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(Cycle XXVII)

Doctoral Thesis

New molecular tools for the study of the sheep tick (*I. ricinus* L.): development, application and epidemiological implications

(VET/06)

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It will be a long list of thanks, but the work carried out needed complex interactions of people and expertise to get to the presented results. So, …

Thanks to Valentina Tagliapietra, Daniele Arnoldi, Ivana Baràková and everyone else helping in ticks collections from all around Trentino and from any catchable vertebrate animal. I am especially grateful to Franco Rizzolli, because without his expertize bird ticks collection would not have been possible; thanks him to introduce me to the fascinating birds world. Thanks to Associazione Cacciatori del Trentino and its wardens and hunters for the collaboration and for their interest in this project.

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A special thanks to Prof. Jeremy Searle, that made me feeling actually part of his research team at Searle lab at the Cornell University, and that made a dream come true; but also to Prof. Giorgio Bertorelle and his research group at the Università di Ferrara for their help in bioinformatics analysis.

Finally, thanks to my wonderful family that encouraged and supported me in any possible way, by sharing doubts, happy moments and ticks collection, and pushing me to strive for, and to wish, all the best from the life.

At last, but not least, a deep thanks to my husband Ilario, that being half of my soul, suffered and enjoyed together with me in this path of growth.

PUBLICATIONS, PRESENTATIONS AND OTHER SCIENTIFIC ACTIVITIES

PEER REVIEWED

Collini M., Albonico F., Hauffe H.C. and Mortarino M., 2015 (in press), Identifying the last bloodmeal of questing sheep tick nymphs (*Ixodes ricinus* L.) using high resolution melting analysis, *Veterinary Parasitology* DOI: 10.1016/j.vetpar.2015.04.007 [IF 2.7]

> All authors designed the research. Under FA supervision, MC optimized the primers and performed the laboratory analyses. All authors contributed to the paper, but MC wrote the first draft, prepared all Tables Figures and Supplementary files, and made the final submission. The developmental procedure is reported here in section 3.3.5.3 and results in 4.3.5.

Baráková I., Derdáková M., Carpi G., Rosso F., Collini M., Tagliapietra V., Ramponi C., Hauffe H.C., Rizzoli A., 2014, Genetic and ecologic variability among strains of *Anaplasma phagocytophilum*, northern Italy [letter], *Emerging Infectious Diseases*, 20: 1082–1085 [IF 7.3]

> MC organized the collection of deer legs and collected many of the feeding ticks from different hosts, especially deer, birds and rodents (section 3.2.2). Feeding and questing ticks were identified morphologically and by molecular analyses by using the newly designed 16SrRNA primers and protocol that MC optimized (see section 3.3.2).

In preparation:

Collini M., Albonico F., Rosà R., Tagliapietra V., Rossi C., Rizzoli A., Mortarino M., Hauffe H.C., Hosts use by *Ixodes ricinus* peri-urban and extensive forests by Real-time HRMA bloodmeal analysis: epidemiological implications *OR* Domestic dogs are important larval wood tick hosts, especially in peri-urban forests [target journal *Parasites and Vectors*; IF 3.25]

Collini M., Trucchi E., Panziera A., Bertorelle G., Tagliapietra V., Arnoldi D., Rossi C., Rizzoli A., Hauffe H.C., High gene flow between I. ricinus populations in the northeastern Alps detected using RAD-Seq analysis

SCIENTIFIC POSTER

Collini M., Hauffe H.C., Masséglia S., Albonico F., Arnoldi D., Bailly X., Bard E., Galan M., Rossi C., Tagliapietra V., Vourc'h G., Rizzoli A., Mortarino M., 2015, Identifying the last bloodmeal of questing wood tick nymphs (*Ixodes ricinus* L.) by DNA amplification: three approaches tested, Heraklion, Crete, Greece, "Genes, Ecosystems and Risk of Infections", 21-23 April 2015.

AWARDED one of two POSTER PRIZES at the International Conference "Genes, Ecosystems and Risk of Infections" (GERI), Heraklion, Greece, in April 2015, for being "very efficiente in addressing a complex issue".

SCIENTIFIC CONFERENCE PRESENTATIONS (speaker is in **bold**)

Collini M., Hauffe H.C., Rizzoli A., Vernesi C., Arnoldi D., Baráková I., Cornetti L., Crestanello B., Girardi G, Konecny A, Rossi C, Rosso F, Tagliapietra V, Albonico F, Mortarino M., Population genetic of the wood tick *Ixodes ricinus* (acari: Ixodidae): new insight into dispersal capacity in relation to host movements - Bloodmeal analysis in questing ticks: application of High Resolution Melting (HRM), Bellaterra, EDENext Annual Meeting, PhD Day, 18 March 2013

Crestanello B., **Collini M.,** Il contributo della genetica della conservazione alla conoscenza e alla gestione di alcune specie di Galliformi alpini: pernice bianca *Lagopus muta*, fagiano di monte *Tetrao tetrix* e gallo cedrone *Tetrao urogallus*, VII Convegno Italiano di Ornitologia, Trento, 13 September 2013

Plantard O., Quillery E., Collini M., Panziera A., Trucchi E., Rizzoli A., Hauffe H.C., Using population genetics to assess tick dispersal, from the mainland to the landscape scale: a review of current knowledge and its utility to design tick-control methods, Heraklion, Crete, Greece, "Genes, Ecosystems and Risk of Infections", 21-23 April 2015 [as coauthor, I provided 4 slides on my research on RAD-Seq for the speaker]

SCIENTIFIC MEETINGS PRESENTATIONS (speaker is in **bold**)

Collini M., **Hauffe H.C.,** Population Genetics of the wood tick Ixodes ricinus (Acari: Ixodidae): new insight into dispersal capacity and host preference. FP7 TBD EDENext Meeting. Bratislava, Slovakia. 25 January 2012. [I could not make the presentation because I was ill and could not attend at the last minute; however, I prepared the talk myself]

Hauffe H.C., Collini M., Population Genetics of the wood tick Ixodes ricinus (Acari: Ixodidae): new insight into dispersal capacity and host preference. FP7 EDENext

Annual Meeting. Izmir, Turkey. 28 March 2012. [I prepared the talk together with my supervisor, H.C. Hauffe]

Hauffe H.C., Collini M., Rizzoli A., Biology of the wood tick Ixodes ricinus. EDENext Annual Meeting, Rovaniemi, Finland. 25 March 2014. [I prepared the talk together with my supervisor, H.C. Hauffe]

Collini M., Presentazione attività PhD project, D-BEM PhD Day, Fondazione Edmund Mach, 6 June 2013.

INVITED PRESENTATIONS

Collini M., Population genetics of the wood tick *Ixodes ricinus* (acari: Ixodidae): new insight into dispersal capacity in relation to host movements, 21 November 2012, Centre for Ecological and Evolutionary Synthesis, University of Oslo, Norway.

Collini M., Population genetics of the wood tick *Ixodes ricinus* (acari: Ixodidae): new insight into dispersal capacity in relation to host movements, 8 April 2014, Searle Lab meeting, Ecology and Evolutionary Biology Dept., Cornell University NY, USA.

PROPOSAL FOR PATENT AT FEM

H.C. Hauffe and M. Collini presented a new technique of possible patent interest to the CRI IP Committee.

Collini M., Hauffe H.C., Mortarino M., Albonico F., 2013, Patent Proposal: Application of High Resolution Melting to identification of host and disease-causing pathogens in wood ticks.

OUTREACH

Collini M., La Notte dei Ricercatori. Participation as high school student guide; Trento, 26 September 2012.

Hauffe H.C., Collini M., Ossi F., Obertegger U., “Ecofiera” di Tione di Trento (TN): Fondazione E Mach. La biodiversità dietro casa... Impariamo a ri-conoscerla! 28.100 visitors, 4 newpaper articles. 3-6 October 2013.

STUDENTS SUPERVISED

Ben Fowle, Kieran David O'Malley, Alessandra Fauri, Francesca Michelon.

COURSES

Gene Expression and Pathway Reconstruction, Phylogeny & Evolution Day, International PhD Programme in Genomics and Molecular Physiology of Fruits (gmpf) (FEM), San Michele a/A, Italy. 26-29 June 2012.

Population and Quantitative Genetics, International PhD Programme in Genomics and Molecular Physiology of Fruits (gmpf), FEM, Viote del Monte Bondone, Italy. 2-6 July 2012.

RADseq experimental design and analysis course, GENECO, University of Lund, Lund, Sweden, 15- 19 ottobre 2012.

International Environment 'Omics Synthesis 2013, Univ. Of Cardiff, UK, 9-11 September 2013.

RAD-Seq library preparation for *Ixodes ricinus* population genetic study. RAD-TAG platform, Centre for Ecological and Evolutionary Synthesis, University of Oslo, Norway, November 2013.

Visiting fellow at Searle Lab, Ecology and Evolutionary Biology Department, Cornell University (NY- USA), 2 April - 29 September 2014.

NEWSPAPER ARTICLES

Collini M., 2011, La Pernice bianca alpina: una sottospecie endemica in declino. *Il Cacciatore Trentino*, 83, 14-15

Collini M., 2012, I piccoli abitanti del bosco, *La Gus dai Buiac* - Comune di Giustino, 15

ABBREVIATIONS

bp: base pairs

DBEM: Department of Biodiversity and Molecular Ecology, Research and Innovation Centre, FEM

dsDNA: double strand DNA

EXTF: extensive forest

FEM: Fondazione Edmund Mach

gDNA: genomic DNA

HRMA: High Resolution Melting Analysis

LB: Lyme borreliosis

MDS: Multi-Dimensional Scaling

mtDNA: mitochondrial DNA

NGS: Next Generation Sequencing

PAT: Provincia Autonoma di Trento/ Province of Trento

PATF: forest patches near to villages

PCA: Principal Component Analysis

PCR: Polymerase Chain Reaction

RAD-Seq: Restriction-site Associated DNA Sequencing

RFLP: Restriction Fragment Length Polymorphism

RLBH: Reverse Line Blot Hybridization

SNP: Single Nucleotide Polymorphism

TBD: Tick-borne diseases

TBEv: Tick-Borne Encephalitis virus

T_m: melting temperature

T_a: annealing temperature

VBD: Vector-borne disease

WP: work package

ABSTRACT

The sheep tick, *Ixodes ricinus*, is the most important zoonotic vector in Europe; its dispersal potential and the relative importance of various vertebrate hosts it exploits, both essential to understand emergence of tick-borne diseases are virtually unknown.

I applied two molecular approaches to 30 *I. ricinus* populations in the Province of Trento, Italy. A novel bloodmeal analysis Real-time HRMA protocol was developed and tested on questing nymphs. RAD-Seq was used for the first time on *I. ricinus* to individually genotype SNP loci in adult ticks.

Bloodmeal analysis confirmed that rodents feed about 30% of tick larvae, but also illustrate that large mammals play a central role in feeding larval ticks. Since birds also feed about 15% ticks, the results of this analysis imply that larval ticks are carried long distances by their hosts; hence dispersal potential is high. In fact, population genetics support that investigated alpine populations are genetically admixed, confirming other phylogenetic studies showing that panmixia of *I. ricinus* population is a general phenomenon. This is the first study showing that dogs are important sheep tick hosts. As dogs feed a higher proportion of ticks in peri-urban forest, they may enhance the contact rate between human and infected ticks, by bringing them into human habitations and urban parks.

My results have added new knowledge to tick dispersal and host use, which will be used to improve models of the spread of *I. ricinus*, and related pathogens, in new climatically suitable areas. My novel bloodmeal analysis protocol, which eliminates previous problems of contamination, could help to identify and explain local TBD dynamics in other areas of the EU.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 3

Chapter 4

LIST OF TABLES

1. GENERAL INTRODUCTION

Ticks (Acari: Ixodidae) are obligate hematophagous ectoparasites that exploit every class of vertebrate worldwide (Hoogstraal and Aeschlimann, 1982). Importantly, they are among the most important vectors for many pathogens affecting humans, as well as wild and domestic animals. Zoonotic spillover to humans has become a key public health concern in the last decades, especially since the incidence of Lyme borrelliosis (LB) (which emerged in the early 1980s) as well as tick-borne encephalitis (TBEv, known from the 1930s in Europe), have risen dramatically in the Northern Hemisphere (Parola and Raoult, 2001; Gray et al., 2009; Randolph, 2009). At the same time, the tick-borne diseases (TBDs) anaplasmosis and rickettsioses are also frequently reported in Europe (Heyman et al., 2010). In a global context, where climate change together with anthropogenic factors (trade, land use, etc.) are affecting TBD epidemiology by modifying tick population dynamics, habitat, hosts, pathogen reservoirs and human contact rate with infected ticks, these diseases are expected to become an increasingly important public health issue (Parola & Raoult, 2001; Heyman et al., 2010; Rizzoli et al., 2014).

In this thesis, I focus on the sheep (or wood) tick *Ixodes ricinus* L., defined as "the most important multi-potent vector in Europe" (Randolph, 2009). Many studies of this species have investigated the ecology of *I. ricinus* habitat, such as the impact of abiotic (e.g. climate) and biotic (e.g. vegetation) variables on its population, and local TDB dynamics and the interconnection between hosts, vector and pathogen (e.g.: Estrada-Peña et al., 2006; Estrada-Peña, 2009; Randolph et al., 2002; Cagnacci et al., 2012; Bolzoni et al., 2012; Rizzoli et al., 2007; Rizzoli et al., 2009; Rosà and Pugliese, 2007; Carpi et al., 2008; Krasnov et al., 2007; Hudson et al., 2001). In contrast, there is

very little knowledge about tick dispersal capacity or host preferences and host-specialization, information that would help the improvement in the modelling of TBD risk and spread (Bolzoni et al., 2012; Medlock et al., 2013; Léger et al., 2013; Gray et al., 2009; Madhav et al., 2004).

As highlighted by De Meeûs and colleagues (2007), the study of zoonotic disease vectors is challenging, since these organisms are generally small, and certain features of their life cycle, usually including diapause, make direct observation of them almost impossible. A molecular genetics approach could provide indirect measures of population parameters (effective size, substructure, dispersal rate, and host-race formation) of the arthropod populations, based on the analysis of patterns of genetic variability with appropriate markers.

Here, feeding behavior and dispersal of *I. ricinus* were investigated using two *state-of-the-art* molecular approaches: high resolution melting analysis (HRMA) and Restriction-site Associated DNA Next Generation Sequencing (RAD-Seq NGS) technology. Since *I. ricinus* movement, and therefore gene flow, is correlated with host vagility, host use and dispersal capacity can be inferred from the comparative analysis of these two molecular techniques. Population genetics patterns can also tell us about population isolation and dynamics, which have epidemiological implications with regards disease emergence and spread.

The PhD project was designed and funded within the framework of the large collaborative FP7-HEALTH.2010.2.3.3-1 EDENext project 'Biology and control of vector-borne infections in Europe' (EDENext) at the Department of Biodiversity and Molecular Ecology, Research and Innovation Centre, Fondazione Edmund Mach (Italy)

in collaboration with the Dipartimento di Scienze veterinarie e sanità pubblica, Università degli Studi di Milano.

1.1 EDENext project

Initiated in 2011, EDENext brings together 46 international partners to investigate the biological, ecological and epidemiological components of vector-borne disease (VBD) introduction, emergence and spread and, using the newly acquired knowledge, to create new tools to control them (Fig. 1). EDENext takes advantage of the results, as well as concepts, methods and tools, of the earlier FP6 project *EDEN Vector-borne disease in a changing European environment*. While the latter focused on the effects of environmental changes on the emergence of VBDs, EDENext aims to explain and to model the processes leading to the introduction, spreading and establishment of VBD and, most importantly, to define the possible control strategies to break the epidemiological cycles of VBDs. The project addresses five groups of vectors and associated zoonoses: rodents and insectivores (RBD), hard ticks (TBD; Acari, Ixodidae), mosquitoes (MBD; Diptera, Culicidae), sand flies (PhBD; Diptera, Psychodidae), and biting midges (CBD; Diptera, Ceratopogonidae). Research activities are organized vertically into Work Packages (WPs) according to the five vector groups, while horizontal themes (Modelling, Data management and Public Health) provide technical input to the WPs and integration of the datasets and results (Fig. 1.1).

My PhD research project, as part of this framework, was designed to address the objectives of the WP 1.1.1 *Emergence and Spread of bacterial and protozoan tick-borne pathogens* for the sheep tick *I. ricinus*, closing gaps in the knowledge of its ecology and dispersal (Group 4). This WP brings together the expertise in population genetics and TBDs of the Fondazione Edmund Mach (FEM, Italy: headed by A.

Rizzoli, in collaboration with H.C. Hauffe) and the Institut National de la Recherche Agronomique (INRA, France: O. Plantard, G. Vourc'h).

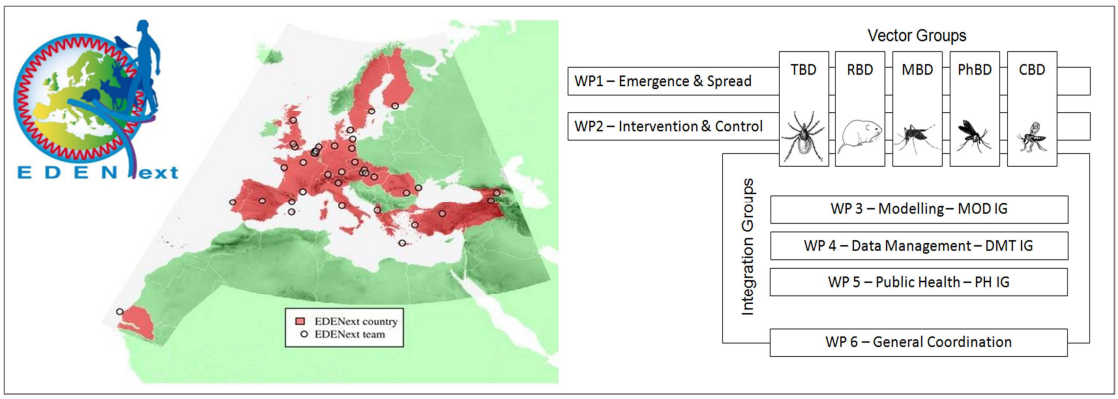


Fig. 1.1 The EDENext project: logo, participating countries and schematic representation of WP organization (reprinted from the EDENext Project).

1.2 The biology of *Ixodes ricinus* L.

Ticks (Suborder: Ixodida) belongs to the Phylum Arthropoda and, as part of the Subclass Acari, Order Parasitiformes, they are closely related to mites (Suborders: Holothyrida, Mesostigmata and Opilioacarida; Nava et al., 2009). The 896 known tick species are distributed worldwide and classified into three Families: the monotypic Nutalliellidae, containing a single species *Nutalliella namaqua*; Argasidae, consisting of 193 species; and Ixodidae comprising 702 species in 14 genera, the most numerous being *Ixodes* (243 species), *Haemaphysalis* (166) and *Amblyomma* (130) (Guglielmone et al., 2010). A recently published study of Mans et al. (2011), together with molecular clock estimates (Jeyaprakash and Hoy, 2009; Dunlop and Selden, 2009), dates the origin of ancestral tick lineages back to the middle Permian (260-270 Mya) in the Karoo-basin (South Africa), and suggests that they parasitised therapsids, based on the

phylogenetic evidence that *N. namaqua*, which feeds on lizards, is at a basal position in relation to the major tick families (Fig. 1.2).

Argasidae, called soft ticks, lack a sclerotized dorsal scutum; nymphs and adults present a ventral capitulum and a highly sculptured integument, and in their life cycles multiple nymphal instars are present. In contrast, Ixodidae, or hard ticks, are characterized by an anterior capitulum, a simple striate integument and a sclerotized scutum, and only one nymphal instar is required before molting to the adult stage. Ixodidae species are divided in two morphological groups according to the position of the anal groove: the Prostriata have the anal groove anterior to the anus, and the Metastriata have the anal groove posterior to the anus. Prostriata are represented only by the genus *Ixodes* and are considered the basal lineage to all the other genera included in the Metastriata (Nava et al., 2009).

I. ricinus L. has been placed, together with 13 other *Ixodes* species, in the *I. ricinus* complex (Xu et al., 2003), a paraphyletic group of closely related and morphologically similar species that are all vectors for Lyme diseases spirochetes. However, the paraphyly of the group suggests that acquisition of the ability to transmit borreliosis agents, distributed worldwide, may have multiple origins (Fig. 1.3).

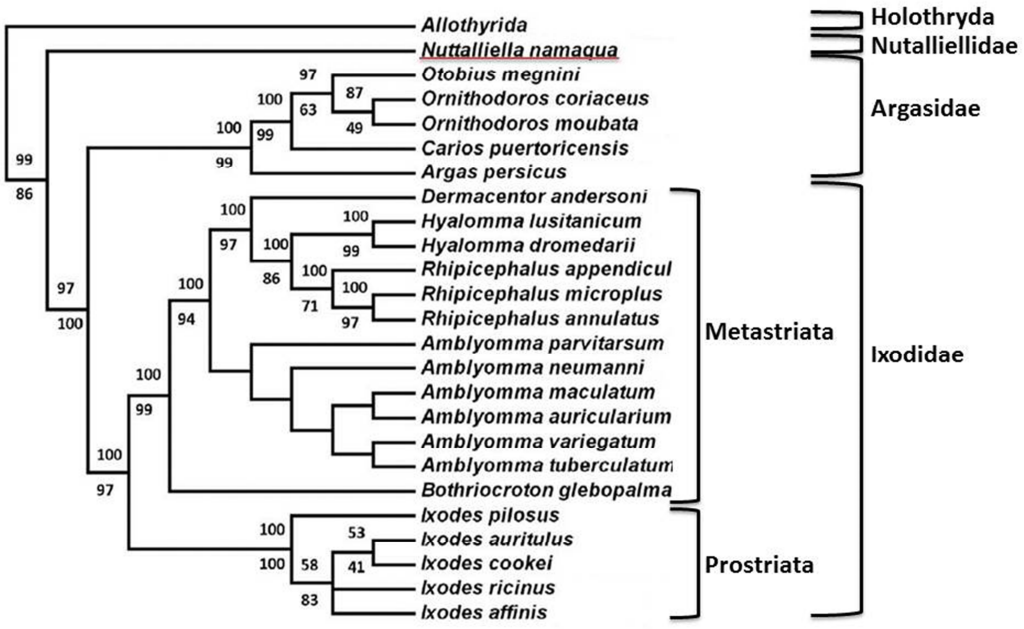


Fig. 1.2 The phylogenetic tree of Ixodida (18S-16S rRNA genes), obtained with Bayesian as well as maximum parsimony analysis. Posterior probability and bootstrap support values are indicated above and below the nodes, respectively (reprinted from Mans et al., 2011).

