosts (*I. ricinus*, *Ha. punctata*, *I. uriae* and *R. turanicus*) presented identical 16S rDNA sequences.

Previous analysis on *Midichloria mitochondrii* s.s., the endosymbiont of *Ixodes ricinus*, allowed gathering information on prevalence of the bacterium in the host, on its way of transmission, on its intracellular and intramitochondrial localization and finally brought to sequencing of the full bacterial genome (Sassera et al., 2011). The presence of *M. mitochondrii* in salivary glands of *Ixodes ricinus* has been undoubtedly demonstrated only recently (unpublished data), since the complex structure of the glands made difficult the unambiguous identification of bacteria from TEM images and the PCR positivity could not exclude a contamination due to the massive presence of the bacterium. The possibility to mark the bacterium with an antibody would make this task much easier but, in 2008, when I started my work, the genome of *M. mitochondrii* was not sequenced and the right targets for antibody development were not available. Considering these limits in research activity and also the fact that we are dealing with an uncultivable intracellular bacterium, the study on *M. mitochondrii* capacity to infect humans and other vertebrates was based on a PCR screening.

The work developed in the first year of PhD research activity was aimed to investigate the presence of *M. mitochondrii* in samples of blood of different mammalian species, including humans, and in biopsies (intestinal tissues) obtained from humans.

The optimal experimental condition to lead a study of this kind would require the analysis of samples obtained from animals and humans with a well documented history of sporadic or continuous contact with ticks (i.e. signs of infestation/tick bites or clinical signs of tick-borne diseases). In this preliminary study these conditions were not guaranteed and we used all the

samples available from other studies. Finally, we investigate the presence of *Midichloria* DNA in vertebrates that can be parasitized by ticks or other hamathophagous insects, in order to provide indirect evidence on the hypothesis of *Midichloria* vector-borne transmission.

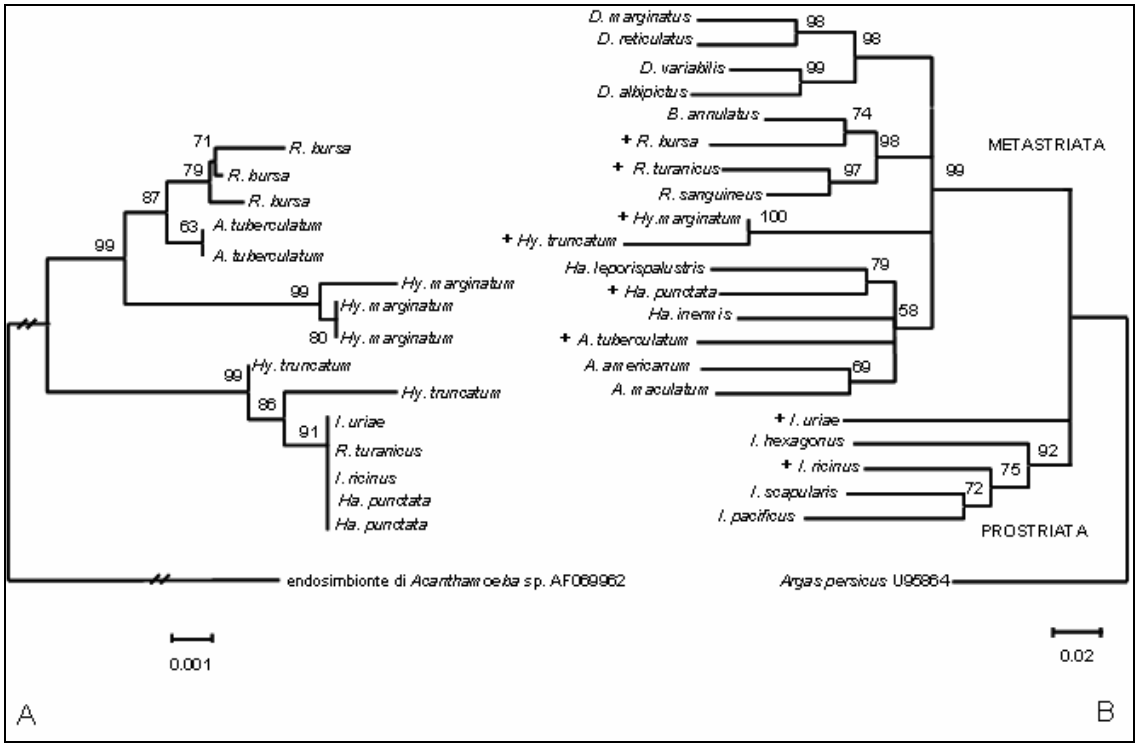


Fig. 4.1 - Comparison of the phylogeny of the symbionts *Midichloria* (left) and Ixodidae ticks (right). Figure A is a phylogenetic tree based on 16S rDNA sequences of the symbionts, indicated as tick hosts. Figure B shows a phylogenetic tree based on 12S rDNA gene sequences of ticks. The symbols + indicate ticks positive for *Midichloria* symbionts. The two trees are generated using the software MEGA (Neighbour Joining; Kimura correction) with 5000 bootstrap repetitions. The values below each node indicate bootstrap percentages. The bars indicate substitutions per site calculated. A sequence of endosymbionts of *Acanthamoeba* (MLO) and one of *Argas persicus* (Argasidae) were used as their outgroup. Other tests were carried out using different outgroups, and using different parameters for the construction of the tree, without finding significant differences.

4.2 Materials and methods

In our study we used DNA extracted from blood and tissues of vertebrates and humans, enrolled in other scientific investigations, according with DNA availability. We analyzed 152 samples of blood from 7 species of mammals (group A), 43 samples of blood from Italian cattle of 4 different breeds (group B), 100 samples of human blood (group C) and 80 bioptic intestinal tissue samples of humans (group D). Details on samples are reported in Table 4.1.

We performed a molecular screening for PCR detection of the presence of *M. mitochondrii* and MLOs; groups A and B represented the more interesting samples. Group A is constituted by vertebrates that are suitable hosts for several tick species (with the exception of cattle that are preferentially parasitized by tick of the genus *Boophilus*, and this tick species, so far, never showed positivity to *M. mithocondrii*).

Human biopses from group D might be expected to contain either contaminating bacteria from the gut content, as well as bacteria that are actually inside the mucosal tissue. The gut mucosa is indeed highly vascularized, and tissue-dwelling bacteria are known to invade this tissue, coming from both the circulation, as well as from the gut lumen. Considering that different forms of inflammatory bowel diseases and colitis in general are suspected to have a bacterial aetiology (or at least a bacterial component as the trigger of the first phases of immune-dysregulation), we thought that search for a novel potential infectious agent was interesting on this type of samples (Eckburg et al., 2005; Frank et al., 2007).

Biological material from groups A-B-C was processed for DNA extraction following standard procedures using a commercial kit (DNeasy Blood and Tissue, Qiagen) according with manufacturer's instructions. All human biopsies (group D) were treated for DNA extraction using RiboLyser Homogenizer (Hybaid) for mechanical tissue disruption. The biopsies were collected by gastroenterologists of three Italian hospitals for a study on presence and circulation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), etiologic agent of Johne's disease and subspected pathogen involved in the etiology of Crohn's disease in human gut. MAP is an extremely challenging bacteria to detect and in order to increase the possibility to extract DNA from the possible few bacteria in the intestines the mechanical disruptor was applied, with certain beneficial effects on the total DNA extraction of the huge diversity of gut bacteria. DNeasy Blood & Tissue commercial kit (Qiagen, Chatsworth, CA) was applied for DNA extraction (see below). Biopsy samples were thawed and 20-25 mg of tissue were transferred to single sterile screw-cap tubes. A volume of 180 µl buffer ATL and 20 mg of glass beads (150–212 µm, Sigma-Aldrich) were added to the vials containing the tissue samples. Tubes were then frozen in liquid nitrogen prior to mechanical disruption in a RiboLyser Homogenizer, then immediately cooled on ice for 5 minutes. Five µl of proteinase K (20 mg/ml) were then added, samples were incubated at 56° C for 3 hours and vortexed briefly at regular intervals. Then we followed kit (DNeasy Blood and Tissues, Qiagen) manufacturer's instructions eluting DNA in 100 ml nuclease-free water.

DNA was extracted both from full heparinized blood samples and from buffy coat derived from the blood samples according with the needs of the previous studies.

Molecular screening for *M. mitochondrii* was performed with a specific primer pair (INT-F and INT-R) previously published (Sassera et al., 2006) targeting the gene coding for the RNA of the small ribosomal subunit (16s rDNA) of *M. mitochondrii*. This primer pair appeared suitable for the screening of the other animal included in the work, but it led to problems on human samples for the formations of aspecific products (also of the expected size) probably due to the presence of alternative annealing sites in human genome. In order to overcome aspecificity PCR problem we considered a different approach.

N. individuals	Groups – Animal species	Provenience
	Group A (blood)	
45	*Equus caballus*	Lazio
15	*Canis familiaris*	Lombardia
47	*Canis familiaris*	Sicilia (Pantelleria)
13	*Bos taurus*	Piemonte
4	*Rubicapra rubicapra*	Lombardia (Lecco)
11	*Ovis aries*	Puglia
21	*Mus musculus*	Trentino
	Group B (blood)	
11	*Bos taurus* (Pezzata Rossa Italiana)	Lombardia
11	*Bos taurus* (Romagnola)	Emilia-Romagna
11	*Bos taurus* (Podolica)	Basilicata
10	*Bos taurus* (Agerolese)	Campania
	Gruop C (blood)	
50	*Homo sapiens*	Piemonte
50	*Homo sapiens*	Sardegna
	Group D (intenstinal biopsies)	
80	*Homo sapiens*	Northern Italy

Table 4.1 – In this table, the four groups of samples (A-B-C-D) are reported. The number of individuals and the Italian region of provenience on the individuals is also reported. Group D comprises patients hospitalized in the Hospitals of Monza and Vercelli resident in Northern Italy.

Since rickettsioses in human are quite rare we decided to design a series of degenerated forward and reverse primers targeting a conserved region of *Gyr*B gene, based on sequences of several member of the family Rickettsiaceae deposited in GenBank. *Gyr*B gene codify for the B sub-unit of DNA girase (Topoisomerase II). We designed the following primers to amplify fragments of *Gyr*B gene:

119F	5' – CCMGGYATGTATATTGGTGA – 3'
561F	5' – AGGAACGGAAGTWACWTTTTTGCC – 3'
913R	5' - ATATTCTCATRATAAWTATCATTCCA - 3'
1413R	5' - ACCSCCAGCAGAATCCCCYTC - 3'
1418R	5' - GCAGAACCTCCAGCSGAAT - 3''

Diverse combination of these forward and reverse primer pairs were applied to amplify the targer gene *Gyr*B. Finally we selected the primers 561F – 1418R as the best pair for our screening. These primers allow to amplify a 857 bp fragment of *Gyr*B gene and, although degenerate, these primers does not produce any aspecific PCR products.

4.3 Results

PCR screening was done by using two primer pairs. The *M. mitchondrii* specific primers (INTF-INTR) were applied for analysis on animals excluding humans, while the new selected primer pair (GyrB561F – GyrB1418R) were used in humans. We also obtained fragments of *Gyr*B sequences using a combination of different forward and reverse primers, thus showing that even other primer pairs worked, but they were not used for this molecular screening.

