

Summary

The research activity I developed during my doctorate can be divided into three parts. First, I investigated the genetic variability of *Ixodes ricinus* in Europe and North Africa also screening for the presence of bacteria belonging to *Borrelia burgdorferi* sensu lato complex. A second part of the work focused on *Mitochondria* the principal endosymbiont of *I. ricinus*. This intramitochondrial bacterium is also present in other tick species but information on the exact intracellular location are not available for the majority of them. I decided to perform a molecular screening for detection of *Mitochondria*-like organisms in vertebrates comprehending humans. Finally, the last part of this work provide information on presence of *Ixodes ricinus* and other tick species in areas (and from hosts) where record of the presence of these arthropods were absent or fragmentary; the ticks collected were also screened for the presence of different tick-borne pathogens.

The genetic analysis on both two mitochondrial loci (COI and COII) and two nuclear loci (Defensin and TROSPA) of *Ixodes ricinus* confirmed 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. In our 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 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. Besides, the result is confirmed from the values of Fu's F_s 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 where 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 Palearctic (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 lato 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 mitochondrial 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 these areas, might be widely diffuse in other European countries (Italy and Czech Republic) and also in Turkey.

Our analyses confirm 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. We suppose that this microorganism might have established the mutualistic association with *I. ricinus* only in recent times and probably experienced a recent increase in population size following the Paleocene 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 through the infection of the host or during co-feeding on the vertebrate hosts (i.e. without the infection of the vertebrate). 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 through 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*.

In these three years, my doctorate work on tick-borne pathogens (TBPs) contributed in providing new information on the presence of ticks and associated pathogenic microorganisms in Italian areas where knowledge was 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*, implicated in development of Lyme disease in humans, and *B. lusitaniae*, also suspected to be pathogenic for humans, in an highly populated area close to the industrial district of Milan (Ticino Park and surrounding area). This investigation allowed to describe human cases of borreliosis in a zone previously not considered 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 signs on tick presence and tick-borne diseases transmitted. Further study in the Ticino Park area detected the presence of *Rickettsia monacensis* and *R. helvetica* (both belonging to Spotted Fever Group-SFG rickettsiae) with high prevalence in *Ixodes ricinus*; and also the circulation of *Francisella tularensis holartica* (Type B) was reported. After this study, SFG rickettsiae and the etiological agent of tularemia, together with Lyme disease causing borreliae

will 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 presence of Lyme disease and TBEV are reported. Our recent survey in this region on the presence of TBPs permitted to include SFG rickettsiae and *A. phagocytophilum*, the etiologic agent of human granulocytic anaplasmosis, to the list of diseases that can be acquired after tick-bites. A study on VCO territory (Piemonte region) produced detailed information on presence of ticks and vectored TBPs: borreliae causing Lyme disease and SFG rickettsiae were detected. We also investigate ticks (and associated bacterial pathogens) present in migratory birds on the autumnal backward journey from central Europe. The preliminary results of our molecular screening allow to detect TBPs (borreliae and SFG rickettsiae). The last work included in this PhD dissertation deals with an outbreak of water-borne tularaemia, in Toscana region. This outbreak was linked to an infected mountain water spring and a subsequent PCR screening was performed in ticks collected in the surrounding areas in order to verify the presence of *F. tularensis*.

Finally, all this investigations on TBPs contributed in designing a complete 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.