SUMMARY

Key words: *Ixodidae*, geographic distribution, ecology, seasonal dynamics, ticks-hosts-RLB, *Dermacentor*, genetic dievrsity

Doctoral thesis: **GENETIC POLYMORPHISM AND ECOLOGY OF IXODID TICKS** was developed within the Doctoral School of „Ion Ionescu de la Brad" University of Agricultural Sciences and Veterinary Medicine from Iași, as part of the project *Perfecționarea și dezvoltarea resurselor umane pentru cercetare și inovare prin școala doctorală*, POSDRU/CPP107/DMI1.5/S/77222, that was cofinanced from the European Social Fund through the Ministry of Labour, Family, Social Protection and Elderly Persons respectively the Managing Authority for the Sectoral Operational Programme Human Resources (AMPOSDRU), under the Sectoral Operational Programme of Human Resources Development 2007 – 2013

The paper was written during four years of study, 1.10.2010 – 30.09.2014 and it is structured in accordance with curent scientific standards in two main parts: the first part, represented by **The current state of knowledge** which includes 33 pages and repredesents 26.19% of the content of the paper and the second part, **Personal contributions** with 93 pages wich represent 73.81%.

The content of the first part of the **Current state of knowledge** is divided into two chapters that brielfy report the information from national and international literature on the subject of this paper.

The first chapter entitled **MORPHOLOGY AND ECOLOGY OF IXODID TICKS** comprise bibliographical studies regarding ticks taxonomy, morphology and data about ecological factors. Taxonomy of ticks, it is still a debate subject among specialists. Last update of ticks species was done recently, in 2010 by Guglielmone et al., where are recognized 896 species organized into three families: Nuttaliellidae, with one species, Argasidae comprising 193 species and Ixodidae formed by 702 species. Here, also we present elements of external morphology, the basis of ticks identification; molecular methods are used particularly to clarify conflicts of sinonimy (Zhaler, 1995, Barker et al., 2002); characterize phylogeography of some

species (Noureddine et al., 2011) and measure genetic diversity (Casati et al., 2007, Chițimia et al., 2009). In this chapter we also described the life cycle of ticks, their hosts, host-vector-pathogen interactions and the main ecological factors influencing their life cycle.

In the frame of Chapter II – **GENERAL CONSIDERATIONS REGARDING GENETIC CHARACTERIZATION OF HARD TICKS,** was done a bibligraphical study regarding genetic characterization of hard ticks in Romania and worldwide and of markers used to measure and describe genetic diversity of ixodid ticks. In Romania few studies were done concerning ticks genetic characterization. In general it was described levels of intraspecific and interspecific relations among four species from Romania (*Dermacentor marginatus, Haemaphysalis punctata, Haemaphysalis parva, Ixodes ricinus*) and one species from China (*Haemaphysalis longicornis*), the gamasid *Dermanyssus galinae* was used as an outgroup. Markers used were the non-coding regions ITS1 and ITS2 and two partial mitochondrial gene: pCOI and pNADH5. Low levels of intraspecific variation were found (0.1 – 1% for pCOI and 0.2 – 1.2% for pNADH5) and higher levels for interspecific variation (15.9 – 27.6 pCOI; 20.3 – 42.4% pNADH5) (Chițimia et al., 2009, Chițimia et al., 2010). These studies regarding genetic characterization of hard ticks from western part of Romania were first of this kind in Romania, they brought useful informations regarding intraspecific and interspecific genetic variability among ticks.