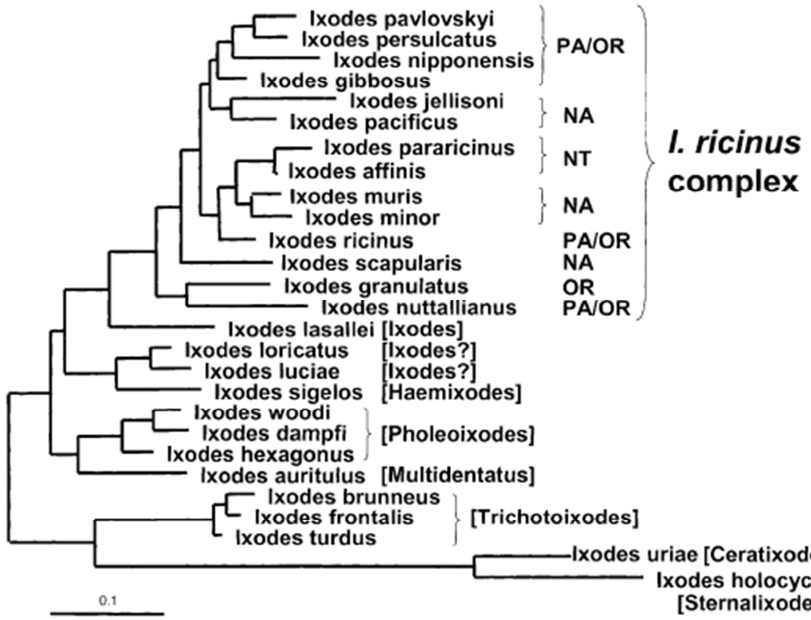


Fig. 1.3 16S Bayesian tree of *Ixodes* species, with special reference to the *I. ricinus* complex; it indicate that members of the *I. ricinus* species complex are closely related despite the fact that they are distributed in different geographic regions of the world (OR=oriental region; NA= Neartic region; NT= Neotropical region). However, the complex is not a monophyletic group unless 3 more species, *I. muris*, *I. minor*, and *I. granulatus*, are also included in it (reprinted from Xu et al. 2003).

The *I. ricinus* life cycle consists of three instars: larva, nymph and adult, commencing from the hatching of the clutch of eggs deposited on the ground by the female (Fig. 1.4). Sexual dimorphism only appears at the adult stage. This species is defined as a 'three-host tick', meaning that each instar takes a single bloodmeal (but see Results and Gray et al., 1999), before molting to the next stage. *I. ricinus* is an exophilic ticks, i.e. actively questing on the vegetation in order to latch on to passing hosts. The feeding phase usually takes several days, and varies between life stages: larva, 2–3 days; nymph, 4–5 days; adult female, 7–9 days. The adult male rarely feeds and never becomes fully engorged. As the majority of their lifespan is spent independently from hosts, questing on the vegetation or developing to the next instar in the ground litter, ticks are very vulnerable to desiccation. Hence, in both the questing and developing phases, ticks can obtain water from sub-saturated air by secreting and then re-ingesting hygroscopic fluid that is produced by the salivary glands. However, this process promotes tick survival only if the relative humidity in their microclimate does not fall below 80%. Accordingly, preferential habitat of the sheep tick includes areas with good vegetation cover and a mat of decaying vegetation on the ground, such that even during the driest periods of the year, a damp microclimate is maintained near the ground. Deciduous woodland, particularly those containing oak and beech, harboring good numbers of large mammals are preferred to conifer forest and cultivated lands, however ticks may also be found in abandoned farmland with dense underbrush, and in patchy vegetation in sub-urban and urban environments (Gray et al., 1998, 1999; Lindgren and Jaenson, 2006; Cagnacci et al., 2012; Rizzoli et al., 2009). Large vertebrate hosts such as wild ungulates, carnivores, cattle and sheep, are the main determinants of tick abundance in preferred habitat, as the female needs a large

bloodmeal to produce eggs, whereas larvae and nymphs are less demanding in their host preference, feeding in addition to large mammals, on a variety of birds, rodents, insectivores, and reptiles (Hoogstraal and Aeschlimann, 1982). Humans are considered accidental hosts (Fig 1.4; Gray et al., 1998; Lindgren and Jaenson, 2006).

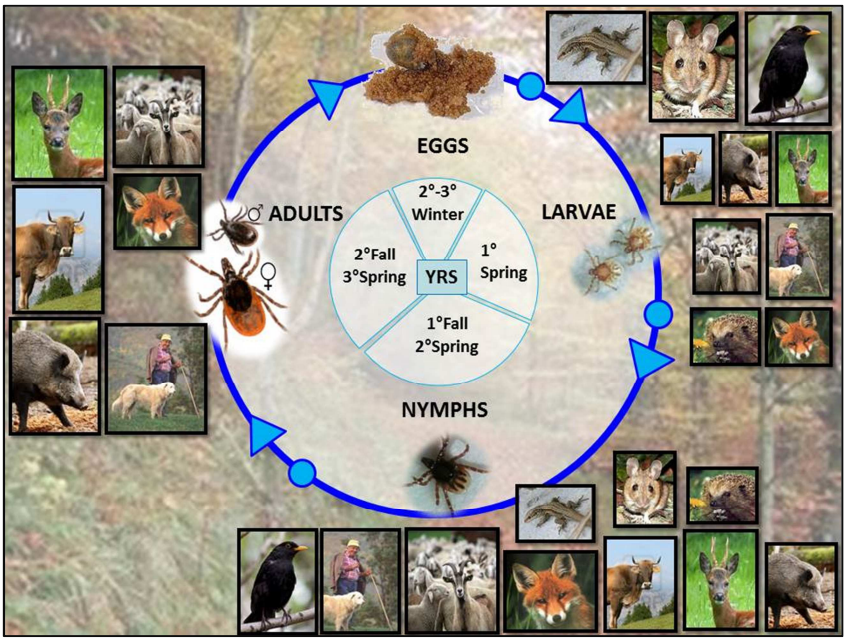


Fig. 1.4 *Ixodes ricinus* life cycle; between each of the life stages (larva to nymph, nymph to adult, adult for producing eggs), the sheep tick needs a bloodmeal. As a generalist ectoparasite, *I. ricinus* uses 350 different vertebrate species as hosts, including, occasionally, humans (Hoogstraal and Aeschlimann, 1982).

The phenology of *I. ricinus*, i.e. the variation in abundance of the three developmental stages over time and space, is known to be greatly influenced by climatic factors, but also host populations seasonal abundance (Tagliapietra et al., 2011; Randolph et al., 2002; Estrada-Peña et al., 2006; Medlock et al., 2013; Randolph, 2008, 2009; Gray et al., 2009; Gilbert, 2010). Ticks finely regulate their questing activity and developmental phases according to these variables, thanks to two diapause mechanisms:

the developmental diapause, involving arrested development of the engorged stages or of eggs, and the behavioral diapause, involving a form of quiescence of the unfed ticks at times when environmental conditions are unsuitable for seeking hosts (Gray et al., 1998). These mechanisms enable the tick to avoid entering host-seeking phases at unfavorable times of the year, such as high summer and winter. Since tick population phenology may be highly variable according to local climatic conditions (Randolph et al., 2002; Tagliapietra et al., 2011), completion of tick development to the adult stage may take from 2 to 6 years.

Ticks are responsible for the transmission of a variety of microorganisms including bacteria, viruses and protozoa, and toxin associated diseases, to wild and domesticated animals, as well to humans. Ticks are recognized as second only to mosquitoes in importance for the transmission of zoonoses (Parola and Raoult, 2001). The significance of *I. ricinus* as a vector is the result of various peculiarities in its biology; in fact, its wide host range permits interaction with potentially infected reservoir hosts in vertebrate communities, and allows different stages to feed on the same hosts and the trans-stadial maintenance of infection, greatly improves its performance as vector (Randolph et al., 2004). Therefore, it is epidemiologically relevant to discriminate, within the host community, between 'tick maintenance hosts' (or amplifying hosts, mainly large bodied mammals) involved only in feeding tick populations and regulating density; and 'reservoir hosts' (rodents and birds for Borrelia spp.; rodents for TBEv), that maintain and amplify the pathogens involved in the zoonotic cycle and are responsible for infecting the ticks, except in the case of co-feeding, where an infected tick could transfer the pathogen to nearly feeding ticks on a non-infected hosts (Gray et al., 1998; Randolph et al., 1997). As vector potential of a

pathogen in a certain area is a function of the vector-reservoir host contact rate, the specific composition of the host community could have a great impact on the epidemiological cycle of TBD. In fact, some studies (Ostfeld and Keesing, 2000; Schmidt and Ostfeld, 2001; LoGiudice et al., 2003) have underlined that the presence of incompetent host species for a specific pathogen could decrease the transmission of the pathogen itself by diverting tick bite (i.e. infection of new ticks) from more competent hosts. This is called the 'diluition effect' and seems to be likely supported by high levels of biodiversity (Johnson and Thieltges, 2013). However, the relationship between biodiversity and zoonotic diseases appears to be case-specific rather than a general dynamic and still needs careful evaluation in disease epidemiology (Pfäffle et al., 2013).

Rizzoli et al. (2014) reports an updated list of the main *I. ricinus* associated pathogens and hosts (Fig. 1.5). Even if the list refers to host-pathogen association retrieved in the European peri-urban and urban environment, it is also valid for zoonotic cycles taking place in natural systems, where, in terms of human health, TBD risk is more meaningful.

Fig. 1.5 (next page) List of the most important host group and species involved in *I. ricinus*-borne diseases systems in peri-urban and urban habitats. Pathogens in bold indicate those for which reservoir competence has been experimentally proven. (reprinted from Rizzoli et al., 2014).

Order	Species	Associated *I. ricinus* stage	Associated pathogens
Rodentia	*Apodemus flavicollis*	L, N	**TBEV** ***Borrelia afzelii*** ***Borrelia burgdorferi* s.s.** ***Borrelia spielmanii*** ***Borrelia miyamotoi*** ***Cand.* N. mikurensis** *Anaplasma phagocytophilum* *Babesia microti*
	Apodemus sylvaticus	L, N	**TBEV** ***Borrelia afzelii*** ***Borrelia burgdorferi* s.s.** ***Borrelia spielmanii*** ***Cand.* N. mikurensis** *Anaplasma phagocytophilum* *Babesia microti*
	Apodemus agrarius	L, N	***Borrelia afzelii*** ***Cand.* N. mikurensis** *Anaplasma phagocytophilum* ***Babesia microti***
	Myodes glareolus	L, N	**TBEV** ***Borrelia afzelii*** ***Borrelia burgdorferi* s.s.** ***Borrelia miyamotoi*** ***Cand.* N. mikurensis** *Anaplasma phagocytophilum* *Babesia microti*
	Microtus agrestris	L, N	**TBEV** *Borrelia afzelii* *Babesia microti* *Cand.* N. mikurensis *Anaplasma phagocytophilum*
	Microtus arvalis	L, N	*Cand.* N. mikurensis *Anaplasma phagocytophilum* *Babesia microti*
	Rattus norvegicus	L, N	***Borrelia afzelii*** ***Borrelia spielmanii***
	Rattus rattus	L, N	*Borrelia afzelii* *Anaplasma phagocytophilum*
	Eliomys quercinus	L, N	***Borrelia spielmanii***
	Muscardinus avellanarius	L, N	***Borrelia spielmanii***
	Glis glis	L, N	**TBEV** *Borrelia afzelii*
	Sciurus carolinensis	L, N	***Borrelia afzelii*** *Borrelia burgdorferi* s.s.
	Sciurus vulgaris	L, N	**TBEV** *Borrelia burgdorferi* s.s. *Borrelia afzelii* *Borrelia garinii*
	Eutamias sibiricus	L, N	*Borrelia burgdorferi* s.s. *Borrelia afzelii* *Borrelia garinii*

Order	Species	Associated *I. ricinus* stage	Associated pathogens
Lagomorpha	*Lepus europaeus*	L, N, A	*Borrelia burgdorferi* s.l. *Anaplasma phagocytophilum*
	Lepus timidus	L, N, A	*Borrelia burgdorferi* s.l.
Soricomorpha	*Sorex araneus*	L, N	**TBEV** *Borrelia burgdorferi* s.l. *Anaplasma phagocytophilum* *Babesia microti*
	Sorex minutus	L, N	*Borrelia burgdorferi* s.l.
Erinaceomorpha	*Erinaceus europaeus*	L, N, A	*Borrelia afzelii* *Borrelia spielmanii* *Borrelia bavariensis* *Anaplasma phagocytophilum*
	Erinaceus roumanicus	L, N, A	**TBEV** *Borrelia afzelii* *Borrelia bavariensis* *Anaplasma phagocytophilum* *Cand.* N. mikurensis
Artiodactyla	*Capreolus capreolus*	L, N, A	*Anaplasma phagocytophilum* *Babesia venatorum*
	Cervus elaphus	L, N, A	*Anaplasma phagocytophilum*
	Dama dama	L, N, A	*Anaplasma phagocytophilum*
Carnivora	*Vulpes vulpes*	L, N, A	*Borrelia burgdorferi* s.l. *Anaplasma phagocytophilum*
	Meles meles	L, N, A	*Borrelia afzelii* *Borrelia valaisiana*

Fig. 1.5 Continued

The two most important TBDs in Europe are Lyme disease (LB) and Tick Borne Encephalitis (TBE). LB is a multi-systemic inflammatory disorder caused by an immune response to the pathogenic genspecies of *Borrelia burgdorferi* sensu lato, and is the most prevalent arthropod-borne disease in the temperate regions of the northern hemisphere, causing approximately 65,500 patients yearly in Europe. As is evident from Fig. 1.5, experimentally confirmed competent reservoir hosts include many common

species of small and medium-sized rodents (mice, rats, squirrels, hares and rabbits), as well as several bird species (especially passerines), reptiles and insectivores. In contrast, large wild and domesticated mammals are considered non-competent hosts for the pathogen, but important vector-maintaining hosts (reviewed in Rizzoli et al., 2011). TBE, instead is caused by the TBE virus of the genus *Flavivirus* within the Flaviviridae family. It is present only on the Eurasian continent with three subtypes (European subtype, the Siberian sub-type and the Far Eastern subtype) which are associated with varying degrees of disease severity. The TBEv cycle involves permanently infected ticks and small mammals, especially rodents; transmission occurs horizontally between tick and vertebrate hosts, but co-feeding of infected and non-infected ticks on the same hosts and trans-stadial and trans-ovarial transmission of the virus, also play a major role in virus transmission (reviewed in Mantke et al., 2011).

Given the low active mobility of ticks in general (Falco and Fish, 1991), *I. ricinus* transportation on hosts while blood feeding is the only means of tick movement and introduction into new regions, along with vectored pathogens. Their survival in the new area is related to the climatic condition, but also on the presence of a suitable hosts community (reviewed in Léger et al., 2013; Gray et al., 2009; Semenza and Menne, 2009).

Concern for the impact of TBDs on human health has arisen from the current altitudinal and latitudinal expansion of *I. ricinus* populations, already widely distributed across Europe (Fig. 1.6; Léger et al., 2013; Medlock et al., 2012), but most importantly from the higher potential contact rate between ticks and humans as a result of increased human exploitation of tick habitat for recreational activities and by reports of infected ticks population in peri-urban and urban green areas of Europe (reviewed in Rizzoli et

al., 2014). Several strongly interlinked factors are favoring the establishment of ticks populations in new environments: climate change (i.e. increase in the mean annual temperature and milder winters) is positively affecting both tick survival at higher altitudes and latitudes, as well as hosts community density; changes in land use (i.e. reforestation; fragmentation) and wildlife management are affecting hosts spatial distribution (Gray et al., 2009; Cagnacci et al., 2012; Medlock et al., 2013).

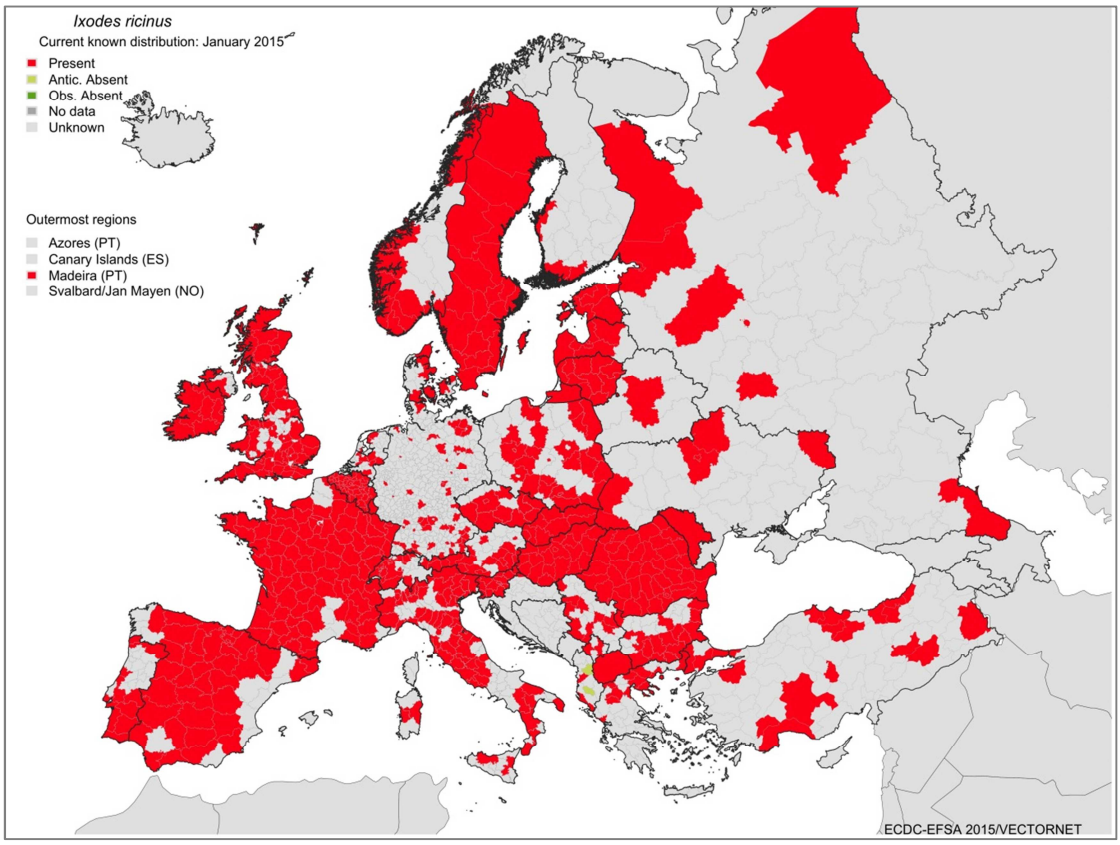


Fig. 1.6 *I. ricinus* distribution updated to January 2015, from www.ecdc.europa.eu.

1.3 Bloodmeal analysis in questing ticks

TBD systems are characterized by a complex network of interactions. Multiple vertebrate hosts are involved, many of them are competent reservoirs for etiological agents of infections (Fig. 1.5). In addition, transmission can occur vertically (e.g. trans-ovarial and trans-stadial), by co-feeeding, as well as by vector transmission between competent infected hosts and non-infected individuals. In this scenario, the knowledge of vector feeding ecology, host community composition and host status (competent reservoirs or amplification host) is of critical importance for understanding the epidemiology of VBD, in order to improve disease control strategies and to model and predict disease risk for public health (Mukabana, et al., 2002; Kent, 2009; Gómez-Díaz and Figuerola, 2010; Bolzoni et al., 2012).

In a generalist tick such as *I. ricinus*, estimation of host exploitation by field observations (i.e. host capture and tick counting) is generally difficult, expensive and may provide an unrealistic assessment, given the fact that *I. ricinus* spend only few days per life stage on the host (Kirstein and Gray, 1996, Estrada-Peña et al., 2005). Serological methods opened the way for indirect study of feeding patterns of hematophagous arthropods by means of analysis of their gut content, simply named *bloodmeal analysis*. But the advent of the polymerase chain reaction (PCR) and molecular genetic markers, and their ability to amplify DNA even in degraded and damaged samples (aDNA, Pääbo et al., 2004), has meant that DNA-based methods have been increasingly used for bloodmeal analysis (Mukabana et al., 2002; Kent, 2009; Gómez-Díaz and Figuerola, 2010), especially in combination with molecular screening for vectored pathogens. A number of methods have been applied to different hematophagous vectors, as summarized in Table 1.1 and reviewed in Kent (2009); these

usually rely on amplification of a target locus of the host DNA and its identification by means of various post-PCR techniques. Sequencing would be a more straightforward and specific method to identify the host, but the cost of this technique limits its application in high-throughput processing for large sample sets; therefore, more cost-effective post-PCR identification methods are usually employed, such as heteroduplex analysis, restriction length fragment polymorphisms (RLFP), and reverse line blotting hybridization (RLBH; Kent, 2009).