In group A we detetcted 8 PCR positive blood samples from 3 horses (*Equus caballus*), 4 dogs (*Canis familiaris*) from Pantelleria kennel and 1 sheep (*Ovis aries*). The sequecences obtained were not identical; they showed high similarity with other 16S rDNA sequences encountered in *Ixodes ricinus* (*Equus caballus*), *Riphicephalus bursa* (*Canis familiaris*) and *Hyalomma marginatum* (*Equus caballus* and *Ovis aries*).

M. mitochondrii DNA was never detected in any human samples (group C and D). The same negative results were obtained with catlle (group B).

4.4 Discussion

All the direct and indirect data collected until now suggest for *M. mitochondrii* the possibility to reach vertebrate hosts trough the tick vector, but the capacity to cause any disease seems improbable, since several new bacterial pathogens are detected and described recently using molecular tools. However, considering the three lines of evidence so far acquired (horizontal transmission between ticks; DNA presence in salivary glands; DNA presence in blood of tick hosts) we can hypothesize that *M. mitochondrii* is able to circulate among ticks, eventually passing through the infection of a vertebrate host. There is no need to suppose that *M. mitochondrii* causes pathology in vertebrates, since horizontal transmission between ticks could be ensured through a transient infection, or even during co-feeding on the same host (Nuttall et al., 1998).

Chapter 5

Tick-borne pathogens in diverse Italian areas

5.1 Introduction

In the last years the land exploitation has dramatically reduced the biodiversity of Italian environment and significantly modified the distribution and abundance of ticks. Recreational activities in wildlife/woodland areas, ecotourism and other outdoor activities such as hunting/hiking influence the frequency of tick bites in humans, hence the risk of tick-borne pathogens (TBPs) infections.

In our country, 36 tick species are present (7 Argasidae and 29 Ixodidae) but only few of them are frequently encountered in different environments and play a role in the epidemiology of tick-borne diseases (TBDs) (Genchi et al., 1999). For this reason, the correct identification of tick species is of fundamental importance for risk evaluation purposes.

Experienced tick taxonomists use morphological features (shape, size and color of body parts and other specific morphological keys) to identify species (Manilla, 1998). Classical morphological identification of species present limits. In fact, if a specimen is damaged or in an immature stage of development, even specialists may be unable to make identifications also due to lack of identificative keys for some taxa. DNA barcoding is a way to identify species using a short DNA target sequence. It is comparable to the way a supermarket scanner distinguishes products using the black stripes of the Universal Product Code (UPC). DNA barcoding allows to identify a species even from a reduced amounts of tissue. Besides, non-experts can obtain a quick identification of unknown specimens. Taxonomists can also use a new molecular tool to investigate complicate cases like identification of cryptic species. In our works we currently apply tick DNA based molecular identification when necessary.

The habitat preferred by ticks is represented by a rich diversity of herbaceous vegetation and shrubs, preferably with a cool and moist microclimate, even if it is not uncommon for their detection in areas with warm and dry climate, and sparse vegetation. The tick *I. ricinus* is the most abundant species in Northern Italy and it is the main vector involved in the transmission of several TBDs (see Chapter 4). The species *Dermacentor marginatus* is found frequently in Mediterranean areas with dense bush and tree cover. This tick is common in vegetation where oak and pine are prevalent, also in warm and dry environments. Larval stage usually feed on small mammals and birds, whereas adult ticks mainly feed on large mammals, commonly on sheep and cattle and occasionally also on humans. The brown dog tick, *Rhipicephalus sanguineus*, presents a world-wide distribution. This tick feeds on mammals, and dogs are the preferred host. In some areas, the population can reach pest proportion in houses and kennels. *Boophilus* spp. ticks parasitize cattle and other domestic and wild animals in many regions of

the world. This genus is unusual among ticks in that it can complete the entire life cycle on the same host. In our country, ticks represent an important veterinary problem. Domestic animals are frequently highly infested in some areas. Beside the direct damage that ticks produce on the host as ectoparasites in terms of blood deprivation, the capacity to vector a wide variety of microorganisms represents a considerable risk also for humans. Lyme disease and TBEV are endemic in various areas of Italy. Other TBPs frequently reported in ticks in Italy include *Anaplasma* spp., *Ehrlichia* spp., *Rickettsia* spp., *Babesia* spp. and *Theileria* spp. and *Coxiella burnetii*. Cattle could serve as the major reservoir for protozoa of the genera *Babesia* and *Theileria* while for bacteria of the genus *Anaplasma* cattle, dogs, sheep and goats may be the most important reservoir species. Bovine anaplasmosis, caused by *Anaplasma marginale*, is endemic in Sicily and results in economic loss to the cattle industry. The role of reservoirs played by wild animals in the epidemiology of TBDs is difficult to assess. More research activity will be necessary in order to delineate and implement measures of diagnosis and control of TBPs transmission to humans and animals. In the following text will be introduced some important zoonoses.

Tularemia

Tularemia is a serious infectious disease caused by *Francisella tularensis*. This bacterium, a Gram-negative, non motile, facultative intracellular cocco-bacillus, presents several subspecies with varying degrees of virulence. Among mammals, rodents and lagomorphs are the main sensitive hosts, also acting as reservoirs and amplifiers. *F. tularensis tularensis* (Type A) is commonly found only in North America, and is highly virulent for humans and rabbits. *F. tularensis holarctica* (Type B) occurs mainly in aquatic rodents (beavers, muskrats) in North America and in hares and small rodents in Eurasia. Type B bacterium is less virulent for humans compared to Type A. The primary vectors of tularemia are ticks, deer flies (*Chrysops* spp.), mosquitoes (*Aedes sticticus*, *Ae. vexans*, and *Ae. punctor*) and possibly other hematophagous arthropods. Tularemia is also reported to occur as a water-borne infection since these bacteria can survive for weeks to months in water and mud (possibly inside amoebae of different genera); outbreaks linked to water source were described in Russia and Italy and when environmental conditions favour sudden increases in rodent populations, as happened in post-war Kosovo in 1999-2000. *Francisella tularensis* is a highly infectious agent that is also known to infect humans during manipulation/slaughtering of carcass of wild animals or trough raptor bird nail scratch (Padashki et al., 2010).

Depending on the site of infection, tularemia has six characteristic clinical syndromes: ulceroglandular (75% of all forms), glandular, oropharyngeal, pneumonic, oculoglandular, and typhoidal.

Tularemia was first recognized in Italy, in the province of Pavia (Lombardia), in hares most probably imported from Eastern Europe (Rinaldi et al., 1964) while the first correctly diagnosed

cases in humans were described in 1966. Since then several cases of disease were diagnosed in humans in different provinces mainly in Northern and Central Italy, in general, linked to contacts with infected hares. In Tuscany and Liguria regions two extensive outbreaks linked to the consumption of water from uncontrolled aqueducts were reported (Greco et al., 1987; Mignani et al., 1988).

Rickettsiosis

Rickettsiosis/rickettsioses indicate a group of diseases caused by several species of *Rickettsia*, a genus of obligate intracellular bacteria. Several types of Rickettsioses are transmitted by ticks, but they can also be transmitted by other arhthropods such as fleas, lice and mites. Rickettsiae are widely distributed throughout the world, but human clinical manifestations vary along the geographical locations depending on the *Rickettsia* species. Rickettsiosis can be classified into two main groups: the spotted fever group (SFG), transmitted by ticks or mites and the typhus group (TG), mainly transmitted by lice or fleas. Rickettsial organisms are classified into several species based on their molecular characters. Around 20 species constitute the SFG group, many of them were identified and isolated in eukaryotic cell lines only in recent years; new species that may cause disease in humans continue to be identified. SFG groups comprise *R. rickettsii*, the agents of Rocky Mountains Spotted Fever (RMSF), *R. conorii*, the etiologic agent of “boutonneuse” or Mediterranean Spotted Fever (MSF) and African tick-bite fever. SFG Rickettsiae cause human disease in six continents. In Europe, *R. conorii* is probably the best known and diffused in the Mediterranean basin and frequently implicated in many cases of rickettsiosis in humans. This bacterium, transmitted by *Rhipicephalus sanguineus*, is endemic in several areas. MSF due to *R. conorii conorii* was considered to be, for long time, the only tick-borne rickettsial disease in Southern and Eastern Europe. However, in recent years, more species or subspecies within the SFG category of the genus *Rickettsia* have been described as emerging pathogens. Tick-borne agents include the two subspecies *R. conorii israelensis*, *R. conorii caspia, R. aeschlimannii*, *R. slovaca*, *R. sibirica R. mongolitimonae* and *R. massiliae* (Brouqui et al, 2007). In addition to these organisms, some other rickettsiae, suspected or proven to cause human disease, have been detected in ticks, including species that are well known to bite humans (Raoult & Roux, 1997; Estrada-Pena & Jongejan, 1999). *R. helvetica* and *R. monacensis*, which are frequently associated with and transmitted by *I. ricinus* in northern and Eastern Europe, and whose pathogenic potential is strongly suspected, have been detected in humans (Fournier et al., 2004).

In the time span between the years 2000 - 2005 in the Emilia-Romagna region 49 cases of tick-borne rickettsiosis were reported by Italian Ministry of Health (Ministero della Salute web site: http://www.salute.gov.it/ricoveriOspedalieri/ricoveriOspedalieri.jsp). The majority of these human rickettsiosis cases were diagnosed as MSF, but probably, a portion of these cases were caused by other SFG rickettsiae (Ciceroni et al., 2006).

Anaplasmosis

The genus *Anaplasma* contains tick-borne pathogens which are transmitted to vertebrates by tick bite; transovarian or vertical transmission does not appear to occur. Anaplasmosis is of growing concern in human and veterinary medicine as an emerging tick-borne pathogen in Europe and in North America, causing an acute disease in several species. *Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae) is the causative agent of the human granulocytic anaplasmosis (HGA) in humans, tick-borne fever of ruminants, and equine and canine granulocytic anaplasmosis (Dumler et al., 2001).

A. phagocytophilum infects a wide range of host including rodents, ruminants, birds, felids, horse and donkeys, dogs and humans (Estrada-Pena et al., 2008). The broad geographic range and the host tropism diversity of *A. phagocytphilum* suggest the presence of complex vertebrate-tick interactions. Several epidemiological studies carried out in Europe have shown molecular evidence of *A. phagocytophilum* in questing ticks and mammals, suggesting in particular the important role of wild ungulates in the maintenance of the infection in nature (Carpi et al., 2009).

Q Fever

Q fever is a human disease caused by infection with *Coxiella burnetii*. This bacterium is an obligate intracellular pathogen that affects vertebrates. This organism may be found in cattle, sheep, goats and other domestic mammals, including cats and dogs. The infection results from inhalation of a spore-like cell, and from contact with the milk, urine, faeces, vaginal mucus, or semen of infected animals. This disease is also vectored by hard ticks (Toledo et al., 2009). Just few bacteria are able to trigger the infection in humans. The incubation period is 9–40 days. The most common manifestation is mild flu-like symptoms with abrupt onset of fever, malaise, profuse perspiration, severe headache, myalgia (muscle pain), joint pain, loss of appetite, upper respiratory problems, dry cough, pleuritic pain, chills, confusion and gastro-intestinal symptoms such as nausea, vomiting and diarrhea. The fever lasts approximately 7 to 14 days. Infected individuals might exhibit no symptoms.

Among animals cattle, goats and sheep can serve as reservoir for the bacteria. Infected animals may show respiratory signs such as pneumonia, but also abortion and infertility. Severe systemic signs such as anorexia and fever may occur concurrently.