Instead, numerous studies regarding genetic structure, genetic diversity and phylogeography of ticks were done in United Stated of America and Europe. Researches made by Norris et al., (1996), Kain et al., (1999), and Qui et al., (2002) on the North continent of America, concerning genetic diversity of *Ixodes scapularis* showed a well defined genetic structure of two separate clades, one in south and one in north. They used mitochondrial markers (12S gene and 16s rRNA) and single stranded conformation polymorphism (SSCP). In Europe majority of studies regarding genetic characterization of ticks concerned the main vector of Lyme disease, namely *Ixodes ricinus*. The interest of describing genetic structure of Ixodes ricinus was high and started more than fifteen years ago, using different sets of markers. Delay et al., (1998) notice significant differences among ticks from Switzerland and one specimen from Tunisia, using allozymes markers. Next, others researchers using microsatellites (De Meeus et al., 2002), mitochondrial markers (Xu et al., 2003, Casati et al., 2008) mitochondrial and nuclear markers (Noureddine et al. 2011, Porretta et al., 2013), MLST typing (Dinnis et al., 2014) contributed enormously to elucidate phylogeography and genetic structure of *Ixodes ricinus*.

Finally after corroborating genetic data, especially the divergence of african subpopulation found by Noureddine et al., (2011) with the ecological factors, scientists confirmed the existence of a new species in North Africa and Iberian Peninsula – *Ixodes inopinatus*.

The second part of this thesis, **Personal contributions,** is composed of five chapters. The **Aim and objectives of the research** motivates the theme of this thesis through the importance of the study and the use of modern methods for genetic characterization of ixodid ticks.

Chapter IV entitiled **GEOGRAPHIC DISTRIBUTION AND SEASONAL DYNAMICS OF IXODID TICKS IN THE NORTHEASTERN ROMANIA**, has as objective the accomplishment of a geographic ditribution map of ixodid ticks collected from the vegetation, in the region of Moldova and Tulcea county; describing ticks communities and evaluations of their seasonal dynamics. Thus, for the fulfillment of the objectives, several tick collection campaigns were made, using dragging method. Ticks were collected from 32 location from the North-East of Romania, for each of them were noted geographical coordinates, altitude and type of vegetation. After, ticks were identified on the basis of their morphological features and conserved in ethanol 70% for further molecular exams.

A total of 1017 ticks were collected from all 32 locations, 7 species belonging to 3 genra were identified: *Ixodes ricinus* (879; 86.26%), *Haemaphysalis punctata* (85, 8.34%%), *Dermacentor reticulatus* (27, 2.65%), *Dermacentor marginatus* (22, 2.16%), *Haemaphysalis inermis* (2, 0.19%), *Ixodes redikorzevi* (1, 0,10%), *Haemaphysalis concinnna* (1, 0.10%). For each species was created a distribution map with each location where it was found.

Ixodes ricinus was the most prevalent tick species, found in all 32 locations (100%). In 10 locations were *Ixodes ricinus* was found together with other species was the dominant species (C.A. Rosetti - IS, Ciric - IS, Cetățuia - IS, Păun - IS, Breazu - IS, Cotu Morii - IS, Solești - VS, Crasna - VS, Gârboavele - GL, Slava Cercheză - TL) and in 17 locations it was the only species found (Copou - IS, Bârnova - IS, Ezăreni - IS, Huși - VS, Adam - GL, Slava Rusă - TL, Grești - VN, Valea Sării - VN, Dofteanca - BC, Poiana Sărată - BC, Podoleni - NT, Mihai Eminesctu -BT, Drancani - SV, Coșna - SV, Șaru Dornei - SV, Pojorâta - SV, Rădăuți - SV). *Ixodes ricinus* was found mainly in deciduous and mixt forest and was the only species present in coniferous forest habitats. This tick species has a great level of adaptability, increasing his distribution to higher latitudes and higher altitudes.

It is worth highlitghting the presence of a significant population of *Dermacentor reticulatus* in the region of Moldova, species vector for canine babesiosis.

We are mentioning that the species *Haemaphysalis inermis* was signeled again after aproximatly half of century, last actualization of this species beiing made in 1965 by Feider Zicman.