In contrast to mosquito vectors, where recently engorged females can be easily captured by using baited oviposition traps, field collection of engorged ticks following detachment from the host is not possible (Sonenshine, 1991). Only questing ticks can be collected easily by the conventional blanket dragging method; therefore, bloodmeal analysis can only applied to questing ticks. Because field-collected questing ticks may have had their previous bloodmeal up to one year before, in the previous instar (Randolph et al., 2002), specific issues must be addressed: i) only a few intact copies of host DNA are likely to be present, stored in the endosome of the tick midgut (Sonenshine, 1991); ii) remnant host DNA quality will be compromised by digestive and hemolytic process (Kirstein ans Gray, 1996; Sojka et al., 2013); iii) the presence of a high concentration of heme molecules inhibits polymerase activity; iv) environmental DNA or human DNA contamination may mask the true DNA bloodmeal signature. Using PCR-based methods, points i) and ii) have been addressed by using short amplicons of mitochondrial DNA. The mitochondrial genome may be present in hundreds to thousands copies in a single cell, making mitochondrial markers an ideal option to robustly amplify vertebrate DNA from the tiny bloodmeal remnants; in addition, vertebrate mtDNA has an evolutionary rate 5 to 10 times faster than the

nuclear genome and a rapid mutation fixation between species, which makes this molecule valuable for species identification, even when short fragment are targeted (Kirstein and Gray, 1996; Kent, 2009). Mitochondrial DNA can be retrieved in all blood cells of birds, while for mammals, having anucleated red blood cells, leukocytes and epithelial tissue still contribute in significant numbers. To cope with PCR inhibitions and contamination problems (points iii) and iv)), a careful protocol design is required, from primers selection to reagent and reaction optimizations. All sample handling procedures must be carried out in sterile conditions and the use of negative controls in DNA extractions and PCR provide a control for contamination.

Kirstein and Gray (1996) were the first to report bloodmeal analysis in questing ticks by means of host DNA amplification with degenerate vertebrate *cytb* primers and host identification with RLFP and RLBH. They proved the ability of molecular methods to reliably amplify host DNA up to 200 days post-engorgement, and defined RLBH has the most feasible method for host DNA identification. Since then, a variety of PCR-RLBH methods have been tested, gradually extending the array of probes for host identification. Pichon and colleagues (2003, 2005, 2006) targeted the multicopy 18S rRNA gene, while Humair et al. (2007) and Morán Cadenas et al. (2007), applying Humair's protocol, targeted the 12S rDNA vertebrate gene. Although partially successful, these methods show a wide variability in bloodmeal identification success (from 26.4% Bown et al., 2009 to 49.4% Pichon et al., 2005 and 62.8% Allan et al., 2010 on *Amblyomma americanum*) according to questing ticks collection time (i.e. time passed since the last bloodmeal), collection site microclimate (affecting speed in digestion) and life stage (quantity of ingested blood) (Morán Cadenas et al., 2007; Pichon et al., 2006). In addition, the taxonomic level of host identification reached in

these studies varies according to the set of probes developed: in the majority of the publications cited above, most hosts were only identified to group or genus level, and rarely to species.

Recently, a PCR-RLFP protocol, targeting the *12S* rDNA gene has been optimized and applied to a large sample dataset (Wodecka et al., 2014). This method appears to provide a higher mean identification success than reported for RLBH (62.8%). However, although the article claims that RFLP patterns are available for about 60 host species, only 19 host species were actually identified in the study.

Alternative methods to DNA-based ones have been proposed, such as protein analysis (Wickramasekara et al., 2008; Laskay et al., 2012), proteomics-based, spectral-matching (Önder et al., 2013), and stable isotope analysis (SIA; Rasgon, 2008), but none of them is well-established or have been applied to large sample of questing ticks.

The large body of tick bloodmeal analysis studies and the range of methods proposed underline the importance of this topic in the epidemiological study of TBDs, but there is still no reliable method free from contaminations and sensitivity problems (Gómez-Díaz and Figuerola, 2010; Estrada-Peña et al., 2013; Estrada-Peña et al., 2005). Within the present thesis, I aim to define a new protocol for bloodmeal analysis, including the application of this new method to the study of feeding patterns of *I. ricinus* larvae in the Province of Trento using a large collection of questing ticks. As reported in the Materials and Methods chapter, we began by testing if there was still room to improve the current DNA sequencing approaches, by designing new primers and by performing a careful optimizations. However, the unsatisfactory results led us to move to a more recent and promising approach: HRMA.

Table 1.1 Comparative overview of current molecular methods for arthropod bloodmeal identification (reprinted from Kent 2009).

Marker	Technique	Benefits	Drawbacks	Applications
COI	DNA sequencing	*Can get specific identity of the host *Sequence database excellent *Can be used to confirm results of another method	*Requires expensive sequencing equipment or contracting services *Sequence data may be missing for some species, potentially leading to bloodmeal mis-identification	*Mosquitoes*: Kent *et al.* (unpublished data)
cytb	DNA sequencing	*Can get specific identity of the host *Sequence database good *Can be used to confirm results of another method	*Requires expensive sequencing equipment or contracting services *Sequence data may be missing for some species, potentially leading to bloodmeal mis-identification	*Mosquitoes*: Kent *et al.* (unpublished data); Hamer *et al.* (2008); Savage *et al.* (2007); Molaei *et al.* (2006, 2007); Kilpatrick *et al.* (2006a, 2007); Richards *et al.* (2006); Cupp *et al.* (2004); Apperson *et al.* (2002, 2004); Hassan *et al.* (2003) *Ticks*: Tobolewski *et al.* (1992); *Blackflies*: Malmqvist *et al.* (2004); Hellegren *et al.* (2008)
	Group-specific primers	*Can obtain broad classification for bloodmeal *Can potentially detect mixed bloodmeals *Only requires PCR and gel electrophoresis	*Conserved primers may be cross-reactive (see Table 1 footnote) *Amplicons often sequenced anyway for more specific results *Multiplexing can sometimes lead to problems with primer interference	*Mosquitoes*: Kent & Norris (2005); Ngo & Kramer (2003); Temu *et al.* (2007); Molaei *et al.* (2006, 2007)
	Heteroduplex analysis	*Sensitive *Many samples can be analysed at once	*Technique difficult to master *Results may be ambuigous and/or need sequence confirmation *Only samples on the same gel can be reliably compared	*Mosquitoes*: Lee *et al.* (2002); Hassan *et al.* (2003); Apperson *et al.* (2002, 2004); Richards *et al.* (2006); Savage *et al.* (2007); *Black flies*: Boake *et al.* (1999); *Tsetse flies*: Boake *et al.* (1999); Njiokou *et al.* (2004); Simo *et al.* (2008)
	PCR–Restriction Fragment Length Polymorphism	*Can target nucleotide substitions or minor sequence differences between organisms	*Requires prior knowledge of polymorphic restriction sites *Requires RFLP profile library to match unknown samples	*Mosquitoes*: Ngo & Kramer (2003); Oshaghi *et al.* (2006a); *Tsetse flies*: Steuber *et al.* (2005); *Ticks*: Kirsten & Gray (1996)
	Terminal RFLP	*Good, user-friendly database for searching results	*Complex, labor-intensive procedure *Sequencing equipment necessary	*Mosquitoes*: Meece *et al.* (2005)
	Real-time PCR	*Highly sensitive and specific *Added ability to quantify starting template	*Need fluorescent probes, real-time thermal cycler and software *Limited number of flurophores available	*Mosquitoes*: Van Den Hurk *et al.* (2007) *Fleas*: Woods *et al.* (unpublished data)
18S, 12S rDNA	Reverse line-blot hybridization	*Can screen many samples for many hosts simultaneously *Potential to identify mixed bloodmeals *Less expensive than similar microarray technology	*May require designing and optimizing novel primers and probes if not already published	*Ticks*: Pichon *et al.* (2003, 2005); Humair *et al.* (2007); Morán Cadenas *et al.* (2007)
Microsatellites	DNA profiling	*Can match bloodmeals to individual hosts	*Dependent on the characterization of microsatellite loci from the host being studied *Requires profiles be generated for all individuals potentially fed upon	*Mosquitoes and people*: Chow-Shaffer *et al.* (2000); Michael *et al.* (2001); De Benedictis *et al.* (2003); Soremekun *et al.* (2004); Scott *et al.* (2006); *Mosquitoes and birds*: Darbro *et al.* (2007); *Tsetse flies and cattle*: Torr *et al.* (2001)

1.3.1 High Resolution Melting Analysis

Invented in 2002 by University of Utah and Idaho Technology, HRMA is a simple, rapid post-PCR method, widely applied for genotyping, mutation scanning and sequence matching, as well as for pathogen screening and identification (Gundry et al., 2003; Wittwer et al., 2003, 2009; Reed et al., 2007). HRMA exploits the fundamental property of double stranded DNA (dsDNA) to melt, that is, to separate into two strands with heat. The quantity of heat needed for the melting to occur strongly depends on the GC content, length and nucleotide arrangement of the amplified dsDNA fragment. Introduced in the 1960's, when melting was first monitored by UV-absorbance, melting analysis became popular from 1997 with the advent of the Real-time PCR LightCycler® and the use of fluorescent intercalating dye (Ririe et al., 1997). Thanks to new instruments and dyes, dsDNA melting can now be monitored at high resolution. The acquisition of high density data, i.e. fluorescence measures per unit time, and precise temperature control, allows detection of small variations in DNA sequences, down to single nucleotide polymorphisms (SNPs).

In HRMA, amplification of the dsDNA region of interest, using conventional or Real time PCR, is performed in the presence of a specialized dsDNA binding dye. The ability of this dye to be highly fluorescent when ligated into the dsDNA, while poorly fluorescent when DNA in the unbound state, is exploited for amplification control in Real time PCR, but in HRMA, it is used essentially to monitor the melting step: after PCR, the amplicons are gradually denatured by increasing the temperature in small increments (0.008 - 0.2 °C). The dsDNA fragments denature gradually, releasing the dye, which results in a drop of fluorescence (Fig. 1.7).

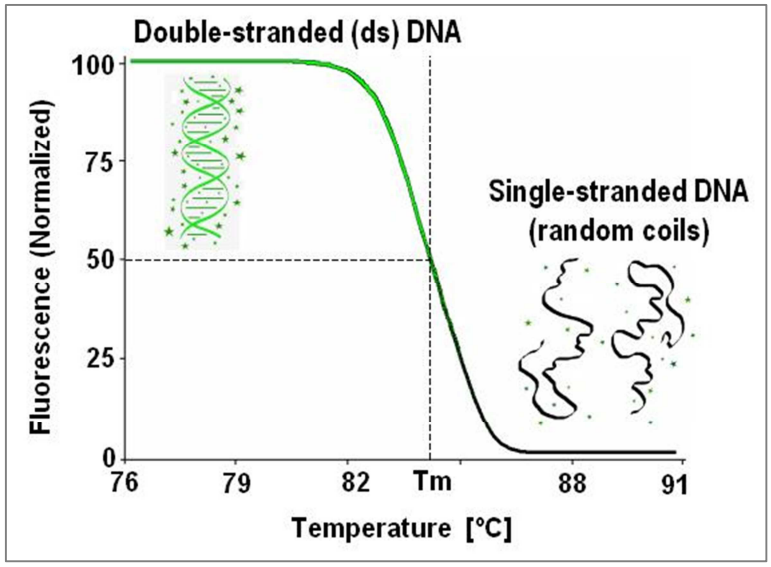


Fig. 1.7 Example of HRMA principle with a normalized fluorescence plot. (https://dna.utah.edu/Hi-Res/TOP_Hi-Res%20Melting.html)

Fluorescence readings and temperature changes collected during the HRM step are compared with specific software. First, visualization of the 'raw melting curves plot' is carried out (see example in Fig. 1.8a). The highest rate of fluorescence decrease is generally at the melting temperature of the DNA amplicon of interest (called T_m), more specifically defined as the temperature at which 50% of the DNA sample is double-stranded and 50% is single-stranded. T_m could be derived by plotting the derivative of fluorescence vs. temperature (-dF/dT against T), or the 'derivative melting plot' (Fig. 1.8b). Since initial fluorescence values on the raw melting profile can be variable between samples making analysis difficult, it is standard to perform a simple normalization of pre- and post-melting regions in order to align data and magnify the differences in melting properties of genetically different samples, visualized as a 'normalized melting plot' (Fig. 1.8c). Another way to visualize melting data is the

‘difference melting plot’, where normalized melting data of a user-defined genotype (reference) are subtracted from the normalized data of all other samples; the reference genotype is visualized as the baseline and the position of the other samples is plotted against the temperature. This process aids the visualization of normalized data (Fig. 1.8d).

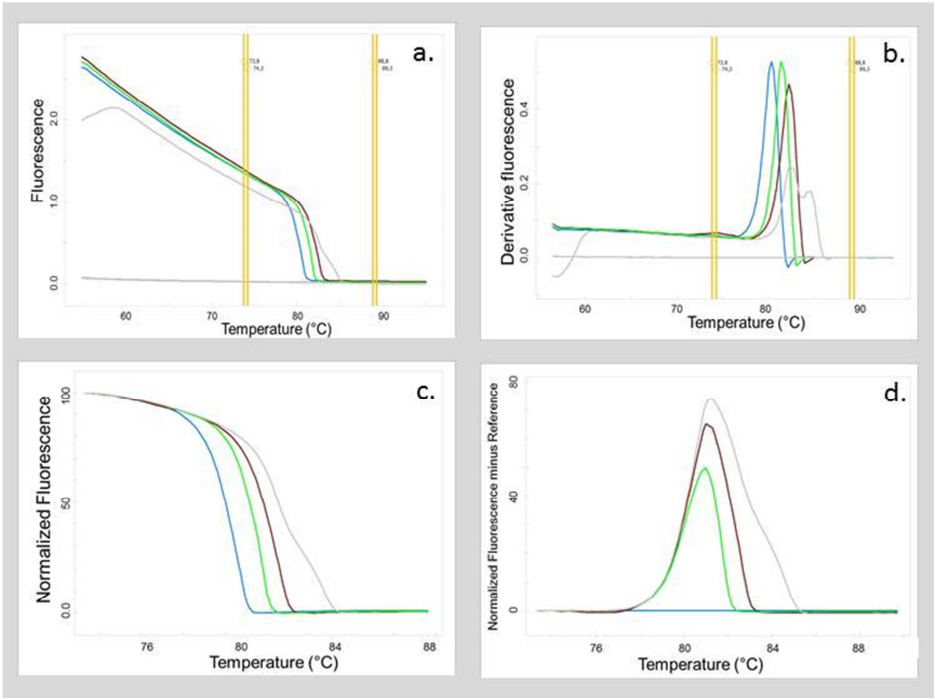


Fig. 1.8 Process of software analysis of high resolution melting data; a.) raw melting curves plot, yellow bars define the normalization area ;b.) derivative melting plot; c.) normalized melting plot; and d.) difference melting plot. (plots from the Real-time HRMA of control samples in bloodmeal analysis).

In the design of a new HRMA assay, the choice of target DNA and primer design are the most critical steps. Several factors should be carefully considered, based on the fact that melting properties are a function of amplicon length, nucleotide compositions and arrangement. Generally, small amplicons are required for maximum sensitivity (optimal 50 to 300 bp) and with known sequence variation. Under these conditions, the number of potential DNA regions for primer design is delimited. In

addition, primers should be highly specific to the target organism, possibly without degenerations; and have a high melting temperature and low probability of dimers formation (www.kapabiosystems.com/assets/Introduction_to_High_Resolution_Melt_Analysis_Guide.pdf).

As mentioned above, HRMA is widely used for genotyping (Erali and Wittwer, 2010) and mutation scanning; however, most importantly for the purpose of this research, HRMA has been applied successfully to sequence matching (Reed et al., 2007), pathogen screening and identification (Do et al., 2008; Lin et al., 2008), and species identification (Winder et al., 2011; Kang and Sim, 2013; McCarthy et al., 2013). Additionally, HRMA has proved to be a sensitive and reliable method even when DNA is degraded and of low quantity, especially when coupled with Real-time PCR (Do et al., 2008). HRMA has already been applied to bloodmeal analysis in the Chagas diseases vector (*Trypanosoma cruzi*; Peña et al., 2012), with bloodmeals identifiable to species 30 days after the bloodmeal. In contrast to RLBH and other currently used methods, the lack of sample processing after the amplification step and the possibility for amplicons to be run on agarose gel after the HRMA and to be sequenced, are additional features of HRMA that are promising for its application to bloodmeal analysis in questing ticks.

1.4 *Ixodes ricinus* phylogeography and population genetics

The analysis of the patterns of genetic variability in molecular markers using appropriate statistical methods can indirectly provide key information about the biology of vectors (i.e. mating system, dispersal and gene flow), demography (effective population size, past demographic events) and evolutionary potential (adaptation, host-race formation and speciation). These features of the molecular ecology approach are particularly important in *I. ricinus*, characterized by an extremely complex life history, living for a few days a year in contact with the host, and of limited physical dimensions (reviewed in De Meeûs et al., 2007).

I. ricinus population structure was firstly investigated by means of allozyme variability by Delaye et al. (1997); a limited number of polymorphisms and absence of differentiation was observed among *I. ricinus* collected from neighbouring populations in Switzerland. Other studies have been carried out using microsatellites markers. Population genetic substructure was suggested by a significant heterozygosite deficit in several analyzed populations: as geographical based genetic structuring or isolation by distance was not significant, the heterozygote deficit was attributed to sex-biased dispersal (De Meeûs et al., 2002; Kempf et al., 2010), and host-parasite association (De Meeûs et al., 2002; Kempf et al., 2011). Using the same microsatellite markers, non-random pairing in males and females (e.g. assortative mating) was proposed in *I. ricinus* (Kempf et al., 2009b). For other tick species (*I. uriae*, McCoy et al., 2003 and Kempf et al., 2009a; *Rhipicephalus micropilus*, De Meeûs et al., 2010), host-race evolution has been hypothesized.

At the European range of the species, phylogeography studies using mitochondrial (Casati et al., 2008), and a combination of mitochondrial and nuclear

markers (Noureddine et al., 2011; Porretta et al., 2013) concluded that *I. ricinus* present one panmictic population at the European level. Noureddine et al. (2011) noted that European *I. ricinus* populations were genetically differentiated from north-African populations, but its species status was not resolved. Further genetic discontinuity was noted between the British and Latvian tick populations using a mitochondrial genes multilocus sequence typing (Dinnis et al., 2014). These results support the hypothesis that there is a high level of gene flow between European populations of *I. ricinus*, presumably as a result of host movements (Noureddine et al., 2011).

As part of the EDENext project, population genetic patterns were to be compared at the European, regional and local levels. While INRA was assigned to study gene flow at the EU and local levels (with some of those results cited above in Nourredine et al., 2011), we were to investigate the level and distribution of genetic variation at the regional level (PAT). Given the number of previously published papers using microsatellite markers for this purpose, we decided to genotype both questing and feeding ticks from across PAT in order to evaluate both the influence of geographical features and host species in shaping genetic variation in the overall population.

However, as later presented in Results, our findings proved that microsatellites were not suitable markers for population genetics studies of *I. ricinus*, as confirmed by Quillery et al. (2013). Given that microsatellites are not abundant in the arthropod genome (Fagerberg et al., 2001), development of new STR markers was not feasible in the time available. On the other hand, Single Nucleotide Polymorphisms (SNPs) have been successfully used in the investigation of genetic variation in populations of non-model species (Helyar et al., 2011). SNPs are the most abundant and uniformly distributed

markers in the genome (Schlötterer, 2004), allowing informative patterns of genetic variation in populations to be analysed (Lao et al., 2006; Paschou et al., 2007).

A SNP dataset was not available at that time for *I. ricinus* (later Quillery et al., 2013) or similar ixodid species and, therefore, we decided to apply the recently developed NGS RAD-Seq technology to obtain one.

1.4.1 Next Generation Sequencing: RAD-Seq

NGS technologies have revolutionized genomic and transcriptomic approaches to biology, but also the study of genetic variation in populations. At the base of this success lies the ability of NGS platforms, such as Illumina, Roche 454 and ABi SOLiD, to produce giga-bases of DNA sequences at minimal cost (Seeb et al., 2011; Davey and Baxter, 2010). It is now possible to produce whole genome sequences of several individuals of a target species and, from their comparison, design new markers for genetic variation analysis, such as microsatellites and SNPs. However, for eukaryotes with large genome sizes lacking an assembled reference genome, whole genome sequencing was still a challenging task, until the introduction of Reduced Representation Libraries (RRL; Seeb et al., 2011; Etter et al., 2011; Davey and Baxter, 2010). RAD-Seq falls into this category (Miller et al., 2007), and is particularly suitable for our purpose as it allows the simultaneous *de novo* discovery and genotyping of tens of thousands of SNPs throughout the genome at limited cost, and requires no prior development of genomic resources (Miller et al., 2007; Davey and Baxter, 2010). The first application of this approach to population genomics (Hohenlohe et al., 2010), SNP discovery and genome mapping (Baird et al., 2008), and phylogeography (Emerson et al., 2010), illustrated the versatility of the method, even in non-model organisms,

without reference genomes and characterized by complex evolutionary histories (Rowe et al., 2011). As presented in Figure 1.9, RAD-Seq combines two molecular biology techniques: DNA fragmentation with a restriction enzyme (as used in restriction fragment length polymorphisms and amplified fragment length polymorphisms methods), and association of each individual/population to a specific molecular identifier (MID), allowing pooling of individuals into a single library to be sequenced in one Illumina sequencing lane. The Illumina HiSeq sequencing technology used here allowed me to read 100 bp of the genomic region flanking the restriction site; paired-end sequencing allows me to extend the genome investigation by 300- 400 bp region downstream of the restriction site (Etter et al., 2011; Fig. 1.9G). In this way, the entire genome is randomly sampled and interrogated, leading to a reduction in the analysis complexity usually associated with whole genome sequencing (Davey and Baxter 2010; Rowe et al., 2011).