5.2 Materials and methods

From early spring 2008 to autumn 2011 more than 3000 ticks (larvae, nymphs, and adults) were collected by dragging or directly on the host and a subset of them (mainly nymphs and adults) were processed in our studies of molecular epidemiology (see below). The sampled individuals were stored in 96% ethanol and identified according to standard taxonomic keys (Manilla 1998). When individuals were not recognizable at species level due to lack of parts of idiosoma/capitulum, when the specimens were damaged or not well preserved we applied a molecular identification based on a fragment of the sequence of the gene coding for cytochrome c oxidase subunit I (COI) to identify specimens with a barcoding approach.

The majority of samples were individually processed and only in few investigations on presence/absence of pathogens in a determined area we processed together pools of individuals. Each individual/pool was broken apart with a sterile needle/pestle and then subjected to DNA extraction by using the Illustra Tissue & Cells Genomic Prep Mini Spin Kit (GE Healthcare, Little Chalfont, UK).

Specific primers were selected and included in official protocols applied for molecular diagnosis at Istituto Zooprofilattico Sperimentale della lombardia e Emilia Romagna (IZSLER – Sezione di Pavia). The molecular screening for presence of bacterial pathogens was performed using the primer pairs reported in Table 5.1 (next page).

In the following text I describe in details the PCR performed for screening on presence of *Midichloria mitochondrii* in ticks. In our molecular screening for pathogens, specific PCRs were experimentally optimized for each microorganism.

PCR amplification conditions for molecular detection of *Midichloria mitochondrii* were performed in 20 ml buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) with 0.2 mM each deoxynucleoside triphosphate, 20 pmol each primer, 1 U Taq polymerase (Invitrogen-LifeTechnologies) and 1 ul DNA sample. The thermal protocol was 95°C for 15 min (time of activation of Hot Stat AmpliTaq Gold Polymerase), followed by 35 cycles at 95°C for 30 sec, 56°C for 30 sec, and 72°C for 60 sec, followed by a final extension at 72°C for 10 min.

In order to identify the bacterial pathogens at species level we sequenced the PCR amplicons. Amplified fragments were sequenced by an automated fluorescence-based technique following the manufacturer's instructions (ABI-PRISM 3130 Genetic Analyzer, Applied Biosystems, Foster City, CA).

Code	Forward Primer	Reverse Primer	Target region	Product Length
1	INT-F 5'-GTACATGGGAATCTACCTTGC-3'	INT-R 5'-CAGGTCGCCCTATTGCTTCTTT-3'	16S rDNA *M. motochondrii*	1070bp
2	BBDL5-fw 5'-ATGCACACTTGGTGTTAACTA-3'	BBDL5-rev 5'-GACTTAATCACCGGCAGTCTTA-3'	16S rDNA *Borrelia* sp.	359bp
3	23Sa-fw 5'-TAAGCTGACTAATACTAATTACCC-3'	23S3-rev 5'-CGACCTTCTTCGCCTTAAAGA-3'	ITS2 (direct) *Borrelia* sp.	428bp
4	Bsl1-fw 5'-CTGCGAGTTCGCGGGAGA-3'	Bsl2-rev 5'-TCCTAGGCATTCACCATA-3'	ITS2 (nested) *Borrelia* sp.	362bp
5	ompA 190.70 5'-ATGGCGAATATTTCTCCAAAA-3'	ompA 190.701 5'-GTTCCGTTAATGGCAGCATCT-3'	*Omp*A *Rickettsi sp.a*	712bp
6	gltA Rpcs877p 5'-GGGGGCCTGCTCACGGCGG-3'	gltA Rpcs1258r 5'-ATTGCAAAAAGTACAGTGAACA-3'	*Glt*A *Rickettsiasp.*	348bp
7	F5 5'-CCTTTTTGAGTTTCGCTCC-3'	F11 5'-TACCAGTTGGAAACGACTGT-3'	16S rDNA *Francisella* sp.	1050bp
8	Tul4-435 5'-GCTGTATACTCATTTAATAAACTGCTG-3'	Tul4-863 5'-TTGGGAAGCTTGTATCATGGCACT-3'	Tul4 gene *Francisella* sp.	327bp
9	TRANS1 5'-TATGTATCCACCGTAGCCAGTC-3'	TRANS2 5'-CCCAACAACACCTCCTTATTC-3'	*Transposon-like region* *Coxiella burnetii*	687bp
10	Msp4 5'-ATGAATTACAGAGAATTGCTTGTAGG-3'	Msp5 5'-TGAAAGCAAATCTTGCTCCTATG-3'	*Major Surface protein 4* *Anaplasma*	849bp
11	LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3'	HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	*COI mtDNA* *Arthropods*	710bp

Table 5.1 – List of primer pairs used in the study on bacterial pathogens in different tick species. The sequence of the primers and the length of the expected products are reported.

5.3 Results

The majority of ticks analyzed in our works were collected by dragging (Fig 5.1). Our research activity indicate that both in Alpine forests and in wild areas of Pianura Padana *I. ricinus* is the species more frequently encountered, often with high population density. Occasionally, adult individuals of *I. hexagonus*, *Dermacentor marginatus* and *Rhipicephalus sanguineus* were also collected. In some locations of the Alpi Marittime and on the mountain areas from 'Riviera di Levante' to forests down the coast of Tuscany, individuals (adults and nymphs) of the genus *Haemaphysalis* can be massively present to be comparable with the density showed by *I. ricinus* in other Alpine/Pianura Padana areas. Other tick species are collected only sporadically and can be considered 'not abundant' in the investigated areas in Northern and Central Italian regions. Molecular identification of unrecognizable tick specimens (DNA barcoding) allows us to both create a data set of sequences representing the genetic variability within a single species and to solve doubtful identification.

We investigate the presence of pathogens in the collected ticks emphasizing different aspect of the molecular epidemiology of these microorganisms. We periodically screened ticks for the presence of *M. mitochondrii*, but so far, we obtained results in agreement with previous investigations. For this reasons these results were not included in the following studies.

Different aspects must be considered during the process of data analysis and interpretations of results. Engorged and unengorged ticks might present various degree of potential risk factors for TBD transmission. Prevalence of infection with bacterial pathogen in nymphs might be linked to the risk of TBD infections in vertebrates, while adult ticks collected on animals will not necessitate an ulterior blood meal on a new host, but females can vertically transmit bacteria to progeny.

In the following section, it will be presented the results of tick research activity emphasizing the epidemiological aspects of some TBDs.

Fig 5.1 – Two operators working for collection of *I. ricinus* ticks by dragging on vegetation in Parco del ticino, Lombardia region, Italy. White protective clothes and insecticidal repellents were used to avoid tick bites on the skin.

5.3.1 Lyme borreliosis in the Pianura Padana, Lombardia (Study n.1)

In late spring 2008, a forestry worker of a natural park West of Milano (Ticino Park) in the Pianura Padana was treated for cutaneous mycosis on the basis of an erythematous rash on an arm. The first diagnosis for cutaneous mycosis resulted to be wrong. Subsequent clinical examination and serologic analyses led to diagnosis of Lyme disease. After this first case of Lyme, a retrospective analysis was conducted on forestry workers in the area. Two workers reporting the appearance of erythematous rash in the previous months underwent serologic analyses and resulted positive for borreliosis.

After these clinical cases, we decided to investigate different areas of the park for the presence of ticks. During May–August 2009, a total of 1,094 *Ixodes ricinus* (576 larvae, 507 nymphs, and 11 adults - 7 males) were collected by dragging. Ticks were collected in rural or suburban areas of the municipalities of Somma Lombardo, Lonate Pozzolo, Magenta, and Pavia (Figure 5.1). These sites are located along the Ticino River, which crosses the counties of Varese, Novara, Milano, and Pavia. A subset of 234 collected nymphs of *I. ricinus* was screened by PCR for Lyme borreliae. The presence of the tick species *Dermacentor marginatus* and *Riphicephalus sanguineus* was also reported in the area. PCR screening for *B. burgdorferi* sensu lato (s.l.) was performed using primers BBLD5′ and BBLD3′ for 16S rDNA (1). Positive samples were examined by using a nested PCR protocol for the 23S–5S rDNA spacer region (2-3) of *B. burgdorferi* (s.l.). In addition, all 11 adults and pools of 10 larvae from Somma Lombardo, Lonate Pozzolo, Magenta (collection sites A, B, and C in Figure 5.1) were screened for *B. burgdorferi* s.l. by using the same procedure.

B. burgdorferi s.l. was detected in 42 (18%) of the 234 nymphs analyzed. One of the 7 adult males was positive; none of the 4 adult females and none of the pools of larvae were positive. The PCR products obtained from the 42 positive nymphs and from the adult male were sequenced by using ABI technology (Applied Biosystems), and the sequences were searched for homology using BLAST on the National Center for Biotechnology Information non redundant database (www.ncbi.nlm.nih.gov/BLAST). 16S rDNA sequences confirmed identification as *B. burgdorferi* sensu lato, whereas rDNA spacer sequences ITS2 showed the highest scores for *B. afzelii* (36/43) and *B. lusitaniae* (7/43) (Figure 5.2). Six rDNA spacer sequences representing the entire variability were deposited in the European Molecular Biology Laboratory database (FN658703–FN658708), then aligned with homologous sequences of *Borrelia* species by using MUSCLE. Neighbor-joining phylogenetic analysis, using SeaView 4.2, confirmed placements of the obtained sequences into the clusters of *B. afzelii* and *B. lusitaniae*.

The 3 patients (workers in the area) resulted positive for *Borrelia* infection confirmed trough the results of the ELISA received the recommended therapy.

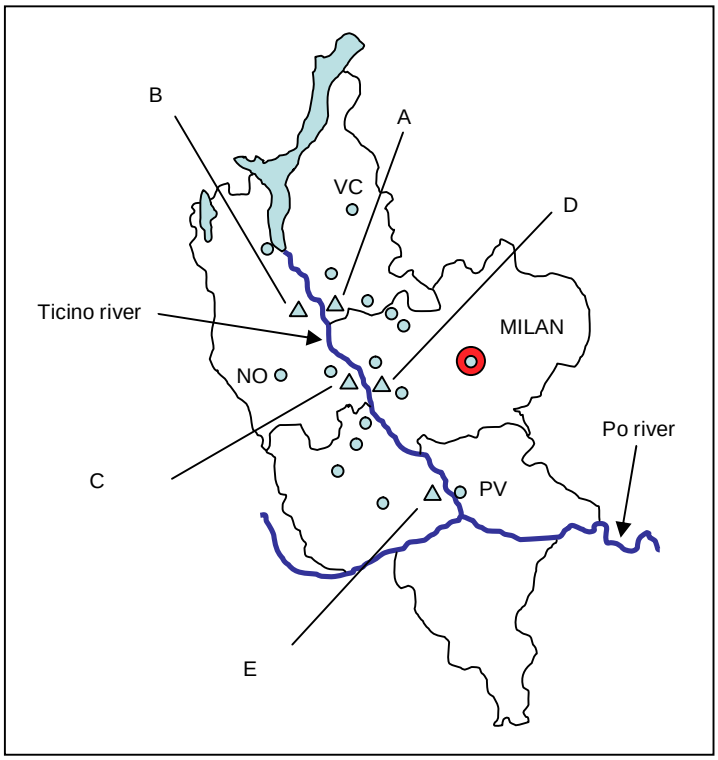


Fig 5.2 -Collection sites (triangles A–E) of *Ixodes ricinus* ticks in the counties of Milano, Pavia, and Varese, Po River Valley, Italy.
Ticks were collected in rural or suburban areas of the municipalities of Somma Lombardo (collection site A 1; 0/19), Lonate Pozzolo (B 0; 0/19), Magenta (C 11; 4/100, D 24; 3/100), and Pavia (E 0; 0/6). The 3 numbers in parentheses for each collection site indicate number of tick nymphs positive for *Borrelia afzelii,* number of nymphs positive for *B. lusitaniae,* and number of nymphs examined by PCR. The adult specimen positive for *B. afzelii* was collected at site D. Empty circles indicate towns with 10,000–50,000 residents; coloured circles indicate towns with >50,000 residents. NO, Novara; PV, Pavia; VA, Varese. Milano and surrounding areas residents = 4.000.000 persons.