Data of the present subchapter 4.1. Geographic distribution of ixodid ticks from North-East of Romania, were the subject of a scientific paper published in the series of Faculty of Veterinary Medicine, Timișoara, Vol XLVII (3), 78 – 83, 2014 ISSN: - 1221-5295, **-"Short survey of questing ticks dispersal (*Ixodidae*) in the North-Eastern Romania".**

The Subchapter **– 4.2 Seasonal dynamics of ixodid ticks from Nord-East of Romania,** aimed to correlate the data obtained in the previous subchapter, with analyses of evaluations of ixodid ticks community and their seasonal dynamics measured in biotops favorable for ticks. Four areas of study were established: C.A. Rosetti, Ciric, Cetățuia, Bucium from Iași city. The type of vegetation usually encountered was formed by deciduous forest, composed mainly by trees as: *Quercus spp.*, *Carpinus spp.*, *Fraxinus spp.*, *Fagus spp*., thereby providing good conditions of humidity and temperature, necessary for a good development of ticks. Fauna found in such type of habitats is formed by: house sparrow (*Passer domesticus*), common blackbird (*Turdus merula*), rodents: striped field mouse (*Apodemus agrarius)*, yellow-necked mouse (*Apodemus flavicollis*), lesser blind mole rat (*Nannospalax leucodon*), red squirrel (*Sciurus vulgaris*), european hare (*Lepus europaeus*), hedgehog (*Erinaceus romanicus*), wildcat (*Felis silvestris*), wild boar (*Sus scrofa*), red deer (*Capreolus capreolus*), red fox (*Vulpes vulpes*) and wolf (*Canis lupus*). Starting October 2013 until September 2014, ticks were monthly collected, by dragging method in four collection sites from Iași urban area. A total of 1526 ticks were collected under this study: *Ixodes ricinus* was the most abundant tick species (92,39%) with 35 males, 26 females 220 nymphs and 1129 larvae, followed by *Haemaphysalis punctata* (6,94%) with 1 male, 12 females, 16 nymphs and 77 larvae and *Dermacentor reticulatus* (0.59%) with 2 males and 7 females. One adult specimen of *Ixodes redikorzevi* was found at C.A. Rosetti recreational area.

Ixodes ricinus had the lead, with 88,59% at CA Rosetti, 99,04% at Ciric, 89,85% at Bucium and 65,51% at Cetățuia.

Haemaphysalis punctata was the second most abundant species found in three recreational areas, with a prevalence of 3% at Bucium 8% at CA Rosetti and 34% at Cetățuia. The high prevalence of *Haemaphysalis punctata* at Cetățuia recreational area may be due to the local ecological factors such as slope exposure, temperature and relative humidity or plant community , this species being adapted to more arid environmental conditions.

Dermacentor reticulatus was the third most abundant species with a prevalence of 1% at Ciric, 3% at CA Rosetti and 7% at Bucium recreational area.

The highest peak density was registered in April for CA Rosetti (6,9 ticks/100m^2), Ciric (9 ticks/100m^2) and Cetățuia (4,4 ticks/100m^2) and in May for Bucium (5,8/100m^2). A second increase of ticks densities was registered in autumn: C.A. Rosetti – 4,6 ticks/100m^2; Ciric – 2 ticks/100m^2; Cetățuia – 1,4 ticks/100m^2; Bucium – 1,2 ticks/100m^2.

Results of the present subchapter were published in the series of Faculty of Veterinary Meidicine, Iași, - **„The Acarological Risk in Iasi Recreational Areas" 2014 Vol. 57 (1-2): 140-145.**

In Iași, the seasonal distribution of host-seeking ticks, shows mainly the pattern of *Ixodes ricinus* activity, representing over 90% of ticks collection. *Dermacentor reticulatus* had the first peak-activity, registered in March, decreasing in April and May and ceasing during the summer. Instead *Haemaphysalis punctata*, had the peak activity in April and *Ixodes ricinus* in April-May. All three species had a second peak in autumn, presenting a bimodal pattern of their seasonal activity.

Seasonal dynamics of species *Haemaphysalis punctata* is similar to that described by Feider in 1965 south of the 10°C annual isotherm. In recent years the annual isotherm recorded in Iasi frequently exceeded the threshold of 10°C.