Genetic variation in terms of SNPs, insertion/deletions, and microsatellites, can be investigated in the 100 bp reads generated from the restriction site (R1) (Etter et al., 2011). The software *Stacks* is a modular pipeline specifically designed for efficiently processing and assembly of the large numbers of short-read sequences originating from multiple samples generated by RAD-Seq and by other RRL protocols (Catchen et al., 2011, 2013). It incorporates a maximum likelihood statistical model to identify sequence polymorphisms and distinguish them from sequencing errors, either *de novo* or with sequences aligned to a reference genome. The basic *Stacks* workflow and the programs involved, are presented in Figure 1.10. According to the RAD-Seq features, this method promised a dataset that could be analysed to answer our biological questions.

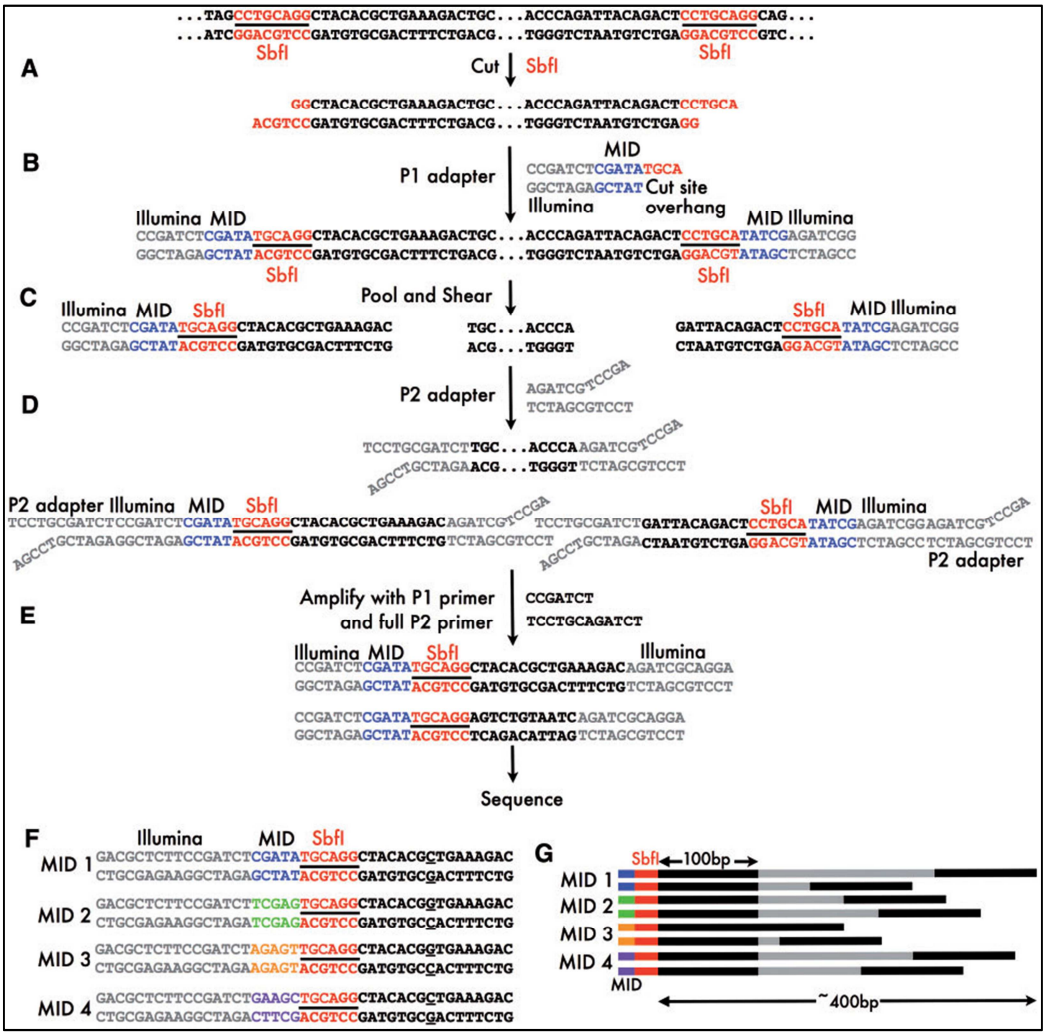


Fig. 1.9 The RADSeq method. (A) Genomic DNA is sheared with a restriction enzyme of choice (SbfI in this example). (B) P1 adapter is ligated to SbfI-cut fragments. The P1 adapter is adapted from the Illumina sequencing adapter (full sequence not shown here), with a molecular identifier (MID; CGATA in this example) and a cut site overhang at the end (TGCA in this example). (C) samples from multiple individuals are pooled together and all fragments are randomly sheared. Only a subset of the resulting fragments contains restriction sites and P1 adapters. (D) P2 adapter is ligated to all fragments. The P2 adapter has a divergent end. (E) PCR amplification with P1 and P2 primers. The P2 adapter will be completed only in the fragments ligated with P1 adapter, and so only these fragments will be fully amplified. (F) Pooled samples with different MIDs are separated bioinformatically and SNPs called (C/G SNP underlined). (G) As fragments are sheared randomly, paired-end sequences from each sequenced fragment will cover a 300- 400 bp region downstream of the restriction site. (Reprinted from Davey and Baxter, 2010).

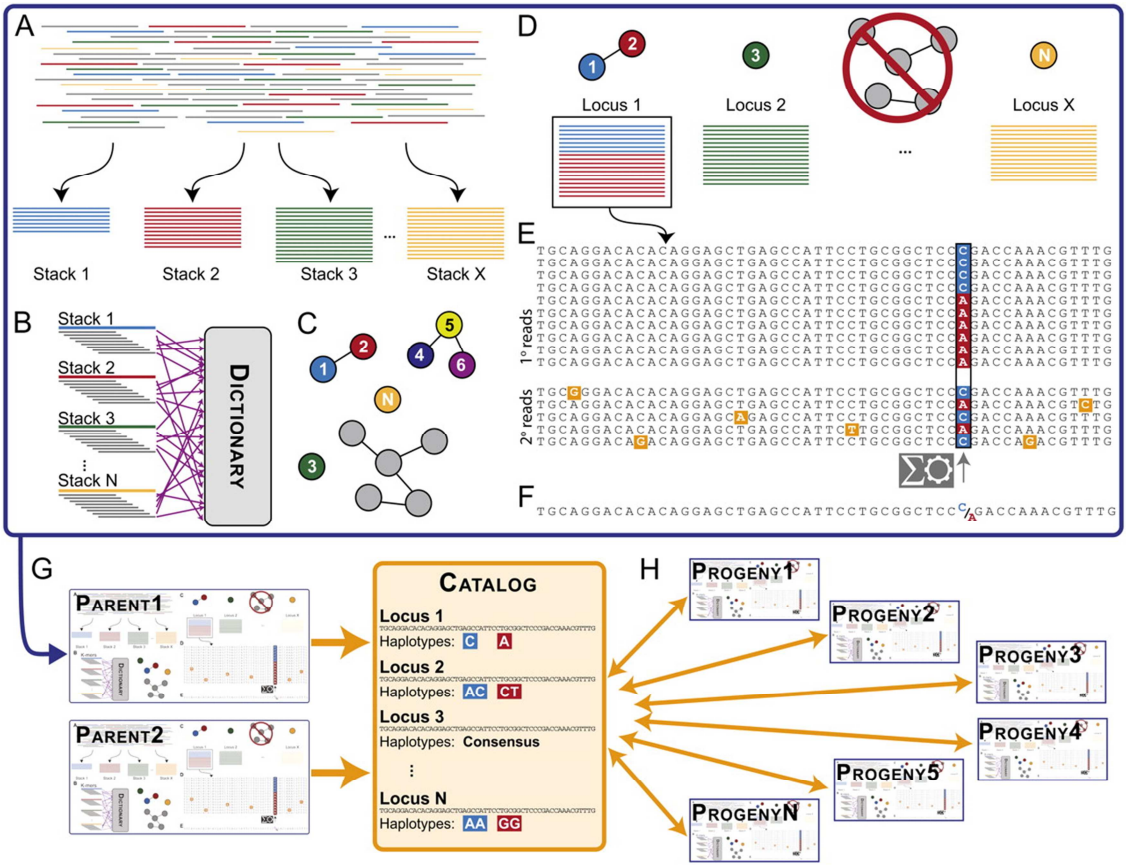


Fig. 1.10 Schematic *Stacks* workflow. (A) The *ustacks* program forms stacks in an individual from short sequencing R1 reads (cleaned by *process_radtags.pl*) that match exactly. (B) The *ustacks* program breaks down the sequence of each stack into k-mers and loads them into a dictionary. The *ustacks* program breaks down each stack again into k-mers and queries the k-mer Dictionary to create a list of potentially matching stacks, which can be visualized as nodes in a graph connected by the nucleotide distance between them. (C) *ustacks* merges matched stacks to form putative loci. (D) *ustacks* matches secondary reads that were not initially placed in a stack against putative loci to increase stack depth. An SNP model in *ustacks* checks each locus at each nucleotide position for polymorphisms. (E) *ustacks* calls a consensus sequence and records SNP and haplotype data. (F) The *cstacks* program loads stacks from the parents of a genetic cross into a Catalog to create a set of all possible loci in a mapping cross. (G) *sstacks* matches map cross progeny against the Catalog to determine the haplotypes at each locus in every individual in the cross. (Reprinted from Catchen et al., 2011)

2. OBJECTIVES

The TBD spread and emergence facing Europe urgently calls for improved knowledge of *I. ricinus* biology, especially dispersal and host-association patterns. The present study focuses on a relatively small geographical area (Province of Trento, Italy), where 30 *I. ricinus* populations were sampled intensively both while feeding and while questing. The main objective of this thesis was to apply *state-of-the-art* molecular approaches to fill this knowledge gap, specifically:

1. Population genetics will be applied to investigate the genetic structure of sheep tick populations, in order to understand if geographical barriers (i.e. mountain chains; rivers) and/or host exploitation affects the rate of gene flow (and therefore, dispersal) on this scale;
2. Bloodmeal analysis of questing tick nymphs will be optimized to define the relative importance of various wild and domestic vertebrate species as larval hosts in two important tick habitats (extensive forest and forest patches near urban settlements).

Since feeding ecology and dispersal are interrelated in sheep ticks (because they move as they feed), I will also meet the main objective by merging the results of the two approaches, such that the epidemiological implications of the research regarding TBD circulation, spread and maintenance, can be discussed.

3. MATERIALS AND METHODS

For reasons that will become obvious below, field and laboratory protocols are listed in the order they were developed and applied. Therefore, because the list of Materials and Methods is rather extensive, a chart is provided below to aid the reader in following the workflow. Red X's indicate methods that were tested, but subsequently abandoned because reliable, repeatable results were not obtainable.

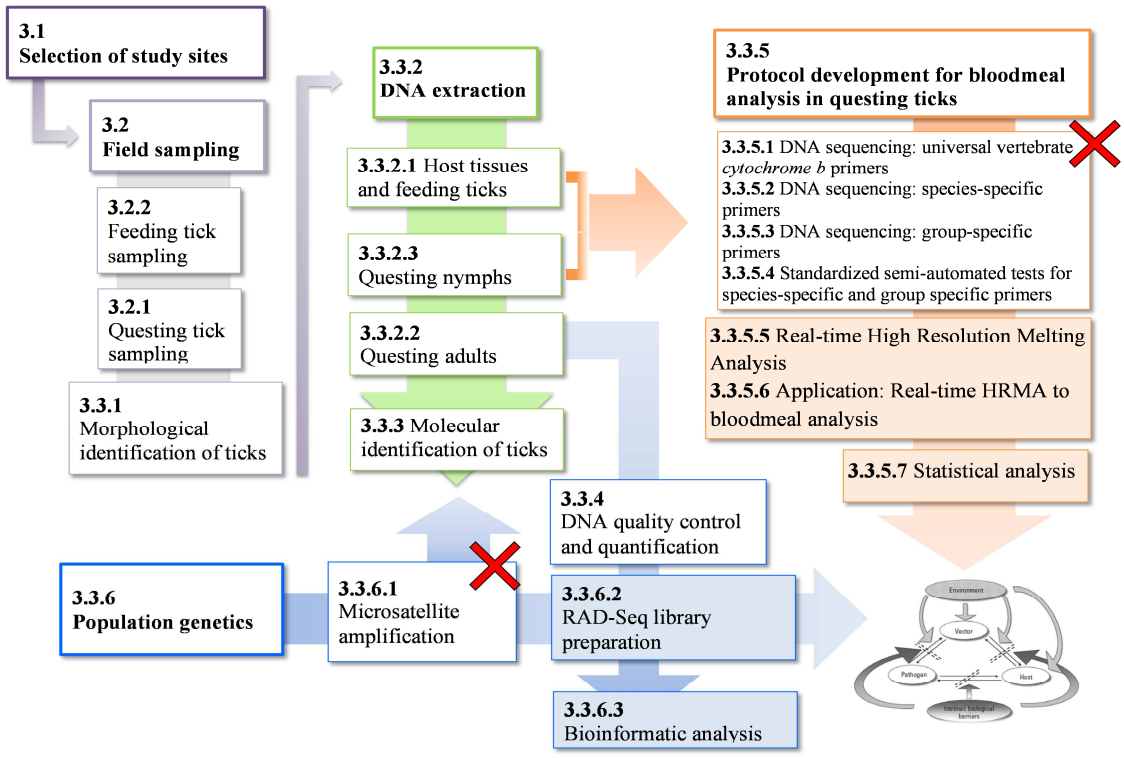


Fig. 3.1 Schematic workflow of material and methods protocol used and their interactions.

3.1 Selection of study sites

The Province of Trento (PAT; 6206 km^2) lies in the heart of the Dolomitic Alps (Fig. 4.1); 77% of the territory is above 1000 m a.s.l.. Its complex geomorphology, consisting of a network of river valleys of various orientations and extent, surrounded by rugged mountain chains, and dotted with lakes, and hills, results in a wide diversity

of local climatic conditions, ranging from sub-mediterranean to continental and alpine, that vary according to exposure, altitude, and prevailing winds. Forest covers about 55% of the region. In the lowest reaches of the southern valleys, thermophile deciduous woods and coppices with hophornbeam (*Ostrya carpinifolia*) and flowering ash (*Fraxinus ornus* L.) prevail; in sub-continental and continental valleys, beech forests (*Fagus sylvatica* L.) are common at the lowest altitudes, replaced by mountain and subalpine Norway (*Picea abies* L. Kasten) and European silver spruce forests (*Abies alba* Miller) along the altitudinal gradient. Much of the coniferous forest is heavily managed; in addition, widespread anthropic disturbance is present across the study area. In fact, although towns and larger urban areas are mainly concentrated in the valley floors, many villages are at higher altitudes, embedded in patchy agro-ecosystems, representing the natural bridge with the described forests. Cultivated crops (especially grape and apple) are a strong feature of the landscape, but agricultural activities at higher altitudes are less profitable and hay meadows and pasture somewhat abandoned. Additionally, tourism, is one of the most important economic driving forces in the area, and increases the exploitation of natural areas for recreational activities. The fauna is characteristic of natural Alpine habitats. Importantly for this thesis, many of the vertebrate species are also recognized as important hosts maintaining sheep tick (*I. ricinus*) populations, such as small mammals (*Apodemus* spp., *Myodes glareolus*, *Sorex* spp. and *Crocidura* spp.), passerine birds (*Turdus merula, T. philomelos, Erithacus rubecula*), wild ruminants (*Capreolus capreolus*, *Cervus elaphus*, *Ovis musimon*, *Rupicapra rupicapra*) and Carnivores (*V. vulpes*; Carpi et al., 2008; Bolzoni et al., 2012; Cagnacci et al., 2012). In addition, some are competent reservoirs for tick-borne pathogens such as *Borrelia burgdorferi* s.l., Tick Borne Encephalitis virus and

Anaplasma phagocytophila (Rizzoli et al., 2004; Mantelli et al., 2006; Pecchioli et al., 2007; Carpi et al., 2008; see General Introduction).

3.2 Field sampling

In order to carry out an in-depth investigation of the host use and genetic structure of tick populations at a local scale, 30 sites were selected in typical tick habitat across PAT. At least one site was selected from each of the main valleys of the Province. Questing ticks were sampled in all 30 sites, while sampling of all stages of feeding ticks from the main host species was carried out in 10 of these (Fig. 4.1 in Results).

3.2.1 Questing tick sampling

Material:

- 1 m^2 white felt blanket;
- measuring tape;
- forceps;
- sterile 2 mL sterile vials with plug seal cap (Sigma);
- 10% bleach;
- gloves.

Questing nymphs and adults were collected by conventional blanket-dragging (Sonenshine, 1993). A 1m x 1 m white felt blanket, attached by one side to a wooden pole, was dragged over leaf-litter, grass and low understory. Every 5 m the blanket was checked for ticks. Ticks were removed from the blanket by researchers wearing gloves and using forceps sterilized with diluted bleach (10%), placed individually in 2 mL

vials, transported live at room temperature and subsequently frozen at -80°C, until DNA extraction. Initially, a 100 m transect was dragged (as part of a long-term monitoring program at FEM), then dragging continued at random in the surrounding area until enough ticks were collected (see below).

Sampling was carried out on dry days during the spring activity peaks of tick populations, i.e. middle of April to June (2012 and 2013) (Tagliapietra et al., 2011). At each site we aimed to collect at least 30 questing nymphs and 12 questing adults, half males and half females. Altitude, exposure and geographical coordinates, as well as predominant vegetation type were noted; tick density was expressed as the number of ticks (adults and nymphs) collected in 100 m^2.

3.2.2 Feeding tick sampling

At the outset of the project, I planned to genotype both questing and feeding ticks from various host species using microsatellite loci in order to compare the genetic patterns of ticks found on certain host species, to determine if different tick genotypes were associated with certain vertebrate hosts ('host races'). Therefore, to collect feeding ticks of the dominant tick-host species in PAT, small mammals and birds were trapped, and large mammals were sampled at hunting inspection stations.

In the 10 sites selected for feeding tick sampling, the aim was to collect at least 30 ticks of any stage from the main tick host species, with a maximum of 5 ticks from the same individual.

Target host species:

- ✓ small mammals: Muroidea: yellow-necked mouse (*Apodemus flavicollis*), wood mouse (*Apodemus sylvaticus*) and bank vole (*Myodes glareolus*), the most

numerous and widespread forest-dwelling rodents in PAT, and competent hosts for several tick-borne diseases; *A. flavicollis* inhabits forested areas from the valley floor to the vegetation limit, *M. glareolus* is also fairly widely distributed, but prefers coniferous woodland up to 1800 m; *A. sylvaticus* is a less common rodent species in PAT;

- ✓ birds: Passeriformes: blackbird (*Turdus merula*), European thrush (*T. philomelos)* and European robin (*Erithacus rubecula*), the most important host for *I. ricinus* in forested and peri-urban habitats (Marsot et al., 2012; reviewed in Rizzoli et al., 2014). These species forage low in the vegetation or on the ground and, therefore, are more prone to *I. ricinus* infestations (Marsot et al., 2012). It is worth noting that *E. rubecula* is predominantly a forest species, preferring dense coniferous or deciduous tree clusters; however, it is commonly found in urban garden and parks; *T. merula* and *T. philomelos*, are also ubiquitous species, inhabiting forest as well as forest edge and more urbanized areas;
- ✓ wild large mammals: Cetartiodactyla: roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and chamois (*Rupicapra rupicapra*). These deer species are widely distributed in the Province, while chamois prefer higher altitudes and more open habitat, where ticks are often absent.
- ✓ domesticated animals: Canidae: domestic dog (*Canis lupus familiaris*); Caprinae: domestic sheep (*Ovis aries*).

3.2.2.1 Trapping of small mammals

Material:

- Ugglan live traps (8x9x23 cm);
- Potatoes cut into chunks;
- whole sunflower seeds;
- polyethylene transparent bags;
- forceps;
- 10% bleach;
- 70% ethanol (Sigma) in 2 mL sterile vials with plug seal cap (Sigma);
- gloves and facial mask with virus filter.

In sites CON, PIN, REV, MEZ and TRA (Table 4.1, Fig. 4.1) trapping was carried out by FEM personnel and myself, after health and safety training in small mammals trapping and handling. At these sites, the only aim of small mammal trapping was the collection of feeding ticks. Trapping sessions took place in spring-summer 2012 and 2013, with traps set on the first day then checked daily for two or three consecutive nights and repeated twice or three times for each site until enough ticks were collected. Live traps were placed in linear transects, when possible, ca. 10 m apart, next to fallen logs, tree roots, rocks and where rodent burrows were visible. A variable number of traps was used: between 42 and 47 for each session.

CAV is a long-term sampling site of DBEM with 4 permanent grids of traps. A single grid is composed of 8 transects with 8 traps each; both transects and traps are ca. 10 m apart. Traps are activated monthly for three nights. Sites LUN and CAD were established as part of the PAT-funded project ROCOALPS (Rodent communities in a changing environment: implications for human health in the Alps; PI: Konečny Adam,

2010-2013) with two 8x8 grids each, similar to those in CAV; here, traps were activated monthly for two nights. Site GRI was trapped as part of a collaborative project between DBEM and MUSE (Trento) for the definition of small mammal biodiversity in PAT; in this case, transects were used and a variable number of traps (37 – 40) was used for each trapping session.