Heavily populated flat regions of Pianura Padana were not considered risk areas for Lyme Disease before this study. Besides, *I. ricinus* ticks had never been reported in these areas in the Lombardia region, one of the most important industrial districts in Europe and an area of intensive agriculture and livestock breeding. Human population density is high; >6 million persons reside in Milano and surrounding counties. In Italian areas where *I. ricinus* ticks are known to be endemic, physicians have appropriate awareness of the risks from tick bite and Lyme disease; outside these areas, awareness is not adequate.

Ticino Park was created to defend the Ticino River and the diverse natural environments of the Valle del Ticino from the attacks of industrialization and urbanization increasingly invasive. The consortium that manages the park, which includes 47 municipalities and 3 provinces, governs a territory of over 91 000 hectares, using a different system of protection for natural areas, agricultural and urban areas and trying to enhance not only the environment but also the historical, architectural, archaeological territory. Ticks were not previously included in the list of animals to manage and control. Our study show that ticks collected from 3 locations along the Ticino river harbored Lyme disease borreliae. In addition, we detected evidence for *B. burgdorferi* sensu lato infection in 3 persons at risk for tick bite who work in the area. One location from which we collected *I. ricinus* ticks (location E) is in the suburban area of Pavia, a densely populated town. The risk of contracting Lyme disease in Italy is thus not limited to mountains and wild areas but extends to the plains, such as the Pianura Padana, and possibly reaches suburban areas. The characteristics of the territory of the sampled area, although in heavily populated counties, are ecologically compatible with the presence of *I. ricinus* ticks

because of the woods and bushes, corridors of vegetation connecting the plains and the river banks to mountain areas, and presence of micro rodents. In addition, the area along the Ticino river that includes collection locations C and D (where most tick specimens were sampled) is populated by roe deer (*Capreolus capreolus*), whose role as a major host for *I. ricinus* ticks is well known. These ungulates were introduced into Ticino Park area 2 decades ago.

The Lyme disease case initially diagnosed as a mycosis and the 2 undiagnosed cases among forestry workers in the area west of Milano suggest that awareness of risks associated with tick bite probably is not adequate among physicians in the region. Moreover, before our investigation, visitors of the wild areas along the River Ticino were not adequately informed about the presence of ticks. Our report provides a basis for supplying proper information to health institutions and physicians in the area, as well for helping park administrators adopt proper precautions.

5.3.2 Parco del Ticino: *Ixodes ricinus* population density and bacterial threats (Study 2)

After the previous report on Lyme disease in the Pianura Padana, in 2008, we continued the investigation on ticks and tick-borne diseases in the area. Inside the territory of the Ticino Park, 15 sampling zones (1ha) were selected; the dragging method was applied to collect ticks. In order to evaluate tick population density we established an abundance value sets on the number of nymphs collected from a single operator in one hour sampling with a dragging tablecloth (1x2 m). We decided to set our quantitative method on nymphs, since this stage present the more casual distribution compared with adults and larvae that normally are more aggregated in limited places. Larvae might remain amassed to quest in proximity of the hatching site (where eggs were laid) while adults might became abundant in the rest sites, nests or burrows of the vertebrate hosts. On the contrary, the intermediate stage (newly molted questing nymphs) derive from engorged larvae, dropped off the host after the blood meal, thus presenting a more casual distribution. We arbitrarily decided to consider an area highly infested when more than 30 nymphs were collected in 1h, while where the presence of *Ixodes ricinus* remain inferior to this threshold we just report its presence (see Fig. 5.3 and Fig 5.4 for details).

The 15 sampling zones presented various degree of urbanization, from wild environments with low degree of human presence and rural buildings to antropized districts in the proximity of densely populated cities and areas (Pavia, Magenta, Abbiategrasso, Aereoporto Internazionale Malpensa). The Ticino Park represents an excellent panorama of wildlife in the area of the Po Valley. Forty-eight species of mammals permanently live in the Ticino Park. The carnivorous predators are represented by fox (*Vulpes vulpes*), badger (*Meles meles*), and other mustelids (*Marets martes*, *Martes foina*, *Mustela putorius* and *Mustela nivalis*) to whom is given the key role in regulating the biological equilibrium. The Lombard Park is also pursuing a plan for the re-introduction of the Eurasian otter (*Lutra lutra*). After two centuries of absence, the ungulates have reappeared in the woods of Ticino. These are represented by the roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*). The latter has colonized the south/central part of the woods following the escape of some animals by a private fence near Besate, in 1974. Their number, in a short time become excessive, causing serious damage to crops: the boars are often encountered in agricultural fields, causing severe complaints of farmers and forcing the authority of the park to operate selective-killing. Another re-introduction, started more than twenty years ago, has the deer as its protagonist. In different periods several dozen of individuals were released in the forests of the central area of the park. Subsequent censuses have confirmed the success of this initiative. All this vertebrates are potential hosts for *Ixodes ricinus*.

In spring/summer 2011 we collected 1245 *I. ricinus* ticks (546 larvae – 684 nymphs – 9 females and 6 males); these samples were processed for DNA extraction (DNeasy Blood and Tissue kit, Qiagen) and successively screened for PCR detection of pathogens. No other tick species were collected in this period, but previous sampling campaign allowed to collect specimens of

Dermacentor marginatus and *Riphicephalus sanguineus*, respectively from areas close to sampling sites D and C-L (see Fig 5.4). The tick *D. marginatus* is frequently encountered on wild boars. This study (n.2) was focused on bacteria of the genus *Rickettsia* and the dangerous pathogen *Francisella tularensis*. On a subset of 10 pools of nymphs (10 individuals each) and 100 individual nymphs, we performed a molecular screening by using two primer pairs (5-6) targeting rickettsial genes *omp*A and *glt*A and primer pairs (7-8) targeting 16s rDNA and *tul*4 gene of *Francisella tularensis.* Tick-sampling campaigns allow to determine a zone in the center of Ticino Park (F) with a population density of *I. ricinus* particularly elevated. Besides, in other four sampling sites (G-H-L-M) in the proximity of F, *I. ricinus* ticks were collected. Also the presence of this species is reported for one locality in the Northern part (A) and one in the Southern part of the territory considered in this study.

Our results show that *I. ricinus* is present on the entire territory of the Ticino Park, on both sides of the Ticino river with variable population density (Fig 5.4). The tick *I. ricinus* is certainly more abundant in the central area, probably due to a series of favourable factors. First of all, one of the main host for adult ticks, roe deer (*C. capreolus*), is present with high number of individuals that could guarantee the completion of the tick life cycle in short time. Besides, the central area is quite rural with limited urbanization level and the suitable habitat of *I. ricinus* is common (Fig 5.3 and Fig 5.4): under forest substrate vegetation (*Rubus* sp. and *Hedera helix*) that also constitute an important part of the diet of roe deer (Tixier et al., 1997), with high degree of humidity assured by several irrigation canals and torrents.

Fig 5.3 – *Ixodes ricinus* and roe deer are frequent in woods with abundant under forest vegetation (*Rubus sp.* and *Hedera helix)* and high degree of humidity (A). On the contrary, in dry enviromments (meadows, pastures and bushes) and pinewood (*Pinus sylvestris*) the presence of *Ixodes ricinus* was not reported.

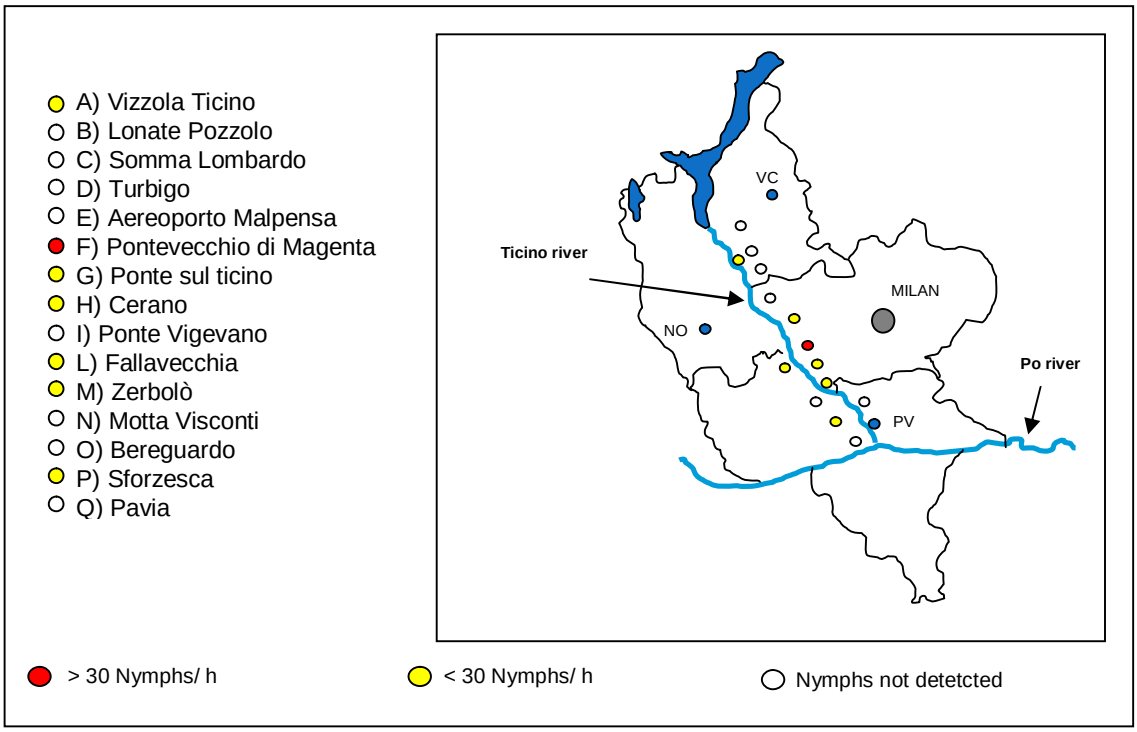


Fig 5.4 – The 15 sampling sites along the Ticino river are reported in the map. The Ticino Park extends in four counties (VC, NO, MI PV). The area were the presence of I. ricinus is very high is marked by a red circle. Yellow circles represent the sites were I. ricinus was collected. White circles sign the points where I. ricinus presence was not reported during this study.

Molecular screening on collected ticks detected PCR positivity for both *Rickettsia* sp. and *Francisella tularensis*. In the 100 individual nymphs we registered a 45% positivity to *Rickettsia* sp. (primer used: 5-6 page 64) that after sequencing of positive amplicons allow to determine the presence of *R. monacensis* (18) and *R. helvetica* (21), while the remaining samples did not produced readable sequences (6): Both *Rickettsia* species belong to Spotted Fever Group (SFG) and were recently included in the list of bacteria causing rickettsiosis in humans (Fournier et al., 2000; Jado et al., 2007; Radulovic et al., 2011). We obtain an higher positive response for *Rickettsia* sp. in pooled samples (8/10). Three of this pools and two individual nymphs also resulted positive for *Francisella tularensis* subsp. *holarctica*. This result evidences that SFG rickettsiae and *F. tularensis* (type B) need to be included in the list of TBDs circulating in ticks and consequentially in vertebrates in the Po river valley.