Seasonal activity of Ixodes ricinus adults followed generally the pattern of the species. A bimodal pattern, , with a peak activity recorded during the spring (April-May) and a second period of much lower intensity in September and October. Nymphs of *Ixodes ricinus* had nearly the same seasonal activity with adults, nevertheless their abundance was considerably higher. The peak activity of nymphs of *Ixodes ricinus* was registered in April at CA Rosetti, Ciric and Cetățuia, and in May at Bucium. Larvae had an allochronic activity in comparison with nymphs and adults, having their peak activity during the summer at C.A. Rosetti, Ciric and Cetățuia areas. Our findings differ from recent reseraches regarding seasonal dynamics of Ixodes ricinus in Romania, where the peak activity of Ixodes ricinus was recorded during May-June (Ioniță etal., 2006, Ioniță et al., 2009, Coipan et al., 2010).

The **Chapter V** entitled **ECOLOGICAL FACTORS INFUENCING IXODID TICKS,** was devoted to evaluate how biotics and abiotics factors will affect *Ixodes ricinus* seasonal dynamics, and to identify the panel of hosts of this species by a modern tool of molecular biology.

Seasonal activity of ticks is strongly influenced by environmental abiotic factors - temperature; humidity; saturation deficit and rainfall. They may extend, reduce or even stop ticks host seeking activity. For example relative humidity is an indicator of the rehydration ticks strength. *Ixodes ricinus*, *Dermacentor variabilis*, *Amblyomma cajennense*, ticks from different continents, can survive only when relative humidity do not fall below 80% for a longer period of time (Pfaffle et al., 2013).

Data regarding daily mean temperature (^{o}C) and relative humidity (%) were obtained from the Regional Centre of Meteorology Moldova.

Analysis of meteorological data and seasonal activity of ticks showed a negative correlation between the activity of *Ixodes ricinus* nymphs and adults along with a increase of monthly mean temperature over 19 ° C associated with a low relative humidity (70%). In three out of four areas studied, larvae had an allochronic seasonal activity compared with nymphs and adults, peaking in July, a seasonal pattern positively correlated with high temperature. These findings are in concordance with other European studies regarding *Ixodes ricinus* larvae seasonal activity, temperature being the most important abiotic factor influencing their host-seeking behavior (Dantas-Torres et al., 2010)

Results of the present subchapter were published in the series of Faculty of Veterinary Meidicine, Iași, - „Seasonal dynamics of ixodid ticks in Iași urban area” 2014 Vol. 57(1-2) :135-139.

Host identification of Ixoded ricinus nymphs were done by multiplex polymerase chain reaction – reverse line blot hybridization (mPCR/RLBH). Through this method hosts are identified by performing a blood meal analysis using molecular biology tools. This method (mPCR/RLBH) was adapted and describe by Humair et al., 2007. The method consists of a single run polymerase chain reaction ampliÞcation of the 12S rDNA molecular marker by using nondegenerate primers followed by a reverse line blot hybridization assay by using specific oligonucleotide probes. The palette of probes allowed us to distinguish major groups of host vertebrates (mammals, small rodents, artiodactyls, birds, lizards) and to identify the bloodmeal sources at the genus or species level.

A total of 96 nymphs from forest Breazu were analyzed by mPCR / RLB to identify their hosts. To validate the multiplex PCR reaction was amplified a fragment of a gene belonging to the species *Ixodes ricinus*. There are 20 probes were used for birds: (*Certhia brachydactyla, Columba palumbus, Corvus corone, Erithacus rubeculla, Fringilla/Pyrrhula,Gallus gallus,Garrulus glandarius, Luscinia megarynchos, Parus, Parus ater, Phasianus colchicus,*

Phylloscopus, Pica, Picus viridis, Prunella, Sitta europaea, Sturnus vulgaris, Sylvia, Troglodytes, Turdus/Parus), one for reptiles (*Reptilia*) and 35 for mammals (*Apodemus sylvaticus, Bos taurus,Capreolus capreolus,Crocidura russula, Equus cabalus, Erinaceus erinaceus, Glis glis, Lepus europaeus, Meles meles, Microtus/Micromys, Mycromis minutus, Microtus agrestis, Microtus arvalis, Mus musculus, Mustella erminea, Mustella putorius, Mustella nivalis, Myocastor coypus, Myodes glaerolus, Neomys, Neomys anomalus, Neomys fodiens, Oryctolagus cuniculus, Ovies aries, Rattus norvegicus, Rattus rattus, Sciurus vulgaris, Sorex, Sorex araneus, Sorex minutus, Suncus etruscus, Sus scrofa, Talpa europaea, Tamia sibiricus, Vulpes vulpes*.)