In all cases, Ugglan live-traps were baited with sunflower seeds and pieces of potato to provide a moisture source. Traps were activated in the late morning or early afternoon and checked the subsequent morning. Small mammals were released from the trap into a clear polyethylene bag and, once confined in the corner of the bag, were taken firmly by the scruff of the neck and tail. Species identification by morphological traits did not allow us to discriminate between *A. flavicollis* and *A. sylvaticus*; therefore sampled individuals are later identified at genus level (*Apodemus* spp.; Michaux et al., 2001). All ticks were gently removed with sterile forceps and placed individually in 2 mL Eppendorfs with 1 ml of 70% ethanol. Ticks coming from the same individual rodent were placed in the same tube, unless damaged during removal.

Small mammal and trap handling were carried out under strict health and safety rules for field work (e.g. using gloves and face mask). Permission to carry out the trapping in the defined areas and all animal handling procedures were authorized by the Comitato Faunistico Provinciale della Provincia di Trento prot. n. 595 04.05.2011. Ethical guidelines concerning animal welfare were followed as defined by the European Commission and detailed in the EDENext project.

3.2.2.2 Bird netting

Material:

- 12x2.5 m mist-nets with 5 shelves, mesh size 16 mm, normal filament;
- 12x2 m mist-nets with 5 shelves, mesh size 16 mm and thin filament;
- 4 m telescopic poles for mist-net support;
- forceps;
- 10% bleach:
- 70% ethanol (Sigma) in 2 mL sterile vials with plug seal cap (Sigma);
- birding morphological measurement instruments;
- gloves.

In order to analyse feeding ticks acquired by hosts in the area of interest (and not outside the Province), our netting regime aimed at capturing resident birds, rather than migrating ones. Therefore, live-trapping was carried out in all but CAV sites during the reproductive period for the bird species of interest. Netting was carried out with the assistance of ornithologist Dott. Franco Rizzolli, a licensed bird ringer. At each site, a minimum of 120 m of mist-nets, divided into two or more transects, were set and maintained for one or two days per trapping session. Trapping efficiency drastically dropped after just one day and consequently, we had to move the nets to a different position daily. In general, ecotonal areas, like meadow-forest borders, or wetlands, as well as areas near food sources (like orchards and bushes with berries) were selected. Mist-nets were activated before sunrise and monitored for trapped birds every hour; they were closed during heavy rain and during the hottest hours in the middle of the day. Only the licensed ornithologist handled the birds; species and sex were identified, standard morphological measurements taken, reproductive and moult condition noted,

and ectoparasites were collected. Ticks were mainly removed from the region surrounding the eyes, ear holes and beak. Once removed with sterile forceps, ticks were placed in 70% ethanol. Ticks from the same individual were placed in the same 2 mL vial, unless damaged. Each bird was ringed before release at the trapping site. At the CAV site there is a permanent ringing station, managed by Mauro Segata (forest warden and ornithologist), who kindly collected ticks from birds netted at this site during the same period.

3.2.2.3 Collection of ticks feeding on large mammals

Material:

- polyethylene transparent bags;
- forceps;
- 10% bleach;
- gloves;
- magnifier lamp;
- 70% ethanol (Sigma) in 2 mL sterile vials with plug seal cap (Sigma).

Ticks were collected from the most common large wild mammals present in PAT (see list above), bagged during the autumn hunting season, when these ungulates are still territorial and consequently, feeding ticks are representative of the animal's area of origin (Carpi et al., 2008). In collaboration with the Trentino Hunter's Association, the wardens of each game reserve encompassing our sampling site, and/or local hunting guards were contacted. The Hunter's Association is responsible for ensuring that the species, sex and age of each bagged animal matches those of the hunter's license. However, such controls are done in two ways: either the hunter is required to bring the

animal to a central checkpoint on the evening of the kill, or the hunter contacts the warden who subsequently goes to the hunter's house to make his report. Therefore, to collect ticks, either:

- ✓ carcasses were checked for ticks in the presence of wardens and/or hunting guards, within 24 h of the kill, by myself or trained DBEM personnel; or,
- ✓ the warden of the game reserve or local hunting guards were trained to remove the forelegs (part of the leg distal to the carpal joint, which mainly hosts tick larva and nymphs), as soon as possible after the kill, and to place them in a sealed plastic bag, which I provided; if the hunting personnel were willing, I also provided sterile vials filled with high grade ethanol for adult ticks. These samples were conserved at -20°C until delivery to FEM. Forelegs were stored at -20°C until tick removal, carried out under a magnifying lamp, following Carpi et al. (2008).

Species, sex and age, as well as altitude, exposure, and location of the kill were registered for each sample.

For PIN and CON sites, ticks were occasionally collected from acquaintances' dogs. Domestic sheep (*Ovis aries*) ticks were collected by contacting local sheep breeders.

Ticks coming from the same individual were placed in the same 2 mL vial filled with 70% high grade ethanol, unless damaged.

3.3 Laboratory methods

3.3.1 Morphological identification of ticks

Material:

- identification key (Cringoli et al., 2005; Estrada-Peña et al., 2004);
- dissecting microscope (Zeiss);
- forceps;
- petri dishes;
- 10% bleach and ethanol for cleaning instruments;
- gloves.

In PAT, the most widespread tick is *I. ricinus*, but on wildlife it is also possible to retrieve *I. hexagonus* and *I. trianguliceps*. Given the specialist, nidicolous life-style of these tick species, I could assume that the vast majority of questing ticks collected by blanket dragging would be *I. ricinus*; however, feeding ticks collected from parasitized wildlife could be both nidicolous and generalist ticks. Therefore, for both questing and feeding ticks, I attempted to identify collected ticks morphologically using a recognized identification keys (Cringoli et al., 2005; Estrada-Peña et al., 2004), at 40X magnification under a dissecting microscope.

Although this procedure was straightforward for most of the questing ticks, morphological identification was sometimes difficult for engorged specimens in the larval and nymphal stages, since some morphological features were damaged or missing as a result of collection, or deformed because of engorgement. In this case I confirmed species identity by molecular analysis (see section 3.3.3).

3.3.2 DNA extraction

DNA isolation from ticks, in general, is a challenging task due to their size (especially larval and nymphal stages) and the hard chitinous exoskeleton; furthermore previous studies have shown that tick DNA is susceptible to degradation (Hubbard et al., 1995; Hill and Gutierrez, 2003; Halos et al., 2004). Additionally, according to the use of DNA from collected ticks (i.e. pathogen detection, bloodmeal analysis, NGS application) with the EDENext project, various DNA qualities and quantities were required. Therefore, I used a number of different protocols and commercial and non-commercial kits in order to meet the post-extraction applications requirements in terms of quantity, quality of DNA, but also with the aim of improving the efficiency of the protocols, in terms of time and cost.

3.3.2.1 Host tissues and feeding ticks

DNA was extracted from engorged ticks collected from the host while feeding (see *Feeding tick collection*) and from tissue samples available from previous or ongoing projects at FEM, using the Qiagen Dneasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). DNA from a large variety of host species was needed to validate bloodmeal protocols (as control samples; see section 3.3.5). Specifically, for Real-time HRMA, host DNA extracted from tissue and engorged ticks was needed i) to test that the primer pairs amplified the target fragments correctly and reliably using conventional PCR; ii) to validate the species-discriminating power of HRMA and; iii) as positive samples in Real-time HRMA of questing ticks with unknown bloodmeal sources. Feeding ticks were also employed for microsatellite genotyping validation.

For engorged ticks, a pre-lysis step, following morphological identification,

included a physical disruption step. A single tick was placed in 2 mL autoclaved Eppendorf tube with a 5mm stainless steel bead and 100 µL PBS (Phosphate-buffered saline) solution. Using a mixer mill, tubes placed in the proper sampler holder, were shaken for 3 min at 30 Hz to completely crush the tick. The homogenate was then transferred to a fresh autoclaved Eppendorf tube, 20 µL Proteinase-K 10 mM/mL (Sigma-Aldrich, Saint Louis, USA) and 180 µL Qiagen ATL tissue lysis buffer were added, and left overnight to digest on a rotary tube mixer placed in an incubator at 56 °C. Spin-column DNA purification was performed following manufacturer instructions for QiaAmp® DNA Investigator Kit (Qiagen, Valencia, CA, USA), *Purification of Total DNA from Nail Clipping and Hair*.

3.3.2.2 Questing adults

To avoid contamination from environmental DNA, human DNA and cross-contamination among samples, all recommended precautions were used: forceps were sterilized with bleach and ethanol; washing and DNA extraction were performed under a biological (UV sterilized) hood; sterile and DNA-free consumables and reagents were used for all methods.

Sample lysis

The sample lysis procedure was common to the different DNA extraction methods used, as follows. After morphological identification, each tick was washed twice in DNA-free distilled water to rehydrate it and to eliminate possible surface contaminants. Briefly, ticks were immerse in 200 µL of RNase DNase free water (Sigma-Aldrich, Saint Louis, USA) in 0.5 mL sterile tubes for at least 20 s; after

agitation of the tube by tipping it up and down, the tick was transferred, using a fresh sterile 200 µL tip, to another 0.5 mL tube and the procedure repeated. The tick was then transferred in the same way to a fresh 0.5 mL tubes, still containing 200 µL RNase DNase free water (Sigma-Aldrich, Saint Louis, USA), until processed for lysis. In 1.5 mL sterile vial containing 180 µL ATL Buffer, each tick was cut in small pieces using a sterile disposable scalped with pointed blade (WVR, Radnor, PA, USA). 20 µL Proteinase K 10 mM/mL (Sigma-Aldrich, Saint Louis, USA) for Qiagen column extraction or 20 µL Proteinase K (KingFisher™ Cell and Tissue DNA Kit; Thermo Fisher Scientific, Vantaa, Finland) for Thermo magnetic-beads extraction, 30 µL DTT 1 M (Sigma-Aldrich, Saint Louis, USA) were added. After vortexing for reagent-sample mixing, tubes were sealed with parafilm, and placed in an incubator for overnight digestion at 56°C.

Before purification with one of the following methods, digested samples were treated with RNase A 100 mg/ml (Qiagen, Valencia, CA, USA). 1 µL for each 50 µL digested sample was added to the lysate and incubated at room temperature for 30 min.

Spin-column protocol

Following indications for obtaining high quality and quantity genomic DNA from the RAD-Seq library generation protocol of Etter et al. (2011), DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA, USA) using the manual protocol *Purification of Total DNA from Animal Tissues* was used to purify DNA from lysed samples. Eleven samples plus a negative extraction control were manually processed at a time. Double elution of genomic DNA in two separate 1.5 mL sterile tubes was performed with 50 µL of the kit elution buffer. To improve DNA release from the column, elution buffer was

heated at 56°C, the sample was added to the silica membrane, and incubated for 5 min. Eluted gDNA was stored at 4°C until DNA quality control and quantification.

Phenol protocol

After lysis and RNAse treatment, 1 volume of phenol (pH 8) was added to the lysate sample, and placed for 15 min on a rotary tube roller, and centrifuged for 15 min at 5000 g. The aqueous phase was transferred to a fresh 1.5 mL Eppendorf tube. These steps were repeated twice. After phenol extraction, 0.4 volume of sodium acetate (3 M, pH 4.6) and 2 volumes of chilled 100% ethanol were added to the sample to precipitate the DNA. Samples were vortexed to aggregate DNA and briefly centrifuged to remove solution drops from the lid, before placing them at -80 °C for 10 min. DNA was pelleted by centrifuging for 14000 g for 15 min. Ethanol was removed from the sample. 200 ml of 70% ethanol was added and the tube gently vortexed. After sample centrifugation at 14000 g for 15 min, as much of the 70% ethanol as possible was removed. To eliminate residual ethanol, tubes were placed in a heated centrifuge under vacuum. DNA was resuspended in 50 µL of ATE buffer (Qiagen, Inc., Valencia, CA, USA). Samples were gently rocked to enhance DNA resuspension. gDNA was then stored at 4°C until DNA quality control and quantification.

Magnetic-beads protocol

The recently-developed magnetic-beads DNA purification technology promises high quality DNA, free of protein, nucleases, and other contaminants or inhibitors. The KingFisher Cell and Tissue DNA Kit with KingFisher™ Flex Magnetic Particle Processors (Thermo Fisher Scientific, Vantaa, Finland) combines the high quality DNA

and efficient extraction with an additional decrease in time of extraction, by employing a robotic handling of samples. KingFisher™ Flex Magnetic Particle Processors allow simultaneous processing of 96 samples, and the purification protocol can be easily controlled and modified with the Thermo Scientific™ BindIt™ Software.

We used the KingFisher Cell and Tissue DNA Kit (Thermo Fisher Scientific, Vantaa, Finland) following the manufacturer's instructions, although the lysis step was performed as described above. Plates and consumables for samples and reagents were UV-sterilized in a biological hood before loading into the instrument under a UV-sterilized biological hood. Final elution volume was adjusted to 80 µL for females and to 60 µL for males to fulfill final gDNA concentration requirements. In order to check for contamination, three negative controls were added in each 96-well plate. Eluted gDNA and negative controls were transferred from the elution plate to 1.5 mL sterile tubes and conserved at 4°C until DNA quality control and quantification.

3.3.2.3 Questing nymphs

Spin-column protocol

After morphological identification, each questing nymph was washed as described above (see *Sample lysis*). The protocol *Purification of Total DNA from Nail Clipping and Hair* of QiaAmp® DNA Investigator Kit (Qiagen, Valencia, CA, USA) was used with minor modifications. Briefly, each nymph was placed in a sterile vial containing 230 µL ATL Buffer (Qiagen) and cut into small pieces with a sterile scalpel; 40 µL Proteinase-K 10 mM/mL (Sigma-Aldrich, Saint Louis, USA) and 30 µL DTT 1 M (Sigma-Aldrich, Saint Louis, USA) were added to the solution. Overnight digestion was performed on a rotary tube mixer placed in an incubator at 56 °C. Prior to

processing, 1 µL Carrier RNA (1 µg/µL in ATE (Qiagen) was added to the lysate, as suggested in the kit manual for low quantity samples to increase DNA binding to the silica membrane. Purification was performed manually following the standard protocol, except that: ATE elution buffer (Qiagen) was heated at 56°C and elution volume was adjusted to 60 µL; incubation time after ATE addition to the column was increased to 5 min. In some cases, as specified below, the same purification protocol was performed using the QIAcube (Qiagen, Inc., Valencia, CA, USA), a robotic workstation for automated purification of DNA, RNA and proteins. To avoid contamination from environmental DNA, all DNA extraction procedures were carried under a laminar flow hood (UV-sterilized); 11 nymphs were processed at a time with one negative control, for cross-contamination control. DNA was stored at -20 °C until use.

Magnetic-beads protocol

Since I obtained good quantity and quality of gDNA from adult questing ticks with the magnetic-beads method (see Results), I decided to apply it to questing nymphs for Real-time HRMA bloodmeal analysis, decreasing the time and funds necessary for identifying bloodmeals the new HRMA protocol. Sample lysis and DNA purification protocols and instruments are the same as those described in section 3.3.3 for adult ticks, except that final elution volume for nymphs was 80 µL. Samples and negative controls were transferred in 96 x 0.2 mL-well PCR plate to allow automated robotic PCR reaction set-up, and stored at -20°C until HRMA.

3.3.3 Molecular identification of ticks

According to Cringoli et al. (2005), the most important tick species found in the Alps, in addition to *I. ricinus*, are *I. hexagonus, I. trianguliceps, I. canisuga, I. frontalis, Riphicephalus sanguineus e Haemaphisalis punctata*. Sequences of the mitochondrial 16S gene for these species were downloaded from the database Taxonomy of NCBI (http://www.ncbi.nlm.nih.gov/taxonomy). Sequence alignment was done with Clustal X v. 2.0. PRIMER3 (www.frodo.wi.mit.edu/primer3), a new primer set targeting a 470 bp region of the 16S gene, allowing species discrimination through BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search, was designed. Primer sequences are as follows:

Ir_16S_681-F CAAAAACATTTCATTTTGG

Ir_16S_1159-R GAACTCAGATCATGTAGGAA

Conventional PCR was performed at a final volume of 20 µL, containing 0.5 µM of each primer, 0.25 mM of each dNTP, 1x HotMaster Taq Buffer, 1.25 U HotMaster Taq (5-Prime), and 1 µL of template DNA. The thermal cycling consisted of 94 °C for 2 min, 30 to 35 (in case of larvae) cycles at 94 °C for 30 s, 51°C for 1 min, 65 °C for 1 min and 40 s, and final elongation at 65 °C for 10 min, performed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Amplification results were checked via capillary electrophoresis on a QIAxcel system (Qiagen, Valencia, CA, USA) using a DNA High Resolution Cartridge and QX 15 bp-3 Kb size marker and by applying the method OM500, and were analyzed using QIAxcel ScreenGel 1.0.2.0. PCR products were purified with Exo-SAP-ITTM (GE Healthcare, Little Chalfont, England) and both forward and reverse strands were sequenced on an ABI 3130 XL using Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA). After

electropherogram check and creation of a consensus sequence using Sequencher v. 5.1 (Gene Codes corp.), a BLASTn search was carried out for species identification.

3.3.4 DNA quality control and quantification

Genomic DNA concentration was measured with the fluorometer Qubit® 2.0 (InvitrogenTM, Carlsbad, USA), as suggested by Etter et al. (2011). Fluorometric quantification is based on the properties of the Molecular Probes® Dyes that emit a fluorescent signal only when ligated to the target molecules. In our case, using the double stranded DNA kit (dsDNA), only double stranded DNA was quantified, even in presence of free nucleotides and degraded nucleic acids. DsDNA HS (high sensivity) Assay kit was used following the manufacturer's instructions; 1 µL of DNA was quantified in a dilution ratio of 1:200 with the kit working solution. The DNA in a sample was concentrated using a vacuum/pressure kit (Concentrator 5301, Eppendorf AG, Hamburg, Germany) when gDNA concentration lower than 10 ng/µL.

The quality of gDNA was assessed by running 3 µL of each sample in a 0.7% agarose gel stained with ethidium bromide, prepared with 10x TAE buffer. Electrophoresis was carried out in a 10x TAE buffer solution at 80 V for 40 minutes and viewed using a UV-transilluminator. High quality sample would present a compact DNA band at high molecular weight and absence of DNA smearing.

3.3.5 Protocol development for bloodmeal analysis in questing ticks

3.3.5.1 DNA sequencing: universal vertebrate cytochrome b primers

Universal primer set targeting about 350 bp of the vertebrate *cytochrome b* mitochondrial gene, described first by Kocher et al. (1989), was first tested on DNA extracted from engorged ticks (see 3.3.2.1 Host tissue and engorged ticks) collected while feeding from different host species (*A. flavicollis*, *T. merula, T. philomelos, E. rubecula, C. l. familiaris*, *O. aries*, *M. glareolus, E. europaeus, S. vulgaris, P. muralis*).

To optimize the conventional PCR amplification protocol, in order to amplify DNA from a number of different tick host species and avoid amplification of contaminant human DNA, different annealing temperature (52 – 62 °C), magnesium concentrations (1.0 – 2.0 mM) and touch-down protocols were tested. The following selected protocol was applied to questing ticks with unknown bloodmeal source. Reaction was performed with AmpliTaq Gold® DNA Polymerase (Applied Biosystem, Foster City, CA, USA) in 50 µL final volume mix containing: 1x AmpliTaq Gold® 360 Buffer 10x, 2 mM $MgCl_2$ 25 mM, 5 µL 360 GC Enhancer, 0.25 mg/mL of Bovine Serum Albumine (BSA) 0.4 µM of each primer, 0.25 mM of each dNTP, 1.25 U AmpliTaq Gold® 360 DNA Polymerase and 4 µL of template DNA. Thermal cycling, performed in a Veriti® Thermal Cycler (Applied Biosystem, Foster City, CA, USA), consisted of an initiation step at 95 °C for 10 min; denaturation at 95 °C for 30 sec, annealing at 54 °C for 30 sec and elongation for 1 min and 10 sec at 72 °C, 50 times; final elongation at 72 °C for 7 min. Negative controls for both DNA extraction and PCR amplifications were included in all amplification reactions. Amplification results were checked via capillary electrophoresis on a QIAxcel system (Qiagen, Valencia, CA, USA) using a DNA High Resolution Cartridge and QX 15 bp-3 Kb size marker with the

OM500 method, and were analyzed using QIAxcel ScreenGel 1.0.2.0. PCR products were purified with the Exo-SAP-IT^{TM} kit (GE Healthcare, Little Chalfont, England) and both forward and reverse strands were sequenced on an ABI 3130 XL using Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA). Following the electropherogram check and the creation of a consensus sequence using Sequencher v. 5.1, a BLASTn search was carried out to identify the species represented by the amplified fragment.

3.3.5.2 DNA sequencing: species-specific primers

A. flavicollis e *C. capreolus* were selected to test the feasibility of a bloodmeal identification approach that uses species-specific primer and sequencing since they are both bloodmeal sources for *I. ricinus* larvae and nymphs, and reservoir hosts for several TBDs.

For *A. flavicollis*, I created an alignment of *cytochrome b* sequences retrieved from Genbank. New primers were then designed using the online software PRIMER3 (www.frodo.wi.mit.edu/primer3) (see Table 4.8). For *C. capreolus* we used the primer F_UNIV2 and R_CAPREOLUS following Garros et al. (2011), to amplify 240 bp of *cytochrome b* in this species.

To validate the primers and optimize reaction conditions, PCR amplification was first performed on control DNA samples of *A. flavicollis* and *C. capreolus*. Optimized protocols (which were then applied to amplification of bloodmeal DNA in questing ticks) are as follows.