5.3.3 Bacterial pathogens transmitted by ticks in an Alpine area in Piemonte (Study n.3)

Various tick-borne pathogens occur in the Eastern mountain regions of Italy, where Lyme disease and TBE virus, transmitted by *Ixodes ricinus*, are regularly diagnosed in humans (Cinco et al., 2008). On the contrary, the epidemiology of Lyme and other TBDs of veterinary and medical relevance, remains still to be defined in the North-western part of Italy where just few data are available and a new area (Parco del Ticino) endemic for the presence of Lyme-causing borreliae has been recently described. Medical awareness on distribution and prevalence of tick-borne diseases is also not adequate.

Recently, in Northern Piemonte (valle Ossola), serological analysis based on detection of specific antibodies of *Coxiella burnetii* showed positivity for this microorganism in wild animals (Viganò et al., 2008). Recently, concern for the circulation of Q fever etiologic agent in diary herds, aroused due to commercialized raw milk in the area.

Valle Ossola, located in the territory of Verbano Cusio Ossola (VCO), present diverse wild environments comprehending two protected areas (Parco Naturale Veglia-Devero and Parco Nazionale Val Grande); wild animals are abundant and *Ixodes ricinus* is present with consistent population density as well. Cattle and other domestic animals might be exposed to this tick species and vectored pathogens.

We developed a two years study (summer/autumn seasons 2009-2010) on *Ixodes ricinus* and associated pathogens in the selected area. Engorged ticks were collected on wild ungulates and domestic dogs, while unengorged ticks were collected by dragging in four different locations. A total of 130 adult *I. ricinus* (24 males and 116 females) were collected on hunter-killed roe deer (34 *Capreolus capreolus*) and red deer (3 *Cervus elaphus*), 313 *I. ricinus* (7 adult females and 3 male, 171 nymphs and 132 larvae) were sampled with the dragging method in four different areas (named after the closest rural village: Pioda, Premone, Simbo and Trontano) in Alpine forests. Finally, 38 ticks of different species (9 males and 29 females) were collected on domestic dogs in the area.

We processed the samples for DNA extraction (as described at the beginning of the chapter) and performed a molecular screening for *Coxiella burnetii* (9), *Francisella tularensis* (7-8), *Rickettsia* spp. (5-6) and *Borrelia burgdorferi* s.l. complex (2-3-4); numbers in brackets indicate primer pairs used.

We processed all the ticks collected on wild ungulates (130), 12 *I. ricinus* found on dogs and 40 nymphs collected by dragging from each of the four localities, for a total of 160 ticks (Table 5.2 – Table 5.3). Our PCR results show that bacteria of the genus *Rickettsia* and *Borrelia* are frequently present in *Ixodes ricinus* from VCO; they were detected in both engorged (collected on wild and domestic animals) and unengorged ticks (collected by dragging). *Francisella tularensis* was detected only on ticks collected on 4 ungulates (*C. capreolus*). The etiologic agent of Q fever, *C. burnetii*, was never detected in these ticks.

Respectively, 23 and 9 ticks out of a total of 160 ticks collected by dragging in the four localities resulted positive for *Rickettia* (14.3%) and *Borrelia* (5.6%). In ticks collected on hunter-killed wild animals *Rickettsia* (47/130=36.9%) and *Borrelia* (19/130=14.6%) bacteria presented an higher prevalence when compared with ticks collected by dragging. See Table 5.2 and 5.3 for details.

Pathogen prevalence derived from engorged ticks can be biased due to acquisition of the microorganism from infected vertebrates, from episodes of co-infection or co-feeding (without passing trough the infection of the host).

Six ticks collected on four roe deer showed positivity to *Francisella tularensis holarctica* (6/130 = 4.6%)

Finally, ticks collected on dogs belonged to two different species: *Riphicephalus sanguineus* (24) and*Ixodes ricinus* (12). We just included in this study these last 12 ticks; 4 females showed positivity for *Rickettsia* sp. (Table 5.2), one of these ticks also resulted positive for *B.afzelii*.

All positive samples were sequenced to determine the pathogen at species level, but few amplicons produced un-readable sequences, possible due to co-infections with more than one *Rickettsia* species at least in a portion of cases.

Host number	**Hosts or (dragging)**	**N. positive / N. examined ticks**	**Positive samples (%)**	***Rickettsia* species**
3	Roe deer	3/8	37.5%	*R. monacensis (1) R. helvetica (2);*
34	Red deer	45/122	36.8%	*R. monacensis (15); R. helvetica (19); Rickettsia sp (11)*
4	Dogs	4/12	33.3%	*R. monacensis* (3); *Rickettsia sp.* (1),
	(Premone)	1/40	2.5%	*R. monacensis* (1)
	(Trontano)	8/40	20%	*R. monacensis* (4); *R. helvetica*(4)
	(Simbo)	6/40	15%	*R. helvetica* (2); *R. monacensis* (4)
	(Pioda)	8/40	20%	*R. helvetica* (4); *R. monacensis* (4)

Table 5.2 – Details on the results of the PCR screening for *Rickettsia* species in ticks collected by dragging and on the vertebrate hosts.

Host number	Hosts or (dragging)	N. positive / N. examined ticks	Positive samples (%)	*Borrelia* species
3	Red deer	3/8	37.5%	*B. afzelii* (2); *B. garinii* (1)
34	Roe deer	16/122	13.1%	*B. valaisiana* (3); *B. garinii* (2); *B. afzelii* (11)
6	Dogs	1/12	8.3%	*B. afzelii* (1)
	(Premone)	0/40	-	-
	(Trontano)	0/40	-	-
	(Simbo)	2/40	5%	*B. valaisiana* (2)
	(Pioda)	7/40	17.5%	*B. garinii* (5); *B. burgdorferi ss* (2)

Table 5.3 – Details on the results of PCR screening for *Borrelia burgdorferi* sensu latu complex bacteria in ticks collected by dragging and on the vertebrate hosts.

Our study reported the presence of *R. monacensis* and *R. helvetica* in the VCO area. These SFG rickettsiae represent a potential threat for human health. *R monacensis* is frequently associated with *I. ricinus* in Mediterranean countries and present a wide range of variability (Jado et al., 2007). Both species were isolated from human patients with febrile illness (Fournier et al., 2000; Nilsson et al ., 2010). Remarkably, this investigation on ticks and TBPs allows to report the contemporary presence of the three main genospecies causing Lyme disease (*B. afzelii*, *B. garinii* and *B. burgdorferi* ss) in the VCO territory. Pathogenicity for human remains uncertain for *B. valaisiana*, but this genospecies had been isolated from patients with clinical signs of neuroborreliosis (Diza et al., 2004). The etiological agent of tularaemia also circulate in the same area. Further analysis will be needed to provide precise epidemiological data on TBPs transmitted by ticks in the VCO area. In fact, the pathogens reported in Ossola valley may also be diffused in other valleys of the Alps for which updated data are not available.

5.3.4 SFG Rickettsiae and *Anaplasma phagocytophilum* in Emilia-Romagna (Study n.4)

Rickettsiae are vectored via tick salivary secretions and are maintained transtadially and transovarially in ticks. Several tick-borne rickettsiae are causative agents of human or animal diseases (Parola et al., 2005). *Anaplasma phagocytophilum* (previously named *Ehrlichia phagocytophilum*) is a Gram-negative bacterium presenting an unusual tropism to neutrophils and causes a disease known as HGA in humans (see above).

Roe deer, European brown hare and wild boar are among the most important big game species in Italy and in other European countries and their abundance and density may play a role in the TBPs transmission and maintaining cycle. (Estada-Pena et al., 2008). Abundance of wild animals in the Emilia-Romagna region suggests a high exposure-risk to tick bite and trasmission of pathogens to humans and other vertebrates. Hence, the aim of this study is to determine the prevalence of Spotted Fever Group (SFG) rickettsiae and *Anaplasma phagocytophilum* in ticks collected on wild animals in order to report TBPs circulating between vector ticks and vertebrates hosts in the Emilia-Romagna region.

In our study, ticks were collected from roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), red deer (*Cervus elaphus*) and European brown hare (*Lepus europaeus*) hunted in the hilly and mountain areas of the region. Animals were sampled after being hunter-killed during the hunting season or during selective hunting in 2008, the locations where the animals were killed are reported in Fig. 5.5. All the visible ticks on the animals were removed and identified following taxonomic standard keys (Manilla 1998). DNA extraction was performed from single adult ticks.

Two rickettsial fragments of the *gltA* and *omp*A genes were targeted in a PCR screening (5-6). For *Anaplasma sp.* DNA detection we used previously published primers (10) which amplify a 849 bp fragment of the major surface protein 4 (*msp*4) gene of *A. phagocytophilum*, *A. marginale*, *A. centrale*, and *A. ovis.* (De la Fuente et al., 2005).

To identify infective species, positive amplicons were sequenced and compared with sequences in the National Central for Biotecnology Information (NCBI) GenBank databases. Phylogenetic and molecular evolutionary analyses were conducted using the neighbour-joining method with MEGA software version 4 (Tamura et al., 2007).

A total of 353 adult ticks (243 *I. ricinus* and 110 *D. marginatus*) were removed from 75 hunted wild animals, 38 roe deer , 32 wild boar, 3 red deer and 2 European brown hare. Animal came from all the Emilia–Romagna provinces except Piacenza and Rimini (Fig 5.5).

Ticks collected were mostly adults (197 females –156 males) (90%) and only 39 were nymphs (*I. ricinus*); no larval stage were found in hunted animals. All 353 adult individuals were processed, but nymps were not included in this work.

I. ricinus tick was the most frequently detected species in our habitats (69.9%). *D. marginatus* resulted to be much more frequent in wild boar (27/28 sampled animals) and was found infesting hare only once. A total of 43 animals carried positive ticks but on these wild animals was never reported the presence of the two different tick species on the same animal.

PCR targeting the *Rickettsia gltA* resulted positive from 89 of the 353 individuals examined. The amplifications obtained from *gltA* and *ompA* primers were further sequenced and homology searches on the data bases sequences confirmed that they originated from four *Rickettsia* species: *R. monacensis, R. helvetica, R. slovaca, R. raoultii*. (Table 5.4).

R. helvetica is one of the few SFG species in which the *ompA* primer set used in this study does not led to amplification of a PCR product (Beati et al., 1993). Therefore the identification of this species was based only on *gltA* gene sequences.

The presence of rickettsiae was detected in 23.45% of *I. ricinus* individuals (57/243) and in 29.09% of *D. marginatus* individuals (32/110) (Table 5.6).

In the majority of cases, the ticks collected on the same animal were infected by a single *Rickettsia* species; only in four cases we found animals infested by ticks that harboured two *Rickettsia* species: (2 roe deer with *I. ricinus* ticks infected both with *R. helvetica* and *R. monacensis* and 2 wild boar with ticks infected with *R. slovaca* and *R. raoultii*).

R. slovaca was detected in *D. marginatus* ticks parasitizing wild boar from Bologna (4/19), Parma (1/10), Modena (11/42) and Ravenna (9/39) provinces. *R. raoultii* was found infecting *D. marginatus* from wild boar in Bologna (3/19) and Ravenna (2/39) provinces (Table 5.4). The sequences obtained for *R. slovaca* and *R. raoultii* did not show any intraspecific variation, and the *omp*A partial sequence that we generated showed 100% identity with the sequences available in the data bases for these species.