From total samples analyzed, 30% responded positively to blood meal analysis. The hosts identified were: *Sus scrofa* – 24%; *Bos taurus* – 14%, *Oryctolagus cuniculus* – 14%; *Aves* – 10%; *Vulpes* – 7%; *Microtus agrestis* – 7%; *Neomys* - %; *Sciurus vulgaris* – 4%; *Apodemus* – 4%; *Fringilla*/*Pyrulla* – 3%, *Microtus*/*Micromys* – 3%; *Turdus*/*Parus* – 3%; *Columba palumbus* – 3%. Knowing the hosts preference of ticks is vital to elucidate eco-epidemiology of diseases that they transmit. Detaching ticks from captured animals may indicate the use of that animal as host, but only with a bias for those animals that are easily to capture. Using this method of identifying hosts on the basis of the blood meal taken by ticks in the previous development stage is more feasible and non-discriminatory, managing to identify host with a tiny amount of blood remained in the gut of nymph or adult, even after 280 days from their moult (Humair et al., 2007). Later on the same samples can be checked the degree of infestation of ticks with different pathogens (Coipan et al., 2009, Scot et al., 2012). This method can be adapted for other blood sucking arthropods. It also allows identification of pathogens hosted by arthropods, identification of reservoir hosts and determining their importance in a given area (Scott et al., 2012).

The population examined by mPCR/RLB is part of a study at European level, implying identification of Ixodes ricinus hosts and evaluation of host-vector-pathogen mechanism.

In Chapter VI entitle **GENETIC DIVERSITY OF THE *DERMACENTOR MARGINATUS* AND *DERMACENTOR RETICULATUS* SPECIES** was aimed the describtion of genetic diversity and the analysis of the phylogeography of these species at European level**.**

A better understanding of genetic diversity of vectors is essential for:

1. Providing the information on taxonomical status of the species or subspecies.
2. To measure the gene flux between and within the population vectors (dispersion

3. Development of methods for prevention and control of tick-borne diseases (anti-tick vaccine, acaricides)

The genus *Dermacentor* is one of the most important, comprising a total of 30 species distributed in Europe, Asia and North America (Soneshine et al., 2002). In Europe there are two important species belonging to the genus *Dermacentor*, namely *Dermacentor marginatus* and *Dermacentor reticulatus*. These species are important vectors for diseases such as canine and ruminants babesiosis, SFG ricketssiosis ("Spoted Fever Group") and TIBOLA (Tick-Borne Lymphadenopathy) (Selmi et al., 2008, Foldvari et al., 2013). Sampling was intended to cover a big area of their geographical distribution. Nineteen ticks of *Dermacentor marginatus* from 9 countries (France, Portugal, Italy, Germany, Hungary, Slovakia, Romania, Turkey, Iran and Tunisia) and nine *Dermacentor reticulatus* from 6 countries (France, Portugal Germany, Slovakia, Romania, Turkey) were included in this study. Mostly all ticks were collected by dragging method, only one specimen from Turkey was collected from host.

For DNA extraction ticks were first cut with a scalpel and than disrupted using a Tissue Lyser (Qiagen, Netherlands), DNA isolation was performed with Macherey-Nagel NucleoMag 96 Tissue Kit following the manufacturer instructions. The DNA was eluted in 100µl of rehydration solution available from the kit and stored at -80°C.