- ✓ *Apodemus*: the PCR reaction was performed with AmpliTaq Gold® DNA Polymerase (Applied Biosystem, Foster City, CA, USA) in 20 µL final volume

mix containing: 1x AmpliTaq Gold® 360 Buffer 10x, 2 mM $MgCl_2$ 25 mM, 2 µL 360 GC Enhancer, 0.5 µM of each primer, 0.25 mM of each dNTP, 1.25 U AmpliTaq Gold® 360 DNA Polymerase and 2 µL of template DNA. Thermal cycling, performed in a Veriti® Thermal Cycler (Applied Biosystem, Foster City, CA, USA), consisted of an initiation step at 95 °C for 10 min; denaturation at 95 °C for 30 sec, annealing at primer set specific T_a °C (see Table 4.8) for 20 sec and elongation for 30 sec at 72 °C, 50 times; final elongation at 72 °C for 7 min.

- ✓ *Capreolus*: the PCR reaction was performed with AmpliTaq Gold® DNA Polymerase (Applied Biosystem, Foster City, CA, USA) in 20 µL final volume mix containing: 1x AmpliTaq Gold® 360 Buffer 10x, 2 mM $MgCl_2$ 25 mM, 2 µL 360 GC Enhancer, 0.375 µM of each primer, 0.25 mM of each dNTP, 1.25 U AmpliTaq Gold® 360 DNA Polymerase and 2 µL of template DNA. Thermal cycling, performed in a Veriti® Thermal Cycler (Applied Biosystem, Foster City, CA, USA), consisted of an initiation step at 95 °C for 10 min; denaturation at 95 °C for 30 sec, annealing at 56 °C for 20 sec and elongation for 30 sec at 72 °C, 50 times; final elongation at 72 °C for 7 min.

Amplification results were checked using capillary electrophoresis with QIAxcel system (Qiagen, Valencia, CA, USA) using a DNA High Resolution Cartridge and QX 15 bp-3 Kb size marker with the OM500 method, and were analyzed using QIAxcel ScreenGel 1.0.2.0. PCR products were purified with Exo-SAP-ITTM (GE Healthcare, Little Chalfont, England) and both forward and reverse strands were sequenced on an ABI 3130 XL using Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA).

If species-specific primers prove to give reliable results, additional primers

would be designed and cost effectiveness of the protocol ameliorate by multiplexing primers and by species identification by means of amplicon length, thus avoiding sequencing.

3.3.5.3 DNA sequencing: group-specific primers

With this approach, we targeted tick host DNA by means of host group specific primers. A first selection of the most important ticks hosts in the investigated area was done, taking into account the availability of genetic resources in public databases, such as GenBank. Clustal X v. 2.0 was used to create sequences alignment of different mtDNA regions of the chosen host species groups (*cytochrome b*, *d-loop*, *cytochrome oxidase I, 16S rDNA*). Primers design was performed using PRIMER3 (www.frodo.wi.mit.edu/primer3).

Each primer set was tested on control samples from engorged ticks or from host tissue of the target species of the primer set. Reactions was performed with AmpliTaq Gold® DNA Polymerase (Applied Biosystem, Foster City, CA, USA) in 20 µL final volume mix containing reagents according to the optimized conditions for each primer set reported in Table 3.1.

Thermal cycling was performed on a Veriti® Thermal Cycler (Applied Biosystem, Foster City, CA, USA), and consisted of an initiation step at 95 °C for 10 min; denaturation at 95 °C for 30 sec, annealing at primer set specific T_a °C (see Table 4.9) for 20 sec and elongation for 30 sec at 72 °C, 50 times; final elongation at 72 °C for 7 min. Optimized protocols were then applied to DNA extracted from questing nymphs in order to identify larval bloodmeal source. Amplification results were checked using capillary electrophoresis with the QIAxcel system (Qiagen, Valencia, CA, USA) using a

DNA High Resolution Cartridge and QX 15 bp-3 Kb size marker with the OM500 method and analyzed using QIAxcel ScreenGel 1.0.2.0. PCR products were purified with Exo-SAP-ITTM (GE Healthcare, Little Chalfont, England), and both forward and reverse strands were sequenced on an ABI 3130 XL using Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA).

Table 3.1 Reaction mix for each host group primer set.

Reagents	**ROD**	**SOR**	**PAS**	**CAN**	**RUM**
AmpliTaq GOLD 360 Buffer 10x (X)	1	1	1	1	1
25 mM $MgCl_2$ (mM)	2.00	1.50	1.75	1.51	2.00
360 GC Enhancer (μL)	2	2	2	2	2
Primer F 10 ρmol/μL (μM)	0.5	0.5	0.5	0.5	0.75
Primer R 10 ρmol/μL	0.5	0.5	0.5	0.5	0.75
dNTP 10 mM (mM)	0.25	0.25	0.25	0.25	0.25
AmpliTaq GOLD 360 Polymerase (U)	1.25	1.25	1.25	1.25	1.25
DNA control samples (μL)	1	1	1	1	1
DNA questing ticks (μL)	3	3	3	3	3
total reaction mix (μL)	20	20	20	20	20

ROD= Rodents; SOR= Soricomorpha; PAS= Passeriformes; CAN= Canidae; RUM= Ruminants

3.3.5.4 Standardized semi-automated test for species-specific and group-specific primers

In order to test the newly designed primers described above using semi-automated procedures, the following protocols were performed as follows:

1. a DNA extraction was performed using spin-columns (see protocol 3.3.2.3 Questing nymphs) and the QIAcube robotic workstation from 94 questing nymphs collected in

the same day from a single site;

2. a PCR reaction was carried out in a 96 well-plate using the QIAgility robotic workstation (Qiagen, Inc., Valencia, CA, USA), with the Rodentia, Soricomorpha, Passeriformes, *Apodemus* (short and long amplicons) primers (see Tables 4.8 and 4.9); each sample was tested three times for each primer set and a control sample and negative PCR control was included in each reaction;

3. PCR amplification results were controlled with QIAxcel® (Qiagen, Inc., Valencia, CA, USA), DNA High Resolution Kit;

4. amplicons in the expected bp range were sequenced and BLASTn search confirmed species identity.

3.3.5.5 Real-time High Resolution Melting Analysis

In High Resolution Melting Analysis (HRMA), the choice of molecular markers is the most fundamental and challenging step. The use of universal primers amplifying a short fragment (about 110 bp) of vertebrate 12S mtDNA would have represented the best solution in terms of number of *I. ricinus* hosts targeted, possibly allowing the discovery of neglected host species in the investigated area. However, *in silico* analysis demonstrated that only the design of degenerate primers would allow us to target the wide range of tick hosts; moreover, HRMA was not able to reliably identify host species of the generated amplicons, because melting temperatures for the species-specific amplicons overlapped. Therefore, we decided to focus our attention to a restricted set of tick hosts and to group hosts such that each Real time HRMA reaction targeted a few species at a time. *In silico* trials allowed me to confirm that this approach could reliably identify host species. As presented in Fig. 3.2 (HRMA optimization workflow), primer

selection was organized in several steps, described in the following paragraphs. Every step was repeated multiple times in order to identify the highest performing primer sets in terms of number of targeted species and taxonomic level of host identifications for each one of the host groups. Then, the Real Time HRMA protocol was tested on field collected questing nymphs.

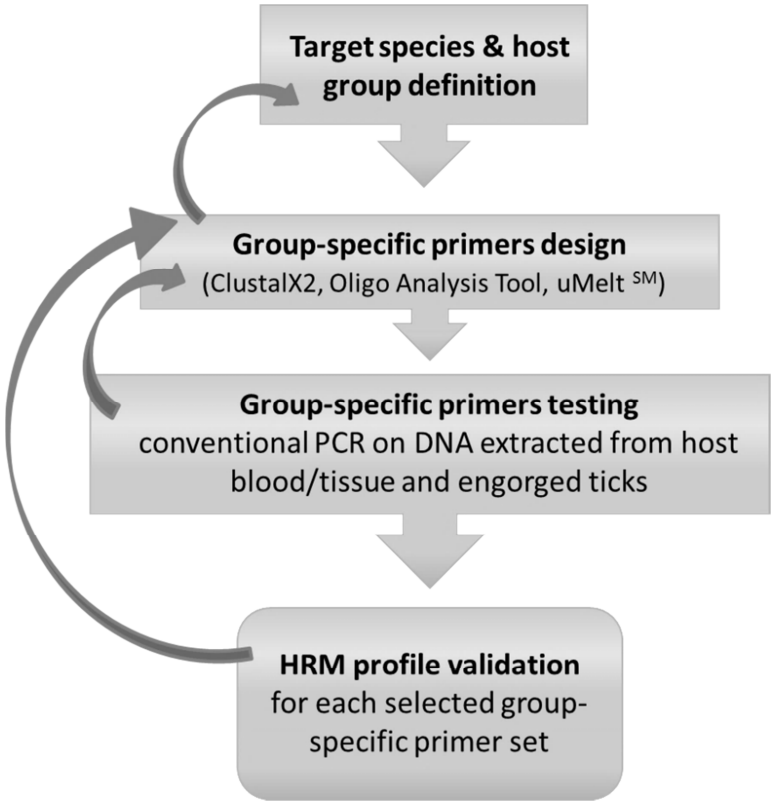


Fig. 3.2 Schematic optimization workflow followed during HRMA primers selection for bloodmeal analysis in questing ticks.

Target species and host group definition

Twenty of the most important vertebrate hosts of *I. ricinus* in Europe were chosen on the basis of their role as maintenance hosts, reservoir competence, occurrence in the Alps, and relevance as livestock and companion animals, as well as availability of control samples and GenBank sequences (Gray, 1998; Morán Cadenas et al., 2007; De

la Fuente et al., 2008; Bown et al., 2011; Marsot et al., 2012; Wodecka et al., 2014). Selected hosts were grouped in the following taxonomic categories:

1. Muroidae (Superfamily): *A. flavicollis*, *A. sylvaticus*, *M. glareolus*, *M. musculus*;

2. Soricidae (Family): *S. minutus*, *S. antinorii*, *S. araneus*, *C. leucodon*, *C. suaveolens*, *C. russula*;

3. Passeriformes (Order): *T. merula*, *T. philomelos*, *E. rubecula*;

4. Canidae (Family): *C. l. familiaris*, *V. vulpes*;

5. Caprinae (Subfamily): *O. aries*, *R. rupicapra*, *C. hircus*;

6. Cervidae (Family): *C. capreolus*, *C. elaphus*.

Humans were not considered a main tick host as suggested from previous studies (e.g. Humair et al., 2007; Pichon et al., 2003; Morán Cadenas et al., 2007; Wodecka et al., 2014). In any case, the risk of human contamination during collection was fairly high (a common problem when working with low quality/quantity DNA), and discriminating contaminant human DNA from that of the larval bloodmeal is not possible with the approach chosen (even after tick washing).

Group-specific primer design

Clustal X v. 2.0 was used to create a sequence alignment of the different mtDNA regions of the chosen host species. Sequences were mainly retrieved from GenBank where possible, but for some species for which no or insufficient sequences were available, sequences were generated by us from host tissues or engorged ticks, using universal 16S and 12S mtDNA primers (GenBank accession numbers: KJ676686 *T. merula*; KJ676687 *T. philomelos*; KJ676688 *E. rubecula*). In order to design primers that did not cross-react with DNA other than the target hosts, sequences of non-target

species and a *Homo sapiens* reference sequence were included in the alignment. In fact, avoiding the amplification of contaminant human DNA when working with extremely low quality/quantity DNA using highly sensitive methods is not easily achieved (Pääbo et al., 2004), even if both field sampling and subsequent handling of ticks are carried out using all possible precautions to avoid contamination. Alignments were checked visually to identify DNA regions that would optimize the discriminating power of HRMA; i.e. highly conserved intraspecific mtDNA regions of about 200 bp with well-defined interspecific variation (at least two single nucleotide polymorphisms, SNPs; see alignment in Appendix 1). Non-degenerate group-specific primers were designed to have melting temperatures (T_m) of about 60 °C and a low probability of dimer formation as predicted by the Oligo Analysis Tool (www.operon.com/tools/oligo-analysis-tool.aspx). Identified mtDNA regions were tested for their species identification potential in HRMA with uMELT^{TM} (https://dna.utah.edu/umelt/um.php; Dwight et al., 2011) using standard parameters and the thermodynamic parameter set of Unified-SantaLucia (SantaLucia, 1998). Finally, each selected sequence was blasted (BLASTn; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to test if the mtDNA fragment would allow unequivocal species identification by sequencing.

Group-specific primers testing

In order to optimize thermal cycling conditions, each primer set was tested on control DNA samples of the target species (see 3.3.2.1, and Table 4.10). In order to verify that non-target DNA amplification did not occur, control samples of some non-target species were included in the conventional PCR of Muroidea (*C. capreolus, C. l. familiaris, S. antinorii*), Soricidae (*C. capreolus, C. l. familiaris, M. glareolus*),

Passeriformes (*C. capreolus, A. flavicollis*) and Caprinae (*C. capreolus, A. flavicollis*). Moreover, each group-specific primer set was tested using conventional PCR on three human DNA templates extracted from a partially engorged nymph collected while feeding, whole human blood, and human hair, including a negative control of the extraction and positive controls for each primer set (see Fig. 4.4 and 4.5 in Results for additional details).

Conventional PCR was performed at a final volume of 20 µL, containing 0.5 µM of each primer, 0.25 mM of each dNTP, 1x HotMaster Taq Buffer, 1.25 U HotMaster Taq (5-Prime), and 1 µL of template DNA. The thermal cycling consisted of 94 °C for 2 min; 40 cycles at 94 °C for 30 s, T_a (°C) of the group-specific primer set (Table 4.10) for 30 s, 65 °C for 1 min; 65 °C for 10 min, and was performed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Amplification results were checked via capillary electrophoresis on a QIAxcel system (Qiagen, Valencia, CA, USA) with a DNA High Resolution Cartridge and QX 15 bp-3 Kb size marker using the OM500 method, and were analyzed using QIAxcel ScreenGel 1.0.2.0. At least one PCR product per group-specific primer set was purified with Exo-SAP-ITTM (GE Healthcare, Little Chalfont, England) and both forward and reverse strands were sequenced on an ABI 3130 XL using Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA); after the electropherogram check and the creation of a consensus sequence using Sequencher v. 5.1, a BLASTn search was carried out to verify the amplification target.

HRMA validation

Real-time PCR coupled with HRMA was conducted on an ECO™ Real-Time PCR machine (Illumina®, San Diego, USA) twice for each sample at a final volume of 15 µL, containing 0.3 µM of each primer, 1x SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA), and 3 µL of genomic DNA. Thermal cycling conditions were 95 °C for 5 min, 50 cycles at 95 °C for 15 s and T_a (°C) of the group-specific primer set (Table 4.10) for 15 s, directly followed by HRMA carried out at 95 °C for 15 s, 55 °C for 15 s, then by an increase of temperature from 55 °C to 95 °C, and 95 °C for 15 s; fluorescence data was collected every 0.1 °C. HRMA was performed using ECOTM v. 4.0 (Illumina®, San Diego, USA). Raw fluorescence plots were normalized by setting pre- and post-melting regions to 100% and 0%, respectively as in standard HRMA. Both normalized and derivative graphs were analysed for melting temperatures (T_m °C) and melting profile shapes. HRMA of control samples was used to define the minimum and maximum T_m for each host species. Note that the observed T_m was generally 2-6 °C lower than that predicted by uMELT™ (Table 4.10).

HRMA testing on questing ticks

The selected group-specific primers and the Real time HRMA protocol were tested on DNA from questing nymphs (see section 3.3.2.3 Questing nymphs - *Spin-column protocol*). Each tick was tested twice for each one of the group-specific primer sets, with minor modifications (i.e. 55 cycles of amplification was needed for the Muroidea and Soricidae primers). In each Real-time HRMA reaction, one positive control for each target species and one negative control were included. Normalized and derivative HRMA plots were obtained using ECOTM v. 4.0 (Illumina®, San Diego,

USA). Amplicons from questing ticks were assigned to species or genera by visually matching their melting patterns (T_m, melting curve shape, number of melting peaks) to those of control samples (see Results for examples). To verify the accuracy of HRMA in identifying the host species providing the larval bloodmeal, the amplicons of all amplified samples were sequenced, both with reverse and forward primers. Amplicons with T_m and melting curve profiles divergent from those of control samples were also sequenced. Using Sequencher v. 5.1 (Gene Codes Corporation, Ann Arbor, USA), consensus sequences were created, visually checked and then aligned in the corresponding group-specific alignment; in addition, a BLASTn search was carried out to confirm species identity.

3.3.5.6 Application: Real-time HRMA to bloodmeal analysis

Once the sensitivity and specificity of the newly designed Real time HRMA for bloodmeal analysis in questing nymphs was proven (see Results), the protocol was applied to questing nymphs collected in the remaining PAT sampling sites, at the same time introducing more automation into the protocol in order to try to save time and lower cost.

Nymph DNA extraction was performed as described above (see 3.3.2.3 Questing nymphs - *Magnetic-beads protocol*). A QIAgility robotic workstation (Qiagen, Valencia, CA, USA) was used for the automated high precision reaction setup. Real time HRMA was performed in a Rotor-Gene™ 6000 real time rotary analyzer (Corbett Life Science) with a 72-well rotor, as reported above, with the following modifications: initiation step 95 °C for 5 min, 55 annealing and elongation cycles at 95 °C for 15 s and T_a (°C) of the group-specific primer set for 15 s, directly followed by HRM with a pre-

melt conditioning step of 70 °C for 90 s, then increasing the temperature from 70 °C to 95 °C, by 0.2 °C steps for 2 seconds each. The Rotor-Gene 6000 Series Software 1.7 was used to analyse amplifications and HRM results by means of both normalized and derivative melting profile shapes and melting temperatures (T_m °C). Initially, amplifications were sequenced to verify their identity; then, following the procedure described in Collini et al. (2015 in press), only amplicons with aspecific melting properties were additionally investigated via capillary electrophoresis and/or sequence analysis. Capillary electrophoresis was carried out using the QIAxcel system (Qiagen, Valencia, CA, USA) with a DNA High Resolution Cartridge and the QX 15 bp-3 Kb size marker, method OM500; results were analysed with QIAxcel ScreenGel 1.0.2.0. For sequencing, Real time PCR products were purified with Exo-SAP-IT™ (GE Healthcare, Little Chalfont, England); both forward and reverse strands were sequenced on an ABI 3130 XL using Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA). Raw sequences were checked and a consensus sequence created using the software Sequencher v 5.1; a BLASTn search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was carried out to verify species identity of each amplicon.

3.3.5.7 Statistical analysis

The results of bloodmeal analysis of questing nymphs obtained from Real-time HRMA using DNA extracted and amplified during both the optimization and application phases of protocol development were pooled in order to have a comprehensive analysis of the feeding behavior of *I. ricinus* larvae in PAT. Statistical analyses were performed with R v 3.1.0 (The R Foundation for Statistical Computing, 2014) in collaboration with Roberto Rosà (DBEM, Animal Ecology research group).

Identified hosts were grouped according to taxonomic orders (Rodentia, Soricomorpha, Passeriformes, Carnivora, Cetartiodactyla). A chi-square test was used to compare EXTF and PATF sites by means of the proportion of identified bloodmeals in the different host groups. A Linear Model was used to assess variation of the number of ticks with identified bloodmeal (identification success) in relation to the explanatory variables DNA extraction method, sampling year, sampling month and habitat type.

3.3.6 Population genetics

3.3.6.1 Microsatellite amplification

At the outset of the project, microsatellites or short tandem repeats (STR) were believed to be the appropriate molecular markers for the genotyping of feeding and questing ticks, because of their wide use and apparently good resolution in population genetics (Beaumont and Brufford, 1999); in addition, previous studies on *I. ricinus* and other Ixodidae, made use of microsatellites (Delaye et al., 1998; McCoy and Tirard, 2000; Fagerberg et al., 2001; Røed et al., 2006; De Meeûs et al., 2002; Kempf et al., 2009b), as presented in the General introduction. Therefore, ten microsatellite (STR) markers for *I. ricinus* were selected among those described by Røed et al. (2006) and Delaye et al. (1998) (see Table 3.2). According to reported data, they lack Linkage Disequilibrium and deviation from Hardy-Weimberg equilibrium. Subsequently, 4 STR markers described for *I. scapularis* by Fagerberg et al. (2001) were tested. Each locus was tested singly and optimization of PCR conditions was performed on template DNA from three questing adult ticks from different sampling sites. Finally, PCR was performed at a final volume of 20 µL, containing 0.1 µM of each primer, 0.25 mM of each dNTP, 1x HotMaster Taq Buffer, 1.25 U HotMaster Taq (5-Prime) and 1 µL of template DNA. Amplification was performed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA); thermal cycling consisted of 94 °C for 2 min; 30-40 cycles at 94 °C for 15 s, Ta (°C) of the STR marker (Table 3.2) for 15 s, 65 °C for 45 sec; 65 °C for 10 min. 1 µL of PCR product was diluted in 18 µL formamide (Hi-Ti Applied Biosystem) with 0.6% GS500LIZ size standard (Applied Biosystems, Foster City, CA, USA) to be visualized on a ABI3130 sequencer (Applied Biosystems, Foster City, CA, USA); microsatellites scoring was performed using the software GeneMapper

v 3.7 (Applied Biosystem, Foster City, CA, USA).

Table 3.2 Features of selected microsatellites loci.