The *gltA* and *ompA* gene sequences from the *I. ricinus* samples showed the presence of *R. helvetica* (8 samples) and *R. monacensis* (33 samples). *R. helvetica* DNA was present in 3 ticks from Bologna, 1 tick from Parma and 4 ticks from Reggio Emilia, all removed from roe deer. *R. monacensis* was found in all the provinces sampled apart Forlì-Cesena, and in all animal species, apart wild boar.

Partial sequences of *ompA* gene of all the positive samples were found closely related with *Rickettsia monacensis* strain Munich, with a nucleotide sequence identity of 99%. Unfortunately, in 18 cases we were not able to identify the *Rickettsia* species.

A. phagocytphilum DNA was found in *I. ricinus* ticks removed from a roe deer in Parma province (N=3), *I. ricinus* from roe deer from Ravenna (N=1). Sequences from the *msp*4 gene show an 99% identity with the *A. phagocytophilum* strain *AP 106.* The four positive samples for *A. phagocytophilum* were also positive for *R. monacensis.*

Emilia-Romagna region is considered endemic for Lyme disease but SFG rickettsiae were not included in the list of potential tick-borne zoonoses transmitted by *Ixodes ricinus*. In Italy SFG rickettsiae have been reported by other authors in different regions: Toscana (Selmi et al., 2009), Friuli Venezia Giulia (FVG) (Floris et al., 2008), Trentino Alto Adige and Veneto (Beninati et al., 2002).

The species *I. ricinus* is one of the most abundant tick species also in Emilia-Romagna, presenting very low host specificity and a record of frequent biting to humans (Manfredi et al.,

1999). *D. marginatus* is most frequently found in Mediterranean areas of Europe; larval stage usually feed on small mammals and birds, while adult ticks mainly feed on large mammals but frequently also on humans. Emilia-Romagna is considered endemic for Lyme disease and our results add SFG rickettsiae to the list of potential tick-borne pathogens.

Our results shows that 4.5% of ticks sampled are infected by *R. raoultii*. High prevalence could be biased by the fact that different ticks were feeding on the same animal, with a possible co-feeding transmission. The sequences obtained for *R. slovaca* and *R. raoultii* did not show any intrapecific variation, and the *ompA* partial sequence that we generated showed 100% identity with the sequences available in the data bases for these species.

We also detected *R. helvetica* from *I. ricinus* removed from roe deer (Fig. 5.5). *R. helvetica* in Italy was detected in *I. ricinus* in the Veneto and in FVG regions (Beninati et al., 2002, Floris et al., 2008). The microorganism *R. helvetica* was isolated from human patients with febrile illness and it may also be associated with serious infections such as central nervous system disorders (Fournier et al., 2000; Nilsson et al., 2010).The role of large mammals in eco-epidemiology of this *Rickettsia* species is still to be defined but some studies demonstrated that cervids may act as reservoir of this organism (Inokuma et al., 2008). In Emilia-Romagna region the most prevalent SFG *Rickettsia* in ticks removed from wild animal is *R. monacensis* (9.1% of ticks examined). DNA of this organism was found in *I. ricinus* removed from the majority of animal species. *R. monacensis* is a recently described member of SFG *Rickettsiae*. It was first isolated in *I. ricinus* ticks from a city park in Munich, Germany (Simser et al., 2002). Recently, two human cases of infection due to *R. monacensis* were documented in Spain, when investigators succeeded in isolating the agent from the blood of two patients with Mediterranean spotted fever-like illness (Jado et al., 2007). In Italy *R. monacensis* was detected in ticks from Trento province, in Toscana and in FVG regions (Beninati et al., 2002, Floris et al., 2008).

The tick-borne bacteria *Rickettsia slovaca* and *R. raoultii* were recently identified as the etiologic agent of Tick-borne lymphadenopathy (TIBOLA), also called *Dermacentor*-borne necrosis erythema and lymphadenopathy (DEBONEL). This rickettsioses is defined as the association of a tick bite, an inoculation eschar on the scalp, and cervical adenopathies (Parola et al., 2009).

Phylogenetic analysis reveal a very low variability among all the sequences. Since the *glt*A gene encodes for citrate synthase, a highly conserved enzyme, the sequences we obtained for this gene are significantly different only between species distantly related to each other.

Furthermore our study demonstrated the circulation of *A. phagocytophilum* in natural environment; although far fewer human cases have been reported, infections in domestic animals are common. Roe deer were demonstrate to be natural host for *A. phagocytophilum* and recently also wild boar was reported to be infected with this organism (Strasek Smrdel et al., 2009). *A. phagocytophilum* strains associated with human disease in Europe have been obtained from *I. ricinus* ticks but not from wild animals such as roe deer. These results suggested that *A. phagocytophilum* strains from ruminants could share some common

characteristics, including reservoirs and pathogenic potential, which may be different from strains that infect humans (De la Fuente et al., 2004).

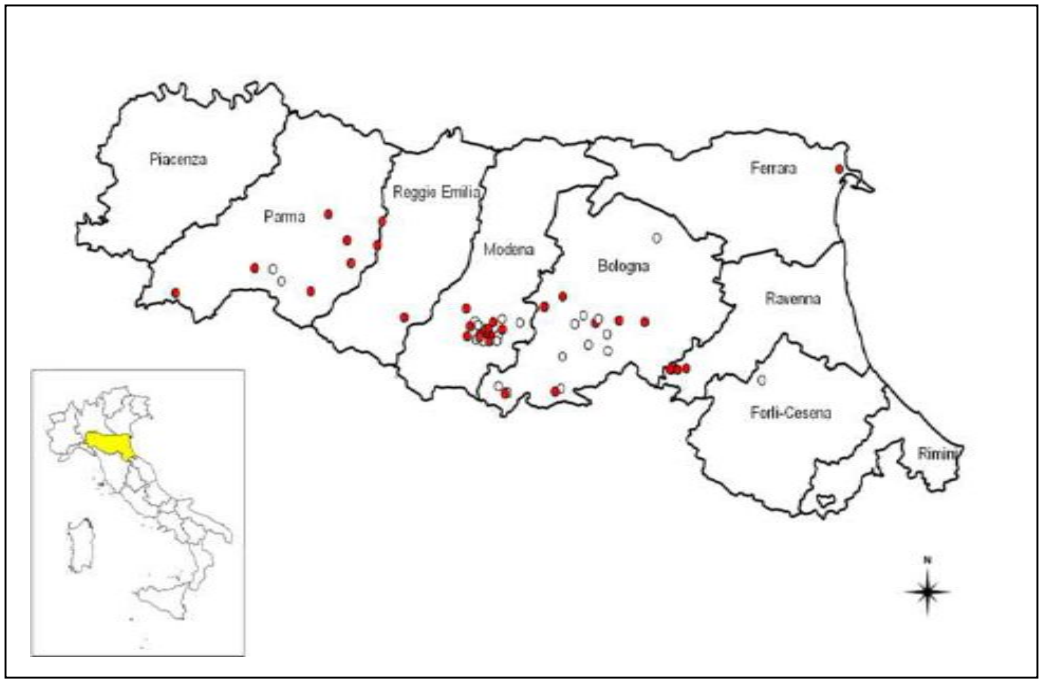


Fig. 5.5 Emilia-Romagna region map with locations where wild animals were hunted (white circles) and sites of detection of SFG rickettsiae (red triangles).

Pathogen species	**Number of positive samples in different provinces (N. positive ticks/ N. examined)**							
	BO	FC	FR	MO	PR	RV	RE	Total
R. helvetica	3/105	0/1	0/3	0/126	1/44	0/44	4/30	8/353
R. monacensis	6/105	0/1	2/3	11/126	6/44	2/44	4/30	31/353
R. raoultii	3/105	0/1	0/3	0/126	0/44	2/44	0/30	5/353
R. slovaca	4/105	0/1	0/3	11/126	1/44	9/44	0/30	25/353
Rickettsia sp.	4/105	0/1	1/3	1/126	11/44	0/44	3/30	20/353
A. phagocytophilum	0/86	0/1	0/3	0/84	3/34	1/5	0/30	4/243

Table 5.4- Number of *Rickettsia* positive sample found in the Emilia-Romagna provinces studied. The table also show the positivity for each Rickettsia species. A total of 20 positive specimens were not identified at species level possibly because the sequences were not readable due to co-infection with more than one bacterial species.

Ticks species	**Wild hosts**	**N. positive / N. examined ticks**	**Positive samples (%)**	**SFG *Rickettsia* species**
D. marginatus	Wild boar	32/105	30.5%	*R. raoultii (5); R.slovaca (25); Rickettsia sp.(2)*
	Hare	0/5	-	-
I. ricinus	Roe deer	40/168	23.8%	*R. helvetica (8); R. monacensis (18), Rickettsia sp. (14)*
	Red deer	6/14	42.8%	*R. monacensis (5); Rickettsia sp.(1)*
	Wild boar	10/59	16.9%	*R. monacensis (9); Rickettsia sp.(1)*
	Hare	½	50.0%	*R. monacensis (1)*
	Total	89/353	25.2%	

Table 5.5 – Details on the results od PCR screening for *Rickettsia* species in ticks collected on wild animals.

5.3.5 Bacterial pathogens in ticks collected on migratory birds (Study n.*4)*

Migratory birds can transport ticks over large distances influencing geographical distribution and population dynamics of these arthtropods and diffusion of vectored pathogens in new areas (Hasle et al., 2011). Nymphs and larvae of *Ixodes ricinus* and other species of hard and soft ticks are known to feed frequently on birds. Here we present the results of an investigation on ticks (and associated pathogens) collected on migratory birds captured in the ringing station 'Fondazione Europea il Nibbio (FEIN)' in Northern Italy. In autumn 2010, 4013 birds were captured with bird-nets in Arosio (Como, Lombardia, Italy) during the back migration from central Europe. All ticks visible on the hosts were removed. The study included a molecular screening for the following pathogens: *Borrelia burdorferi* sl complex (2-3-4), *Francisella tularensis* (7-8), *Coxiella burnetii* (9) and *Rickettsia* sp. bacteria (5-6). The number in brackets refers to primer pairs listed at page 64 of this thesis.

In details, 134 birds of 4013 (3.3%) collected were found to be parasitized by ticks. We report the presence of ticks on 9 different bird species: *Turdus philomelos*, *T. merula*, *T. iliacus*, *Erithacus rubecula*, *Sylvia atricapilla*, *Parus major*, *Fringilla coelebs*, *Anthus trivialis*, *Coccothraustes coccothraustes.*

All the collected ticks were morphologically identified as larvae and nymphs of *Ixodes ricinus*. Besides, using COXI gene of ticks (11) we confirmed the morphological identification of *Ixodes ricinus*, and we could easily classify specimens (even partial or damaged) with a DNA barcoding approach. In this study, *Turdus philomelos*, was the bird more frequently parasitized (84 individuals out of 1185 collected, average tick number per individual 1.59), followed by *Erithacus rubecula* (16 individuals out of 544 collected, average tick number per bird 1.28).