One forward primer (5'GGGAGATGAGCTGGAATAATAGG3') and two reverse primers (5'AAATATAAACCTCAGGGTGGC3'; 5'ATATACTTCTGGATGCCCGA3'), were designed to amplify a fragment of 669 bp of the mitochondrial gene cytochrome oxidase subunit I. The PCR reaction was carried out in a 20µl final volume, containing 4µl Tick DNA template, 0.5µM of each primer, 0.2 mM of nucleotide mix, 1x Buffer, 3 Mm $MgCl_2$ and 1.25 units of Taq polymerase (EurobioTaq, Eurobie, France). Amplifications were performed using the following program: (i) one denaturation cycle at 94°C for 5 min, (ii) 35 cylces with 30 s at 94°C, 1 min at 59 °C and 1 min at 72 °C, (iii) final step at 72 °C for 10 min. PCR products were analyzed by electrophoresis on 1% agarose gel under ultraviolet light after staining with ethidium bromide. Subsequent to electrophoresis, PCR products were purified using the Nucleospin Extract® II kit (Macherey-Nagel, Germany) and sent for sequencing.

Sequences were manually inspected and edited with the software Bioedit v 7.2.5 (Hall, 2005) and alignments were made with Geneious v 6.0.6. Phylogenetic analyses were conducted with Mega 6 software (Tamura et al., 2007) using Neighbour-Joining methods (NJ) with Maximum Composite Likelihood distances (MCL) (Tamura et al., 2004). Branch supports were calculated by boostrap analyses with 1000 (NJ) replicates (Felsenstein, 1985). *Rhipicephalus*

sanguineus GeneBank accession number AF132839.1 was used as outgroup to root the trees. The number of segregating sites, the number of haplotypes, haplotype and nucleotide haplotype were obtained using the software package DnaSP version 5.1 (Rozas et al., 2003) and Rarefaction Index of Haplotypes was calculated with Past v 3.03 (Hammer et al., 2001).

We obtained nineteen sequences from nineteen individuals of *Dermacentor marginatus* and 9 sequences from *Dermacentor reticulatus* of 542 bp post trimming of cytochrome oxidase subunit I mitochondrial gene. For comparison 21 sequence of cytochrome oxidase subunit I from 21 individuals ticks belonging to Ixodes ricinus species were trimmed at the same length and included in this study.

Twelve haplotypes were found for *Dermacentor marginatus* in 19 individuals analyzed, identified by 15 variable sites (14 synonymous and 1 non-synonymous), 5 singleton variable sites and 10 parsimony informative sites. Three haplotypes were found for *Dermacentor reticulatus* in 9 individuals analyzed, identified by 2 singleton variable sites (both synonymous). The haplotype diversity was high for *Dermacentor marginatus* (0.95322) and *Ixodes ricinus* (0.8952) and moderate for *Dermacentor reticulatus* (0.41667). Nucleotide diversity was also calculated for each species, showing similar values for *Dermacentor marginatus* (0.00671) and Ixodes ricinus (0.0699). Instead nucleotide diversity registered for *Dermacentor reticulatus* was extremely low (0.00082).

This study represents the first attempt of describing genetic diversity of the two species of the genus *Dermacentor* in Europe, namely *Dermacentor marginatus* and *Dermacentor reticulatus.*

Both species showed low level of nucleotide diversity, even if *Dermacentor marginatus* haplotype diversity was high, predominantly due to single point mutations. Similar findings were recorded for *Ixodes ricinus* , while *Dermacentor* reticulatus exhibit an impoverished genetic variability with 0.00082 Nd. The wide geographical distribution along with a pronounced variety of host used by *Dermacentor marginatus* can explain the high haplotype diversity and nucleotide diversity registered by *Dermacentor marginatus* compared with *Dermacentor reticulatus*. Instead the low values of haplotype diversity and nucleotide diversity registered by Dermacentor reticulatus can be attributed to the low variety of hosts used to complete his life cycle and to the narrower geographical distribution compared with *Dermacentor marginatus.*

All the molecular tests were performed at **"UMR INRA - ONIRIS , BioEpAR 1300", Nantes, France under supervision of Senior Scientist Olivier PLANTARD**

The thesis ends by presenting the **General Conclusions** in **Chapter VII**.