STR	Repeat motif	Primer sequences and fluorescence label	T_a (°C)	Size range
IRN-4[a]	$(CA)_{14}$	F: NED_GCCATTTTATGTGCCGTTTT R: CTTTGAGTGCGTGCGTGT	54	148-168
IRN-7[a]	$(CA)_{13}$	F: PET_CGGATGATCAATAGTCGATTCC R: CCTAGTCACAAACTCTACCAAGTTA	52	85-101
IRN-8[a]	$(CA)_{15}$	F: PET_CGCTTCGAAGACGACTAAACA R: TGCGAACAATGACAAACAGA	50	169-179
IRN-12[a]	$(GT)_{14}$	F: FAM_GACAAAGGCTGTCAAAGGCTGCATCATA R: CGAGGAAGCCACGACTTGCAGAACTATT	50	151-225
IRN-17[a]	$(GT)_7AT(GT)_{12}$	F: VIC_CATGAGTGTTATATTCGCATTT R: GCTATTACGTCGACGATTTT	55	180-212
IRN-28[a]	$(CA)_{23}$	F: VIC_AGCCACGCTAGTTCTGAGA R: CCTGTTGTGTTTTGTTGGTC	56	100-128
IRN-30[a]	$(GT)_6(GC)_4(GT)_3\ldots(GT)_6$	F: FAM_GCAATTGCTATTCTTTGT R: AGTCTACTAAATCGTCACCA	45	101-109
IRN-37[a]	$(GT)_{19}TT(GT)_2CT(GT)_2$	F: NED_CGGGGCGTTTTTCTTTATTCT R: GAAGCGTCAGACTCCGTAACAG	49	96-122
IR32[b]	$(AG)_{12}$	F: FAM_TCGACAAGTGCAGTGGAGAC R: GTTTCCTACCACAGATTCTCC	61	233-250
IR39[b]	$(AG)_9$	F: PET_ATACCCGTAGAACGAGAG R: GTTTTTCAAGATTTCCGCC	59	121-149
IsAC4[c]	AC	F: AAGCGTATCCGATTTGCCCTTCAT R: GGGTCCCAACGATTGCTAAACCAG	var	n.a.
IsAC8[c]	AC	F: GAGCTACCCCTTTCATCGTCTTCG R: TCTTCCCGCTGCTGTCTCGTATTC	var	n.a.
IsAG25[c]	AG	F: AAATGTCCGAACAGCCTTAT R: GCCCTTGAGTCTACCCACTA	var	n.a.
IsGATA4[c]	GATA	F: CAGACAATGTCATTCAATCGCA R: CGCACAATGCAAAACAAATCTA	var	n.a.

[a]Røed et al., 2006.

[b]Delaye et al., 1998.

[c]Fagerberg et al., 2001; this primer has been tested unlabelled.

3.3.6.2. RAD-Seq library preparation

As reported in Results, STR proved not to be reliable markers for the study of *I. ricinus* population genetics and consequently we moved to SNPs genotyping. As no reference SNPs database is available for *I. ricinus*, we decided to use the recently developed Restriction site Associated DNA sequencing, NGS approach, to *de novo* identify SNPs loci and, at the same time, genotype *I. ricinus* ticks of PAT population (see General introduction).

Restriction enzyme and RAD-Seq strategy choice

To choose the most appropriate RAD-sequencing strategy in terms of restriction enzyme, coverage and number of samples per lane, we used *radcounter v4* (GenePool, Edinburgh). Our target was to paired-end sequencing about 10 individual for each of the 30 sampling sites in PAT (Figure 4.1), for a total of 300 genotyped ticks. Since there is no reference genome currently available for *I. ricinus*, genome data of *Ixodes scapularis* (Geraci et al., 2007) was used with the software, with following sequencing parameters:

- Genome size: 2300 Mbp
- Mean GC%= 45%
- Sequencing technology: HiqSeq2000, 130 million reads per lane;
- plexity (individuals per lane): 46
- coverage: 40x.

R*adcounter v4* analysis suggested *SbfI* as the most appropriate restriction enzyme. It is a non-frequent cutter that identifies the palindromic sequence CCTGCA*GG as the restriction site; it was estimated to be present about 10 times every Mb in the *I. scapularis* genome and therefore it was expected to produce 45136 tags. In such a

RAD-Seq setting, the maximum number of pooled individuals could be 72.

Library preparation

I optimized the RAD-Seq library generation protocol of Etter et al. (2011) for *I. ricinus* at the RAD-TAG platform of the Centre for Ecological and Evolutionary Synthesis (CEES), Oslo University, in collaboration with Emiliano Trucchi in 2012. It was then transferred to the Animal Genetics Laboratory of DBEM-FEM to complete *I. ricinus* genotyping.

Feeding ticks were not used for the RAD-Seq experiment since host DNA would have been over-represented in comparison to *I. ricinus* DNA, reducing RAD-loci coverage; furthermore, bioinformatics analysis would have been extremely complex, since few reference genomes are present for wild host species and none for *I. ricinus*, so that non-tick sequences could not be removed from the dataset. Only adults questing ticks provided to have enough high quality genomic DNA for the application of individual RAD sequencing. However, long trials have been conducted with the different DNA extraction methods described in section 3.3.2.2 in order to select the most efficient, as briefly described in Results. After quality assessment, samples showing high molecular weight and highly concentrated DNA were employed in NGS of RAD-tags.

Briefly, 250 ng of genomic DNA per sample were used, instead of the suggested 1 µg, in the enzymatic *SbfI* digestion (SbfI-HF™, New England BioLabs®). Decreasing gDNA input allowed to use ticks samples for which the DNA concentration was as low as 10 ng/µL. Forty-six Illumina P1 adapters and the P2 adapter were provided us by the RAD-TAG Platform (CEES, University of Oslo). Illumina P1 barcoded adapters were

ligated to digested samples. The 5-nucleotide barcode allows sample identification in bioinformatics data processing and each one differs by at least 2 bp, to limit erroneous sample assignment due to sequencing error. Once barcoded, samples were pooled in libraries of multiple individuals. Pooled samples were sheared by sonication (Bioruptor® Plus, Diagenode) to a mid-size range of 300-600 bp and, after concentration to 25 µL using the QIAquick PCR purification Kit (Qiagen, Valencia, CA, USA), they were size selected on a 1% agarose gel stained with ethidium bromide. Purification steps after the P2 adapter ligation were performed using DNA capture on magnetic beads following the manufacturer's instructions (Agencourt AMPure XP, Beckman Coulter, MA, USA) instead of the standard Qiagen column based purification, at the following ratio *beads solution : DNA = 0.8 : 1*, in an attempt to improve the quantity of retrieved DNA and reduce the carry-over of un-ligated adapters. PCR amplification was performed in 8 x 12.5 µL aliquots, using 16 µL of library template, and setting 21 to 23 replication cycles. To obtain the enriched library concentration requested for sequencing (10 µL library at 10 ng/µL), it was necessary to perform multiple distinct amplifications for each library and combine them before the final concentration step. Amplification product was quantified using the fluorometric-method (Qubit® 2.0), after concentration with magnetic beads, as above, the 260/280 ratio was measured with Nanodrop 8000 UV-Vis Spectrophotometers (Thermo Scientific).

A preliminary library was prepared while optimizing the protocol at the CEES, Oslo and sequenced in November 2012; forty-two adult questing ticks, coming from five different sampling sites and extracted with three different methods (spin-column; phenol protocol and magnetic-beads), were sequenced in a single lane (details in Table 3.3) on a Illumina HiSeq2000 at the Norwegian Sequencing Center, University of Oslo.

In 2014 at FEM, twelve libraries were prepared from 256 adult questing ticks from PAT questing adults, two females from Finland were added to the analysis; males and females, randomly chosen by sampling site and gDNA concentration, were processed in separate libraries, as for the two sex gDNA concentration was significantly different. As two libraries of male individuals did not give good amplification results, probably as a result of low quality/quantity DNA (erroneous gDNA quantification), only 10 libraries were sent for sequencing (2 males and 8 females libraries). Those were pooled two by two in equimolar ratio prior to sequencing, in order to maintain proportional RAD-loci representation for each individual (details in Table 3.4; Fig. 3.3). Paired-end sequencing was performed on a Illumina HiSeq2000 at the Norwegian Sequencing Center, University of Oslo.

Table 3.3 Samples sequenced in the preliminary 2012 RAD-Seq library. Sampling site, sex (F=female; M=male), DNA extraction method used (A=spin-column; B=phenol protocol; and C=magnetic-beads) are reported, as well as the final concentration (ng/ µl) of the enriched library sent for sequencing at the Norwegian Sequencing Center, University of Oslo.

	Samples		**DNA extraction**				**Concentration**
Site	**F**	**M**	**A**	**B**	**C**	**Total**	**(ng/µL)**
CAV	5	3		8		8	
LAM	7	2	6		3	9	
CON	7	2	5		4	9	
REV	6	1		1	6	7	
CVS	7	2	1		8	9	
			Final library			42	14.0

Table 3.4 Wet lab results and sample composition (number of individuals and sex) for the 2014 RAD-Seq libraries. Sex (F=female; M=male), final concentration (ng/ µl) and 260/280 ratios are reported.

Lane	Library name	n° ind	sex	concentration (ng/µl)	ratio 260/280
1	LIB-1	21	F	10.3	-
	LIB-12	22	F	21.8	1.78
2	LIB-2	21	F	9.6	-
	LIB-3	21	F	12.6	-
3	LIB-4	22	F	22.0	1.87
	LIB-5	21	M	10.0	1.75
4	LIB-7	22	F	10.1	-
	LIB-8	22	F	10.9	1.74
5	LIB-10	21	M	10.2	-
	LIB-11	22	F	12.2	1.85

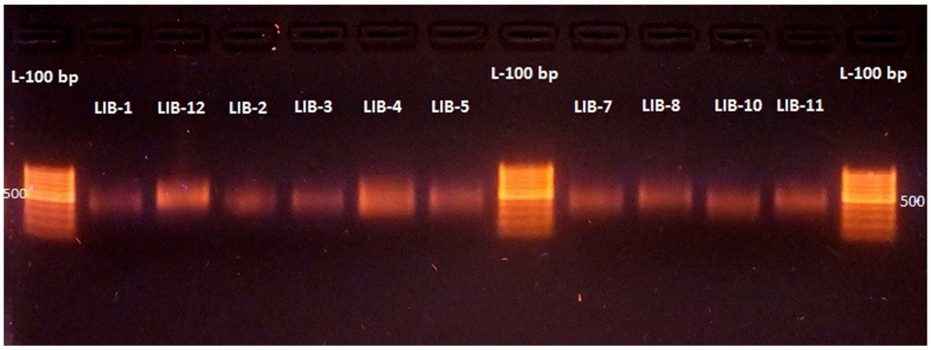


Fig. 3.3 Photograph of the gel electrophoresis of the final 2014 RAD-Seq libraries after amplification and concentration. Medium library size is 500 bp.

3.3.6.3 Bioinformatic analysis

Preliminary de novo genotyping analysis on first sequenced lane

Stacks v. 0.99997 (Catchen et al., 2011, 2013) was used for analysis of the preliminary library of 42 individuals, to investigate the efficiency of the different methods used for DNA extraction. Using the *process_radtags* program, raw data were demultiplexed, according to assigned individual barcodes and quality filtered setting a

minimum Phred quality score of 10 (90% of base call accuracy) in a sliding window of 15% of the sequence. No trimming of the sequences was set at this point. Sequences with no scored nucleotides (N), ambiguous nucleotides in the barcode region or in the restriction site were automatically discarded. As a standard in *de novo* mapping and genotyping RAD-Seq experiment, only first reads (the ones originated at the restriction site and containing the barcode R1), were used and fed in the pipeline *denovo_map.pl;* SNP identification and definition of diploid individual genotypes at each nucleotide position as performed in a maximum likelihood statistical framework (Hohenlohe et al., 2010). Briefly, the pipeline first identifies "*stacks*", identical sequences from a single individual, and then searches for the same stack in all other individuals and merges them to form a locus. In this putative locus, genetic variation is examined and genotype called (see also General Introduction Fig. 1.10). Parameters for the assembly were set as follows: minimum stack depth 5 (-m), maximum distance between stack 3 (-M), maximum distance to align secondary reads 5 (-N) and maximum number of stacks allowed for *de novo* locus formation 3 (default). The genetic variability model type was set to SNP and the alpha significance level to call a SNP was left as default (0.05). Deleveraging algorithm (-t), allowing identification of loci deriving from repeat region and PCR artifact, was enabled. For parameter definitions and effects on *de novo* formation of *stacks* and loci, please refer to http://creskolab.uoregon.edu/stacks/param_tut.php.

Full data set de novo *genotyping analysis*

As no reference genome for *I. ricinus* is available, *de novo* RAD-Seq loci building and SNP calling was performed; at first following the standard procedure (A.)

described in (http://creskolab.uoregon.edu/stacks/) and then, once we had identified some anomalies in the RAD-Seq sequence dataset (i.e. PRC clone; low number of RAD-loci; see Results), more conservative approaches were used (B.)

A. De novo *SNP calling with Stacks; no PCR duplicates removal*

Raw sequences were trimmed to 80 bp using *fastx_trimmer* (FASTX-Toolkit v 0.0.13.2) and then demultiplexed and quality filtered, setting a minimum Phred quality score of 20 (99% base call accuracy), using the *process_radtags* command of Stacks v. 0.99997 (Catchen et al., 2011); sequences with uncalled nucleotides and more than one ambiguous nucleotide in the barcode or in the restriction site sequence were discarded (Catchen et al., 2011, 2013). Simultaneous identification of SNPs and individual genotyping was performed without a reference genome using the *denovo_map.pl* pipeline of Stacks v 1.12. Parameters for the *denovo_map.pl* pipeline were set as follows: minimum stack depth 5 (–m), maximum distance between stack or within individual distance 2 (-M), maximum number of mismatches between loci when building the catalog 2 (–n) and maximum number of stacks allowed for locus 2 (-X "ustacks:--max_locus_stacks"). SNP calling from secondary reads was disabled (-H). The genetic variability model type was set to SNP and the alpha significance level to call a SNP was left as default (0.05). Deleveraging algorithm, allowing the identification of loci deriving from the repeat region and PCR artifact was enabled (-t). The pipelines *export.pl* or *population.pl* were used to export the genotyping results in different file formats.

B. De novo *SNP calling with Stacks; PCR duplicates removal*

According to investigative analysis showing an extremely high rate of PCR clones per individual in the sequence data (>85%, expected 20-50%; see Results), we

decided to remove these duplicate as they may introduce bias in the SNP calling process.

Raw reads were trimmed to 90 bp using *fastx_trimmer* command line of FASTX-Toolkit v 0.0.13.2 and then demultiplexed and cleaned using *process_radtags.pl* program of *Stacks* (v 0.99997, Catchen et al., 2011); sequences with a medium Phred Quality score under 20 (99% of base call accuracy) in a sliding windows of 15% of the sequence itself, uncalled nucleotides and more than one ambiguous nucleotide in the barcode or in the restriction site sequence were discarded (Catchen et al., 2011). Individual paired-end reads were cleaned of PCR-duplicates with c*lone_filter* program. It identify a PCR clone (PCR duplicate) as a pair of reads that match exactly, because a paired-end reads from two different DNA molecules will nearly always be slightly different in length. Individual loci and SNPs were called *de novo* with a maximum-likelihood function by feeding R1 reads in the *denovo_map.pl* pipeline of *Stacks* v 1.12. Parameters for the assembly and SNP calling were set as following: minimum stack depth 2 (–m), maximum distance between stack or within individual distance 2 (–M), maximum number of mismatches between loci when building the catalog 2 (–n) and maximum number of stacks allowed for locus 2. Calling of SNP from secondary reads was disabled (-H). The genetic variability model type was set to SNP and the alpha significance level to call a SNP was left as default to 0.05. A deleveraging algorithm, allowing the identification of loci deriving from repeat regions and PCR artifacts, was enabled. A table including all loci having at least 1 SNP was built using *export_sql.pl* in *Stacks*. Genotype outputs were generated in *.plink* and *.vcf* formats using *population* in Stacks.

As a preliminary analysis showed a steep increase in the number of SNPs

identified from the 80th bp on (Fig. 3.4), only SNPs identified up to the 79th bp were retained in the following analyses.

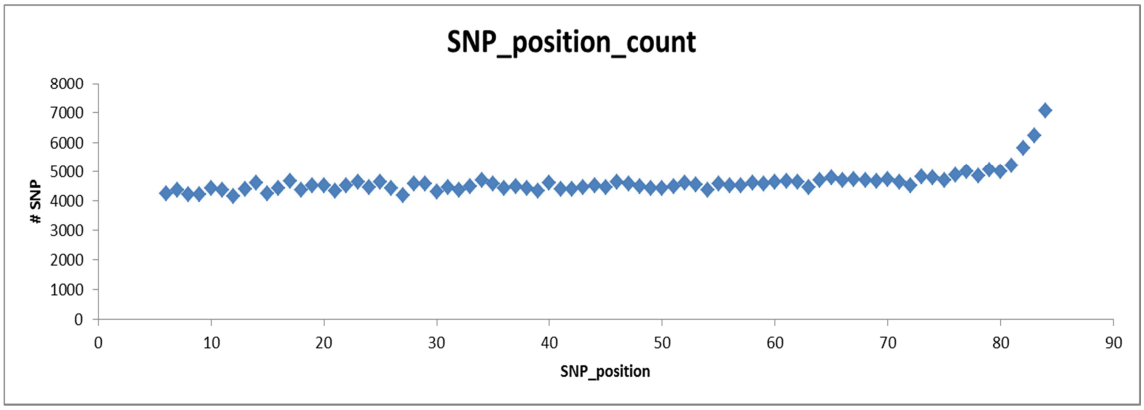


Fig. 3.4 Number of SNP per nucleotide position. Note that there is a significant increase in number of SNPs in the last five nucleotides, suggestive of sequencing errors.

C. SNP calling with alignment to reference genome

The following analyses were carried out in collaboration with the Department of Life Sciences and Biotechnology, University of Ferrara, in the research group led by Giorgio Bertorelle, with the FEM-UNIFE co-funded PhD student Alex Panziera (who is developing and testing analyses of RAD-tag data) and the bioinformatician Andrea Benazzo.

Given the peculiarities of the starting raw data (i.e. high level of PCR duplicates; low number of identified stacks; low genome coverage; see Results), a SNP calling approach with alignment against a reference genome was attempted, in order to obtain more robust SNPs and a higher quality genotyping dataset. Since there is no reference genome for *I. ricinus*, I aligned *I. ricinus* sequences against that of *I. scapularis* (even though *I. scapularis* is not considered a sister species of *I. ricinus*, it is the most similar genome available), thanks to software specifically developed to deal with genome alignment of distantly related species. The IscaW1 (GenBank accession number:

ABJB000000000.1; assembly version: 2008) reference genome is composed of 369492 scaffolds, totalling 1.76 Gb, with a supercontig N50 size of 72 Kb. In the alignment process, repeat regions were masked and only contigs with a length higher than 10k bp were used (17 000 contigs retained out of 369492), by filtering them with a specifically written script. For each *I. ricinus* individual, paired-end sequences were demultiplexed and cleaned (trimmed to 80 bp) and, both R1 and R2 were aligned against the filtered set of scaffolds of IscaW1 using *Stampy* (Lunter and Goodson, 2011), setting a mutation rate of 10%, estimated by aligning nuclear gene sequences from *I. scapularis* and *I. ricinus* retrieved from GenBank, and an insert size of 300 bp. Only sequences aligning with a quality score of 20 were retained for the following steps. After the indexing step of alignment files, performed with SAMtools (Li et al., 2009), PCR duplicates were filtered out of the dataset using *Picard* (http://broadinstitute.github.io/picard/). Finally, GATK (McKenna et al., 2010) was used to check alignment in correspondence of *indel* polymorphisms. For each individual only 0.01 – 0.50 % of reads were *not* PCR duplicates and resulted to be properly aligned. GATK was used to call and identify SNPs; only SNPs having a coverage of at least 4 reads and an alignment quality score higher than 20 were considered. Only a few usable SNPs were retrieved using this approach; therefore, a PCA was performed using genotype probabilities, instead of actual genotypes, computed with the software *ANGSD* (Korneliussen et al., 2014).

Population genetics analysis

VCFtools (Danecek et al., 2011) was used to filter full *.vcf* SNPs dataset. By means of individual missing rate, site missing rate and to convert *.vcf* files to *.plink*. PGDSpider 2.0.5.2 (Lischer and Excoffier, 2012) was used in file format conversions.

The PCA was computed with *smart_pca* of the EIGENSOFT package (Patterson et al., 2006). The *lsqproject* algorithm was always enabled, as suggested for datasets with a high proportion of missing data. Plots of the top two principal components were built with *ploteig* or within the Excel environment, according to different grouping strategies. Significance of the computed eigenvalues was assessed by running *twstat* that calculate Tracy-Widom statistics (Patterson et al., 2006). MultiDimensional Scaling (MDS) analysis was computed with R (The R Foundation for Statistical Computing, 2014), using a custom script wrote by Andrea Benazzo (University of Ferrara); plots were created in Excel. MDS is a robust analysis for genetic structure investigation, even for a dataset with a high rate of missing data.

PCA and MDS were computed on individual genotypes, but also on consensus genotypes built for each of the 30 PAT and the Finland one (which were added for comparison). The consensus genotypes were built from individual genotypes of the defined site; the following rule were followed: at SNP locus level, the more frequent genotype will be chosen, in case of missing genotype in all individuals from a site will also be missing also in the consensus in other sites, otherwise, the more frequent in the other individuals of the site will be chosen. Consensus genotypes were created with a custom script provided by Alex Panziera.

Building mini-contigs from paired-end sequences

Using the command lines *sort_read_pairs.pl* and *exec_velvet.pl* implemented in Stacks v 1.12 and following the procedure described at http://creskolab.uoregon.edu/stacks/pe_tut.php, mini-contigs were built from individual paired-end sequences (90 bp trimmed). Resulting contigs were blasted with default

parameters (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and MEGAN v 5.9.1 (MEtaGenome Analyzer, D. Huson, University of Tübingen) was used to visualize results; only sequences having a BLASTn e-value of 10^{-5} and a minimum score of 80 were retained in the analysis.