So far, just a selected subset of the collected ticks was processed. A total of 85 ticks from 51 birds, belonging to six different species were included in this work. We report tick positivity to *Borrelia burgdorferi* s.l. spirochetes and *Rickettsia sp.* bacteria (Table 5.6). In order to identify the pathogens at species level we procedeed with the sequencing of the PCR amplicons obtained. Preliminary results on the subset of the ticks investigated allow to determoine the presence of the species *Rickettsia helvetica* (8.2%) and spirochetes (16.4%) of the species *Borrelia garinii* and *B. valaisiana*. In details, *B. garinii* and *B. valaisiana* accounted for 78.5% and 21.5% of the infections detected in *Borrelia*-positive ticks, respectively. These two *Borrelia* genospecies are known to be mainly associated with birds (Michalik et al., 2008). Our results are in agreement with previous studies suggesting that some genospecies within the *B. burgdorferi* s.l. complex are indeed associated with blackbird and song trush (*Turdus* sp.) and other avian species and these birds may support the circulation of *B. garinii* and *B. valaisiana* under natural conditions (Humair et al., 1998; Poupon et a., 2006). The pathogens *Francisella tularensis* and *Coxiella burnetii* were never detected in these ticks.

Host species (N. individuals)	N.positive / N.examined ticks	Rickttsia sp.	*Borrelia* sp.
Turdus philomelos (22)	14/35	*R. helvetica* (4)	*B. valaisiana* (2); *B. garinii* (8);
Parus major (4)	4/12	-	*B. valaisiana* (1); *B. garinii* (3)
Fringilla coelebs (6)	2/9	*R. helvetica* (2)	-
Erithacus rubecula (14)	2/18	*R. helvetica* (2)	-
Anthus trivialis (4)	0/9	-	-
Sylvia atricapilla (1)	0/2	-	-

Table 5.6 – Details on the results of the PCR screening for *Rickettsia* and *Borrelia* species in ticks collected on migratory birds.

The species *Ixodes ricinus* shows affinity for avian hosts and this fact might influence TBP/TBDs epidemiology. In this study, all the birds infested with ticks are ground-feeding and at higher risk of tick infestation. Adult ticks rarely infest small and medium-sized birds, in fact, in the present study all the ticks collected were larvae and nymphs. The great majority of pathogen-positive ticks were nymphs that could get infected on the bird host or could get infected also on the previous hosts. Migratory birds, hitch-hiking ticks over large distances, might contribute to the spreading of TBDs in new areas and also might be partly responsible for the heterogeneous distribution of *B. burgdorferii* sensu latu complex in Europe. It is now clearly established that birds play a role as reservoir hosts in the ecology of Lyme borreliosis (Humair, 2002).

Fig 5.6 – Ticks feeding on *Turdus philomelos*. Larvae and nymphs of *I. ricinus* are visible close to the eye and the beak of the bird.

5.3.6 Tularemia outbreak in Toscana region (S*tudy n.6)*

Francisella tularensis is a highly infectious, facultative intracellular bacterium which causes epidemics of tularemia in both humans and mammals at regular intervals. The last large outbreak of Tularemia occurred in Italy in 2008, in Toscana region.

Small water springs are common in mountain areas in Italy, and are used as a source of water both during recreational activities, as well as for home consumption. These water supplies are characterized by a rather common structure: the water filtering from stones and soils is recovered into small basins and then flows out continuously trough a short tube. In several cases, these springs escape microbiological and chemical analyses, and the risk associated with water consumption from these sources can be high.

In December 2007, 19 cases of tularemia were reported to have occurred in the county of Pistoia, diagnosed at the Division of Infectious Diseases of the Pistoia Hospital, and notified at the local health authority (ASL 3, Pistoia), according to Italian legislation (D.M. 15 December 1990). Following the diagnosis of these 19 index cases, an outbreak investigation team was established. During the following months, from January to the end March 2008, 25 further cases of tularemia were diagnosed and notified, for a total of 44 cases. The source of infection was identified within a few weeks from the diagnosis of the first cases: a mountain spring. Out of the 44 patients that presented clinical signs and positive serology for *F. tularensis*, 39 referred to have consumed water from the mountain spring in the period December 2007 – March 2008. There was thus a strong epidedemiological evidence for the mountain spring as the source of the infection, for at least 39 out of the 44 infected persons. The cement basin of the spring was demolished at the end of March 2008, and no further cases were diagnosed after this month.

Water from the mountain spring was subjected to specific analyses for the detection of *F. tularensis* by molecular tools, microbiological culture methods, and mice inoculation.

In addition, ticks of the species *Ixodes ricinus* collected in the area, both in the months preceding the outbreak (recovered from 27 *C. capreolus*, for a total of 58 females and 18 males) and after the outbreak (by the dragging method, collecting 138 larvae and 163 nymphs in the late spring 2008, and 172 nymphs in the late spring 2009) were examined for molecular detection of *F. tularensis*.

All of the subjects that presented clinical signs of tularemia and positive serology for *F. tularensis* were subjected to accurate clinical visits, a panel of haematological analyses and proper antibiotic treatment. The most frequently recorded clinical sign was cervical lymphoadenopathy. Tonsillitis and faringitis were observed in a limited number of cases. The overall mild symptomatology can be attributed to the generally prompt administration of antibiotic treatment (streptomycin, ciprofloxacin, levofloxacin or doxycyclin - in some cases with co-administration - for 10-25 days). In seven cases it was however needed a drainage of lymph nodes.

The ticks collected in the study area where analzed in order to verify the possible circulation of *F. tularensis* in zoonotic cycle. A molecular screening by using specific primers (1) applied on a total of 250 *I. ricinus* nymphs revealed that none of the examined ticks was PCR positive for *F. tularensis*.

In the case here described the majority of infected persons consumed water from the same spring, and this water actually contained living *F. tularensis*. It is well known that *F. tularensis* can survive for long periods inside aquatic amoebae (Abd et al., 2003), similarly to *Legionella pneumophila*. While the mountain spring can be identified as the source of the infection for humans, the source of the infection for the spring is still undetermined. On the one side we can assume that *F. tularensis* found a suitable environment for survival in the spring for at least a few months (i.e. form the onset of the outbreak to the last isolation of the microorganisms from the water). This likely implies the presence of amoebae suitable for *F. tularensis* persistence. On the other side, we should assume that the infection of spring occurred at a give time in the weeks/months preceding the outbreak, considering that no cases of tularemia had been notified before

Considering the presence of *F. tularensis* in the mountain spring, we can thus propose two possibilities, i.e. that *F. tularensis* persisted in the region in the natural environment for several years, or that this bacterium re-invaded the area, e.g. as a consequence of the importation of game animals from endemic countries.

This report emphasize the risk connected with the consumption of natural spring water, were controls on the safety and quality of the water is not adequate. The evidence that *F. tularensis* likely persisted in the spring for several months (and thus the continuity of the risk of infection) point at the importance to establish and maintain over the years adequate controls on the quality of drinking water. Moreover our report emphasizes the need for further studies, to understand how a pathogen likely persisted in an area without causing infections in humans for years. In addition to the control of the water from sources at risk, different components of the environment should be monitored, from vertebrates to arthropods, from soil samples to amoebae. In this respect the analysis on the ticks collected on wild animals living in the surrounding of the spring were aimed to determine the circulation of *F. tularensis* between vertebrates and arhtrhopod hosts. The negativity of results might suggest that *F. tularensis* bacteria remained confined to the spring area, making the risk of infection to humans limited to those that used the water from that source.

Chapter 6

Conclusive remarks

6.1 Midichloria mitocondrii and *Ixodes ricinus*

Our analyses confirm that the genetic variability of *I. ricinus* in Continental Europe and in Northern Africa is limited. The genetic analysis were based on two mitochondrial loci (COI and COII) and two nuclear loci (Defensin and Trospa) of *Ixodes ricinus* and were in accordance to recognize the existence of two distinct groups of haplotypes showing a clear geographic pattern. The first group comprises individuals collected in the European continent, while the second group comprises individuals collected in Tunisia, North Africa. The existence of two groups of populations genetically differentiated in the two continents is also supported by the spatial analysis of molecular variance (SAMOVA), congruently for both mtDNA loci and the two nuclear loci. In previous studies on European and Northern African populations the lacking of data from intermediate areas did not allow determining the entity and the nature of the observed discontinuity (DeMeeûs et al., 2002; Noureddine et al., 2010). In this study, in addition to a considerable number of individuals from Central and Northern Europe, Southern populations of *Ixodes ricinus*, sampled in Italy (Sicily) were included. These individuals belong to European group, hence marking the existence of an abrupt and strong genetic discontinuity. Several hypotheses had been proposed to explain this pattern. It could be linked to geographic discontinuity due to the presence of the Mediterranean sea separating the two continents. Anyway, the absence of discontinuity in the entire European continent and the possibility for *I. ricinus* to cover large distance feeding on migratory birds, might make questionable this hypothesis. Besides, there could be implicated ecological factors linked to *I. ricinus* biology and interactions with its hosts. Another hypothesis, not necessary excluding the previous ones, considers the role of interaction between species. Indeed, *I. ricinus* is a parasites and a vector of other parasites/pathogens, interacting both with its hosts and with the transmitted pathogens, and this fact might be the cause of the genetic difference in the two populations. All these selective pressures might have contributed to the insurgence of the observed differences between European and Tunisian populations of *I. ricinus*. Nuclear and mitochondrial loci are concordant in evidencing a genetic discontinuity between the two continents, but the two markers show different patterns. For mitochondrial DNA, no Tunisian haplotypes were encountered in Europe and vice versa. Nuclear loci show a degree of sharing of haplotypes of the two different haplogroups, probably due to long-distance migration of avian hosts. Further interesting ecological and evolutionary scenarios, in a speculative way, might be suggested to explain such differences. For example, genetic drift is able to drive the loss of genetic variants in a totally random process. The flux of haplotypes might involve not only nuclear genes, but also

mitochondrial genes, but these ones are lost due to genetic drift that on this marker is stronger compared to nuclear loci. An alternative hypothesis proposes the existence of differential migration of males and females, where males do not transmit the mt genome. Indeed, females of *I. ricinus* resulted more philopatric compared to males and less incline to dispersion. Host preference with different dispersion capacity might explain this pattern, males tend to parasitize more often birds and females are more frequently encountered on large mammals. Another hypothesis might be linked to a selective disadvantage of females in the two geographic regions. In conclusion, the European population of *I. ricinus* does not present any phileogeographic structure. I would suggest that the pattern of low genetic diversity observed at nuclear and mitochondrial loci is a consequence of historical and contemporary factors. Both markers show traces of demographic expansion, in fact, mismatch distribution resulted unimodal and not deviate from the model expected in case of demographic expansion. Besides, the result is confirmed from the values of Fu's *Fs* index that are negative and significant. Over crossing the actual distribution of the species *I. ricinus* and the distribution of ice in glacial phases in European continent it is reasonable to suppose that the demographic expansion started from glacial refugia. The value of parameter τ of mismatch distribution of mitochondrial DNA, suggests that this expansion happened almost 20.000 years ago. Thus, evolutive event might be linked to deep climatic changes in temperate areas during glacial/interglacial phases in late Pleistocene. During this period, according to the general model of expansion/contraction, populations of thermophil species, like *I. ricinus*, to defend from ice advancing, moved to lower latitudinal refugia in suitable areas for survival, identified in the Southern Mediterranean peninsulae. In these refugia, various *I. ricinus* populations survived during glacial ages, and after ice melting and temperature rising, migrated to northern areas. Previous studies on *I. ricinus*, hypothesized that this model, already validated for other species, is also valid for this parasite. But the results of this study suggest a different evolutive scenario. In fact, even if the genetic data support the hypothesis of an event of demographic and spatial expansion after glacial ages, on the contrary, the absence of different lineages localized inside the European continent, as the absence of a clinal variation of haplotypes frequencies do not support the glacial genetic fragmentation. The pattern observed is more adapted to explain a scenario were populations of *I. ricinus* remained inter-connected probably due to continuity offered by its multiple hosts, as suggested for other species with wide distribution in western Paleartic (or we could even hypothesize that the species survived the glacial age into a single refugium). In the case of *I ricinus* is of fundamental importance to consider the ecological characteristics of the species in order to determine the response in front of the Pleistocenic climatic changes. The present analysis consents to evidence an historical component at the origin of actual pattern of distribution of genetic diversity, but also a variety of actual processes that acted on populations. The migration on long distances mediated by different *I. ricinus* hosts in different stages of its biological cycle might be implicated in the absence of correlation between genetic and

geographic distances, the presence of haplotypes shared by populations distantly located and the lack of areas with major and minor diversity. On the contrary, the presence of specific and private haplotypes in areas in close vicinity suggests a restricted genetic flux on small geographic scale.

The PCR screening for *Borrelia burgdorferi* sensu latu on ticks collected in Europe and North Africa allows to determine the presence of 4 species of borreliae causing human diseases (*B. burgdorferi* ss, *B afzelii*, *B.garinii* and *B. lusitaniae*) in the analyzed ticks. Due to the small number of individuals included in this study it was not possible to derive statistical correlations between the presence of spirochetes and specific mithocondrial or nuclear gene alleles (COI, COII; Defensin and Trospa). An interesting result of this study evidence that *B. lusitaniae*, a genospecies diffused in Portugal, Southern Spain and Northern Africa with a reduced distribution outside this areas, might be widely diffuse in other European countries (Italy and Czech Republic) and also in Turkey.

The previous analyses show that the genetic variability of *I. ricinus* in Continental Europe and in Northern Africa is limited. Correspondingly, the genetic variability of its principal endosymbiont *Midichloria mitochondrii* is also negligible (Epis et al., 2008). The biological role of these microorganisms (with a prevalence close to 100% in females of *Ixodes ricinus*) is still largely unknown. We suppose that this *M. mitochondrii* might have established the mutualistic association with *I. ricinus* only in recent times and probably experimented a recent increase in population size following the Paleocenic population expansion of its tick vector host. So far, this idea is not yet supported by proper investigation. *M. mitochondrii* was detected in various ticks species that could have acquired the bacteria during blood meal passing trough the infection of the host or during co-feeding on the vertebrate hosts (i.e. without the infection of the vertebrate) (Nuttall et al., 1998). Besides, *M. mitochondrii* is present in several tick species apparently without remarkable variability (i.e., even identical 16s rDNA sequences are observed in different species), thus suggesting for *M. mitochondrii* the possibility to reach new hosts/vectors trough horizontal transmission. The presence of various 16S rDNA gene sequences with high similarity to that of *M. mitochondrii* in other Metazoa, including in environmental microbial mats suggests the existence of a family of *Midichloria*-like organisms (MLOs). Besides, the detection of DNA of MLOs in other hematophagous arthropods suggest a possibility of transmission and “circulation” of these bacteria between vectors and vertebrate hosts. The lack of congruence between ticks and *Midichloria* bacteria phylogenies is a further indirect evidence of possible horizontal transmission. Even in the case of infection of the host, at present, there is no evidence supporting the pathogenic role of *M. mitochondrii* in vertebrates, but it is important to remind that several human mitochondrial dysfunction/pathologies are currently of unknown etiology. Our molecular screening in blood and tissues of vertebrates (including humans) detected 8 PCR positive blood samples from 4 horses (*Equus caballus*), 3 dogs (*Canis familiaris*) and 1 sheep (*Ovis aries*), out of a total of 293 mammalian blood samples examined. The sequences

obtained were not identical; they showed high similarity with the 16S rDNA of MLOs detected in species different from *I. ricinus*.

6.2 Tick-borne pathogens and diseases in Italy

In recent years, new bacterial tick-borne diseases (TBDs) have been recognized, as agents of diverse Spotted Fever-like and Lyme-like diseases, due to different species of *Rickettsia* and *Borrelia* respectively. Besides, rare and accidental human cases of erlichiosis/anaplasmosis and tularemia, appear to be increasing worldwide as well as Crimean Congo hemorrhagic fever and Q fever. It is widely documented that TBDs are spreading across new areas over their natural range, re-emerging or increasing their prevalence in endemic areas as well as emerging in new territories and countries with no previous record of these diseases (Gould and Higgs, 2009).

Ticks are ectoparasites that heavily impact global health by transmitting a wide variety of pathogens to vertebrates. All TBDs are zoonoses that mainly affect animals but may cause severe diseases in humans (De la Fuente et al., 2008). In Italy a variety of microorganisms vectored by ticks represent a possible treath for human health. Although largely present in Italy, the relatively low number of TBD cases reported yearly, probably due to an high rate of asymptomatic disease and to an under-reporting of the symptomatic ones, makes very difficult to assess the real impact of the TBDs on the public health in Italy.

In the period 1992-1998, more than 1000 cases of Lyme disease occurred in Italy (Circolare n. 10 del 13 luglio 2000, Ministero della Sanità). The regions most affected by LD are Friuli-Venezia Giulia, Liguria, Veneto, Toscana, Emilia-Romagna and Trentino Alto Adige. Reports from Southern regions and islands are sporadic probably because the woods are dry and the weather is not favourable to support consistent populations of *I. ricinus.*

Serological analysis showed a higher positivity in categories considered at risk of tick bite compared with general population and the infection in domestic and wild animals is also well documented (Anderson et al., 1989). Erythema migrans was described in Italy only in 1971 although Italian dermatologists were already familiar with it. In 1983, the first case of Lyme borreliosis with multisystem involvement was identified.

Rickettsial diseases are still the cause of serious health problems. In the period 1992-1998, about 1200 cases/year of rickettsiosis have been notified to the Ministry of Health, with an average incidence of 2.1/100,000 inhabitants. Observing the distribution of cases, it is evident that some regions of central and southern islands appear particularly concerned by the rickettsioses. Morbidity rates above the national average are observed in the period under review, in four regions: Sardinia (11.9), Sicily (10), Calabria (4.7) and Lazio (3.9). From 1998 to 2002, 4,604 clinical cases were reported, with 33 deaths in the period from 1998 to 2001. Almost all the cases reported in Italy are cases of MSF. In 2004, three cases of a mild form of

rickettsiosis were serologically attributed to *Rickettsia helvetica*. Serological evidence of rickettsiae circulation in humans was also reported in Italy (Ciceroni et al., 2006).
In the period 1992 to 1998, were reported to the Italian Ministry of Health 61 cases of tularemia, with average incidence of 0.02/100,000 inhabitants.
In Italy the incidence of tick-borne zoonoses has increased over the last years (De Meneghi, 2006). Some sporadic cases of human granulocytic anaplasmosis (HGA) have been reported in North-eastern Italy (Beltrame et al., 2006) and the microorganism was detected in molecular screening on ticks and domestic animals (Carpi et al., 2009) but the infection is probably more frequent than assumed.
The report to Ministry of Health of human cases of TBDs is mendatory but this measure help to provide only indirect epidemiological data. Proper investigation on the risk factors and surveillance of TBDs can be considered effective only in endemic areas of some regions, but in non endemic regions the knowledge on the presence of tick species and associated/vectored TBPs can be inadequate as demonstrated form the misdiagnosed human cases of Lyme disease in the Parco del Ticino in the Po river valley (see chapter 5).
In these three/four years, my doctorate work on TBPs contributed in providing new information on the presence of ticks and associated pathogenic microorganisms in Italian areas were knowledge were scanty, incomplete or obtained indirectly from hospitalized patients presenting clinical manifestations due to a specific TBD.
In the framework of the investigation on tick-borne pathogens, I reported the presence of spirochetes of the species *Borrelia afzelii* and *B. lusitaniae*, both implicated in development of diseases in humans, in an highly populated area close to the industrial district of Milan (Ticino Park). This investigation allowed to describe human cases of borreliosis in a zone, previously not consider at risk of tick infestation or endemic for Lyme disease. We also contributed in the process of valuation of the risk and management of the public health problem, providing correct information to clinicians, workers/tourists of the area exposed to the risk of tick-bite and defining the 'red' zones/hot spots at high risk where it is necessary to position informative plaques on tick presence and tick-borne diseases transmitted. Further study in the Ticino Park area detected the presence of *Rickettsia monacensis* and *R. helvetica* with high prevalence in *Ixodes ricinus*; both species are considered emerging human pathogens (Fournier et al., 2000; Jado et al., 2007; Nilsson et al., 2010)
In addition, in the same study area the circulation of *Francisella tularensis holarctica* (Type B) was reported. After this study, SFG rickettsiae and the etiological agent of tularemia, together with Lyme disease causing borreliae must be included in the list of TBDs present in the Ticino Park and possibly in other flat areas of Po river valley. In Emilia-Romagna region endemic areas for the the presence of Lyme disease and TBEV are reported. Our recent survey on other TBPs permitted to add SGF rickettsiae and *A. phagocytophilum*, the etiologic agent of human granulocytic anaplasmosis, to the list of diseases acquired after tick-bites. A study on VCO

territory (Piemonte) produced detailed information on presence of ticks and vectored TBPs: borreliae causing Lyme disease and SFG rickettsiae were detected. We also investigate the bacterial pathogens present in birds net-captured during the back migration autumnal from central Europe. The preliminary results of the molecular screening allow to detect TBPs. Further development of this work plan to collect ticks on birds during their spring migration from Southern Europe/Northern Africa for a comparative study on TBPs. Besides, it would be interesting to investigate the presence of vector-borne viruses (Crimea-Congo haemorrhagic fever and tick-borne encephalitis viruses) in these ticks. The last work included in this PhD dissertation deals with an outbreak of water-borne tularaemia and a subsequent study on the presence of *F. tularensis* in ticks collected in the surroundings area. Indeed, the PCR detection of bacterial pathogens in ticks represented a first step that later allow to apply this diagnostic molecular methods in other context. For example the protocols optimized on ticks were also used to detect pathogenic bacteria directly in human matrices (e.g. *B. burgdorferi* s.l. sprirochetes in skin biopsies and cerebrum-spinal fluid of hospitalized patients), animals (e.g *Rickettsia* sp. in blood of dogs) and environmental sources (e.g. *Francisella tularensis* in water samples) for the interest respectively of IRCCS (humans) and IZSLER.

Finally, all this investigation on TBPs contributed in designing a molecular screening protocol applicable on ticks removed on human patients in hospitals. This TBP screening, although not diagnostic for TBD in humans, provide important guidance information that in combination with clinical manifestations of the patient can support clinicians for decision on the correct antibiotic therapy.

Unfortunately for a large number of people at risk of tick-bite, occupational and recreational exposures to tick infested areas occur with such high frequency that avoidance of exposure is impractical for prevention. Vaccination (TBD) might be recommended in endemic areas. Prophylactic administration of wide-range antibiotics simply may delay onset of the illness (for example, tetracycline or chloramphenicol are just rickettsiostatic). When in tick-infested areas, frequent careful self-inspection for ticks is indispensable to prevent tick-bite. In addition, wearing of white clothes with sleeves which can be closely fastened around the wrists and tucking pants into boots impede ticks to reach the skin. Permethrin or other repellents provide further protection. Ticks attached to the skin must be promptly removed (entirely) with forceps and the wound cleaned with antiseptic.

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