4. RESULTS

4.1 Sampling sites

As described in the Materials and Methods, 30 sampling sites were identified in PAT (Fig. 4.1 and Table 4.1). In order to explore possible epidemiological implications, such as differential host use, specifically for the bloodmeal analysis (see 4.2.3), we chose sites in extensive forests (N=18; EXTF) and forest patches surrounding human settlements (N=12; PATF) (Table 4.1 and Fig 4.1). Instead, in order to interpret population genetics patterns, it was also important to distribute site locations according to relevant geographical barriers (i.e. mountain chains; rivers) that may influence gene flow between tick populations. In PAT, previous genetic studies on large mammalslike roe deer and red deer have shown that the Adige River Valley, that cuts north-south through the Trento-Alto Adige Region, is a major barrier for gene flow between populations on the West and East side of the river. Since these same species may have an important role in tick dispersal, sampling sites have also been grouped in East (13) and West (17) Province of Trento in some analyses (Table 4.1 and Fig. 4.1).

Hence, the sites were chosen from a range of altitudes, from the lowest, Ala, at 253 m a.s.l, to the highest, Lundo (San Giovanni), at 1264 m a.s.l., evenly distributed throughout PAT, with at least one site in each major valley in typical tick habitat. Ticks were mainly sampled in high stand mixed (coniferous and broad leaved forest) or broad leaved forests, highly favorable tick habitat (Rizzoli et al. 2009; Gray 1998); in two cases, Val Genova and Revò, conifers were predominant, but ground cover, maintaining a sufficient level of humidity for ticks survival, was guaranteed by a rich understory.

Table 4.1 Sampling site parameters. Area (EAST or WEST) is in reference to the Adige River (see Fig. 4.1). EXP: exposure; EXTF: Extensive forst; PATF: Forest patch near urban area. Tick sampling was carried out at each site for questing ticks (Q) or both questing and feeding ticks (Q & F).

Area	Site code	Site (locality)	Coordinates DMS		m a.s.l.	EXP	Brief site description for questing tick sampling	Forest type	Habitat type	Sampling type
EAST	TRA	Transacqua	46° 9'52.09"N	11°49'57.10"E	850	NW	mountain roadside in forest	high stand mixed forest	EXTF	Q & F
	CAO	Caoria	46°11'27.15"N	11°40'59.16"E	940	flat	rural area	meadows and high stand mixed forest	EXTF	Q
	ALA	Ala (Sdruzzinà)	45°44'2.85"N	10°58'29.26"E	253	N	walking path in forest	high stand broad-leaved	EXTF	Q
	VOL	Volano	45°54'55.71"N	11° 5'9.81"E	255	N	walking path in forest	coppices	EXTF	Q
	TRE	Trento (Parco Casteller)	46° 2'7.20"N	11° 8'21.10"E	400	W	urban park	coppices	PATF	Q
	GRI	Grigno	46° 0'42.37"N	11°37'23.61"E	254	flat	SIC area	coppices	EXTF	Q & F
	LEV	Levico	46° 1'10.77"N	11°17'55.83"E	533	S	walking path near the village	coppices	PATF	Q
	PER	Pergine	46° 3'28.68"N	11°12'38.41"E	570	S-E	walking path in forest nearby meadows	high stand broad-leaved forest	PATF	Q
	TEL	Telve	46° 4'12.39"N	11°27'49.05"E	831	SW	walking path in forest	high stand mixed forest	EXTF	Q
	TES	Tesino	46° 3'12.67"N	11°37'54.98"E	749	SW	walking path in rural area	meadows and high stand mixed forest	PATF	Q
	SEG	Segonzano	46°11'6.24"N	11°15'56.30"E	809	SW	walking path in forest	high stand broad-leaved forest	PATF	Q
	GIO	Giovo	46° 9'25.23"N	11° 9'31.92"E	556	-	walking path in forest (houses nearby)	high stand mixed forest	PATF	Q & F
	CVS	Cavalese	46°16'53.97"N	11°27'23.10"E	870	SW	walking path in forest nearby meadows	high stand mixed forest	PATF	Q

Table 4.1 Con'd

Area	Site code	Site (locality)	Coordinates DMS		m a.s.l.	EXP	Brief site description	forest type	habitat	sampling type
WEST	PIN	Pinzolo (Giustino)	46° 9'13.37"N	10°46'22.15"E	895	SW	walking path in forest (houses nearby)	high stand mixed forest	PATF	Q & F
	LUN	Lundo - San Giovanni	45°58'58.80"N	10°53'21.63"E	1264	SE	random sampling in forest	high stand mixed forest	EXTF	Q & F
	VGE	Val Genova	46°10'0.39"N	10°39'40.61"E	1130	S	walking path in forest	high stand mixed forest (mainly coniferous)	EXTF	Q
	TIO	Tione di Trento	46° 2'33.59"N	10°43'59.72"E	566	SW	walking path in rural area	high stand mixed forest	EXTF	Q
	CON	Condino (Calamara)	45°53'5.71"N	10°36'9.01"E	454	W	walking path in rural area	meadows and coppices	PATF	Q & F
	PDU	Passo Durone	46° 1'48.93"N	10°48'17.57"E	956	SE	walking path in forest nearby meadows	high stand mixed forest	EXTF	Q
	LED	Ledro	45°54'15.35"N	10°43'56.86"E	822	W	walking path from the village to the forest	meadows and high stand mixed forest	PATF	Q
	PIE	Pietramurata	46° 0'56.23"N	10°55'27.34"E	677	E	random sampling in forest	high stand broad-leaved	EXTF	Q
	LAM	Laghi di Lamar	46° 7'55.31"N	11° 3'50.47"E	730	SW	walking path along the lake	high stand broad-leaved	EXTF	Q
	CAD	Cadine	46° 5'51.77"N	11° 4'27.45"E	550	W	random sampling in forest	coppices	EXTF	Q & F
	CAV	Cavedine (Dos gaggio)	45°59'7.36"N	10°57'48.26"E	776	flat	walking path in forest	meadows and high stand broad-leaved forest	EXTF	Q & F
	BRE	Brentonico (Castione)	45°50'6.51"N	10°57'45.66"E	615	NW	roadside in rural area	coppices	EXTF	Q
	MOL	Molveno	46° 7'21.99"N	10°57'58.08"E	981	W	walking path along the lake	high stand beech forest	EXTF	Q
	DIM	Dimaro	46°19'6.88"N	10°52'0.03"E	950	N	walking path in forest (mountain houses nearby)	high stand mixed forest	PATF	Q
	MEZ	Mezzocorona	46°14'25.57"N	11° 3'33.16"E	270	flat	SIC area	coppices	EXTF	Q & F
	REV	Revò	46°25'5.90"N	11° 4'44.28"E	827	SE	walking path in forest (apple orchards nearby)	high stand mixed forest	PATF	Q & F
	VER	Vervò	46°18'32.33"N	11° 7'30.48"E	965	SE	walking path in forest	high stand mixed forest (mainly *Pinus* spp.)	EXTF	Q

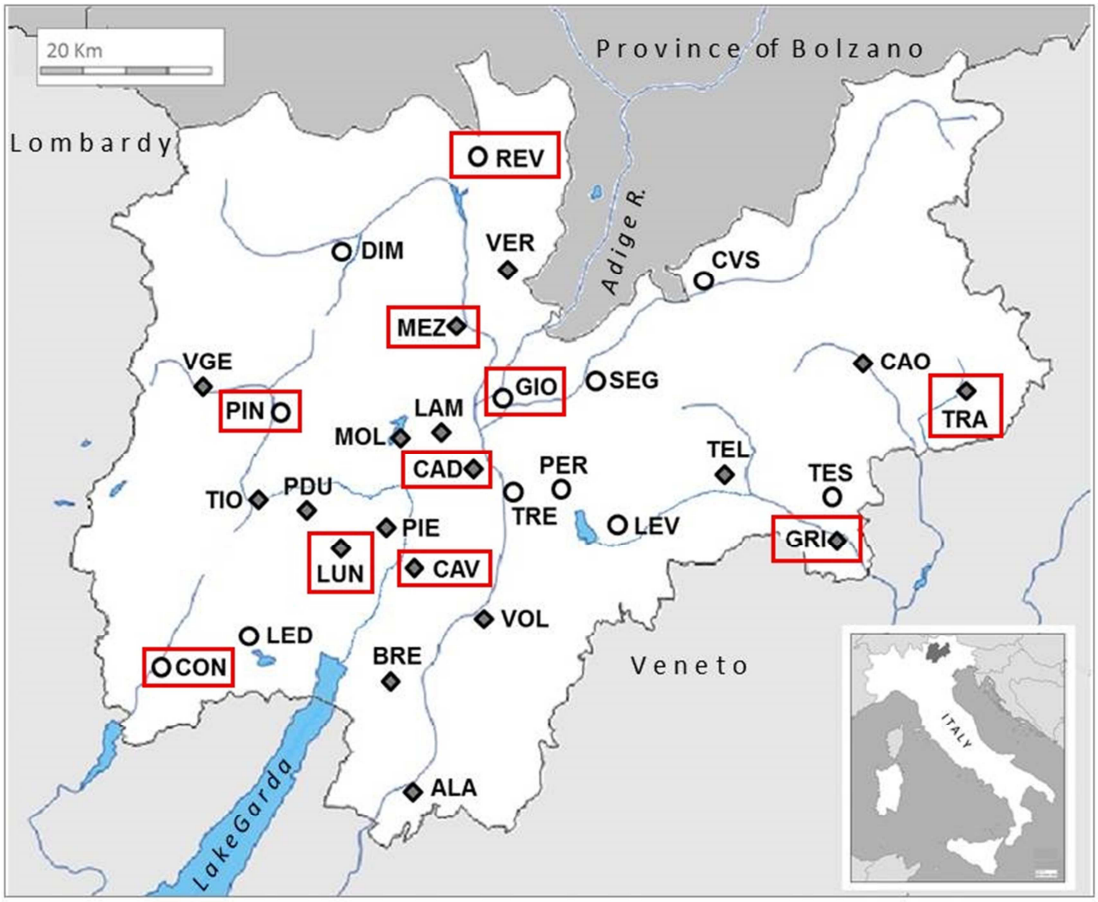


Fig. 4.1 Location of sampling sites in PAT (northeastern Italy: see inset). Note that the Adige River cuts longitudinally across the Province. Sites in which both questing and feeding tick were collected are marked by a red box. Open circles represent sites in forest patches (PATF), while solid diamonds correspond to sites in or at the edge of extensive forests (EXTF).

4.1.1 Questing tick sampling

Generally, the sampling target for each site was reached (see section 3.2.2), except for the sites VGE, DIM, LED, LUN and ALA, where density of adult ticks was particularly low (i.e. even after repeated sampling it was not possible to reach the goal of 12 adults individuals; Table 4.2). In total, 1887 ticks were sampled (314 females, 269 males and 1304 nymphs). Ticks density varied from 1/100 m^2 for TES to 63/100 m^2 for ALA.

Table 4.2 Questing tick sampling results (F=adult females; M=adult males and N=nymphs). For site codes, see Table 4.1.

Site code	Collection date	Tick density (ticks/100 m^2)	F	M	N	Total
ALA	10 May 2012	63/100	5	4	55	64
BRE	10 May 2012	17/100	15	11	54	80
CAD	07 May 2012	21/100	7	16	56	79
CAO	24 April 2013	18/100	10	12	54	76
CVS	23 May 2012	6/100	11	3	23	37
CAV	17 April 2012; 16 April 2013	-	24	14	102	140
CON	27 April 2012	55/100	18	3	48	69
DIM	02 June 2013	8/100	4	9	40	53
GIO	23 April 2013	17/100	11	10	56	77
GRI	24 April 2013	13/100	8	10	50	68
LAM	22 May 2012	46/100	11	10	41	62
LED	25 May and 22 June 2012	6/100	5	4	44	53
LEV	26 April 2012	19/100	15	12	37	64
LUN	14 May 2012	4/100	3	5	35	43
MEZ	23 April and 03 June 2013	-	11	10	37	58
MOL	24 May 2012	2/100	14	6	36	56
PDU	14 May 2012	-	10	7	27	44
PER	26 April 2012	9/100	4	8	38	50
PIE	07 May 2012	6/100	9	10	59	78
PIN	11 May 2012	4/100	8	5	32	45
REV	29 May 2012	20/100	12	12	30	54
SEG	23 May 2012	53/100	8	14	41	63
TEL	21 June 2012	3/100	7	6	21	34
TES	25 June 2012	1/100	10	10	37	57
TIO	11 May 2012	12/100	19	11	37	67
TRA	05 June 2012	19/100	8	9	43	60
TRE	16 April and 02 May 2013	16/100	24	15	53	92
VGE	26 May 2013	12/100	5	4	36	45
VER	30 May 2012	5/100	8	7	37	52
VOL	22 May 2012	20/100	10	12	45	67
		TOTALS	**314**	**269**	**1304**	**1887**

4.1.2 Feeding tick sampling

4.1.2.1 Trapping of small mammals

As expected, because of its ubiquity in all forest types, *Apodemus* spp. were captured in all sites, whereas *M. glareolus* was captured only at LUN, GRI and PRI sites (Table 4.3). A total of 865 ticks from *Apodemus* spp. were collected and the minimum target of 30 samples for each site was reached. For *Apodemus* spp., the highest tick load (number of ticks/parasitized animal) was found at CON, with a mean of 17 ticks/individual, mainly larvae, whereas at other sites load ranged between 2 (LUN) and 7 (MEZ). This result could related to the specific date of sampling (mid August) when the larval stage normally has a density peak (Tagliapietra et al., 2011). 95% (825/865) of the sample were larvae, while nymphs and adults represented only a small fraction of the ticks parasitizing small mammals. Other small mammals species were also captured; specifically in LUN, *Muscardinus avellanarius* was trapped carrying 2 larvae, as well as 9 *Sorex* individuals, carrying a total of 44 larvae.

Table 4.3 Feeding ticks collected from small mammals (l=larvae; n=nymphs and a=adults).

	Species									
	Apodemus spp.					*Myodes glareolus*				
Site	Ind*	l	n	a	total	Ind*	l	n	a	total
CAD	24	104	8	0	112	n.r.[#]				
CAV	33	172	12	0	184	n.r.				
LUN	17	29	2	2	33	14	21	2	0	23
GRI	78	-	-	-	> 200	4	-	-	-	>10
CON	8	134	3	0	137	n.r.				
PIN	39	115	1	0	116	n.r.				
REV	18	75	1	0	76	n.r.				
PRI	20	66	0	0	66	9	29	7	0	36
GIO	13	63	5	0	68	n.r.				
MEZ	11	67	6	0	73	n.r.				
Totals	261	825	38	2	865	27	50	9	0	59

*number of trapped animals parasitized by ticks; -: number of tick/stage not available; [#] n.r.: species not retrieved in this site.

4.1.2.2 Bird netting

In all sites other than MEZ, the three focal host species were present. *T. merula* and *T. philomelos* presented the highest tick load (number of ticks/parasitized animals) with 5 ticks/individual; *T. merula* carried twice as many nymphs as larvae, while *T. philomelos* was infested almost equally by larvae and nymphs (Table 4.4). On the other hand, *E. rubecula* carried a mean of fewer ticks (2 ticks/individual) and mainly larvae; consequently, for this species, I was unable to reach the target number of feeding ticks except where a high number of *E. rubecula* were trapped (CAV and GIO), and REV (a single individual carried 14 larvae). No adult ticks were found feeding on birds.

Table 4.4 Feeding ticks collected from birds for the most frequently netted species (tick l=larvae; n=nymphs and a=adults) at the 10 selected sampling sites in PAT.

Species	***Turdus merula***					***Turdus philomelos***					***Erithacus rubecula***				
Site	Ind*	l	n	a	total	Ind*	l	n	a	total	Ind*	l	n	a	total
CAD	14	23	61	0	84	13	33	17	0	50	8	15	6	0	0
CAV	97	·	-		428	7	4	19	0	23	27	26	15	0	41
LUN	7	6	16	0	22	4	-	-	-	37	2	1	1	0	2
GRI	8	3	29	0	32	7	13	36	0	49	3	4	2	0	6
CON	4	11	25	0	36	9	37	32	0	69	4	6	3	0	9
PIN	10	62	9	0	71	4	23	23	0	46	4	3	5	0	8
REV	15	43	21	0	64	6	25	4	0	29	8	34	1	0	35
PRI	9	8	29	0	37	7	15	2	0	17	9	19	3	0	22
GIO	8	25	37	0	62	10	17	33		50	20	63	7	0	70
MEZ	26	20	48	0	68	5	0	10	0	10	n.r.[#]				0
Totals	198	244	560	0	904	72	167	176	0	343	85	171	43	0	171

*number of trapped animals parasitized by ticks; -: number of tick/stage not available; [#] not retrieved.

In addition to these three species, a large variety of other birds were netted and found parasitized by ticks (Table 4.5), even though some of them are not ground feeding species. It is interesting to note that *Sylvia atricapilla* and *Parus major*, netted at a lower frequency in all the sites, were also frequently found infested by ticks, although with a low number (mean load 1 or 2 ticks/infested individual), while three *Garrulus glandarius* carried in average 6 ticks each, an higher load possibly related to the greater body size of this species (Marsot et al., 2012).

Table 4.5 Feeding ticks on bird species that were less frequently trapped and/or are not normally considered important tick hosts (l=larvae and n=nymphs).

Species	Ind*	l	n	Total
Sylvia atricapilla	16	16	6	22
Parus major	11	14	8	22
Garrulus glandarius	3	5	12	17
Luscinia megarhynchos	7	5	10	15
Poecile palustris	2	1	8	9
Troglodytes troglodytes	3	7	1	8
Parus scopaiola	5	1	7	8
Pyrrhula pyrrhula	2	0	5	5
Fringilla coelebs	4	2	2	4
Aegithalos caudatus	1	0	3	3
Turdus viscivorus	1	1	1	2
Phylloscopus collybita	1	1	1	2
Phoenicurus phoenicurus	2	1	1	2
Motacilla cinerea	1	0	2	2
Acrocephalus palustris	1	0	2	2
Sylvia borin	1	0	1	1
Sitta europaea	1	0	0	1
Phoenicurus ochruros	1	0	1	1
Muscicapa striata	1	1	0	1
Lanius collurio	1	1	0	1
Hippolais icterina	1	0	1	1
Coccothraustes coccothraustes	1	1	0	1

*number of trapped animals parasitized by ticks.

4.1.2.3 Collection of ticks feeding on large mammals

C. capreolus and *C. elaphus* provided most of the ticks from wild large mammals, with 1204 and 242 samples collected, respectively (Table 4.6). *C. capreolus* provided the highest number of ticks since population sizes permit many more hunting licenses for this species than for *C. elaphus* in PAT; in additional, there were high numbers of tick larvae on the collected roe deer forelegs (808). *R. rupicapra* individuals

were found to be parasitized by *I. ricinus* only in GRI; at this site, from 10 individuals, a total of 30 ticks (5 larvae, 2 nymphs and 23 adults) were collected. In PIN, an *Ovis aries musimon* was controlled for ticks and 1 nymph and 3 adults were collected.

Table 4.6 Feeding ticks collected from large mammals (l=larvae; n=nymphs and a=adults).

Species	***Capreolus capreolus***					***Cervus elaphus***				
Site	Ind*	l	n	a	total	Ind*	l	n	a	total
CAD	25	124	18	20	162	3	0	0	10	10
CAV	16	244	67	20	331	1	5	6	14	25
LUN	9	107	27	0	134	3	5	9	1	15
GRI	13	13	6	21	40	5	0	0	16	16
CON	7	40	14	25	79	1	0	0	2	2
PIN	20	13	7	42	62	12	0	1	38	39
REV	16	118	25	30	173	9	8	4	32	44
PRI	5	7	3	9	19	6	16	4	23	43
GIO	11	111	27	20	158	2	15	5	28	48
MEZ[#]	7	31	11	4	46					0
Totals	129	808	205	191	1204	42	49	29	164	242

*number of animals checked (carcasses and/or forelegs) and parasitized by ticks; [#]*C. elaphus* not hunted in this site.

I sampled ticks from domestic sheep (*O. aries*) in a single flock in CAV; 16 *I. ricinus* females were collected from 16 animals.

Ticks were also collected from domestic dogs. In REV, 4 ticks adults were collected simultaneously from a dog living in a farm surrounded by patches of forest and apple orchards. In CON and PIN, ticks were collected over time from two dogs for a total of 11 and 3 adults, respectively; both were family pets frequently visiting forests.

4.2 DNA extraction

Here, I briefly report the most challenging problem we had to face during DNA extraction, i.e. obtaining good quality and highly concentrated gDNA for the application of RAD-Sequencing to *I. ricinus*.

As reported in section 3.3.2.2 for adult ticks, three different approaches were used. With the spin-column, Qiagen Blood & Tissue kit, we obtained good results both in terms of quality and quality of DNA when we applied this method to fresh ticks, or ticks that were frozen for only a few weeks (Fig. 4.2a); however, when the same kit was applied to samples preserved at -80 °C freezer for 2-3 months, insufficient quality and quantity gDNA was obtained (Fig. 4.2b). With the phenol-phenol protocol, we obtained good results even from ticks frozen for a longer period of time, but since the results of L. Cornetti (personal communication) suggested that samples extracted with this method did not produce good RAD sequencing data, we did not use this DNA protocol DNA for further extractions.

Subsequently, we tested the recently acquired magnetic-based DNA purification reagents and instrumentation. This method allowed gDNA extraction from frozen adult ticks, and remarkably, the quality and quantity of extracted DNA was significantly improved in comparison to the Qiagen kit used on fresh ticks (Tab. 4.7; Fig. 4.3), as the Mann-Whitney test for statistical difference between the two samples (i.e. different extraction methods) highly supports (Table 4.7).

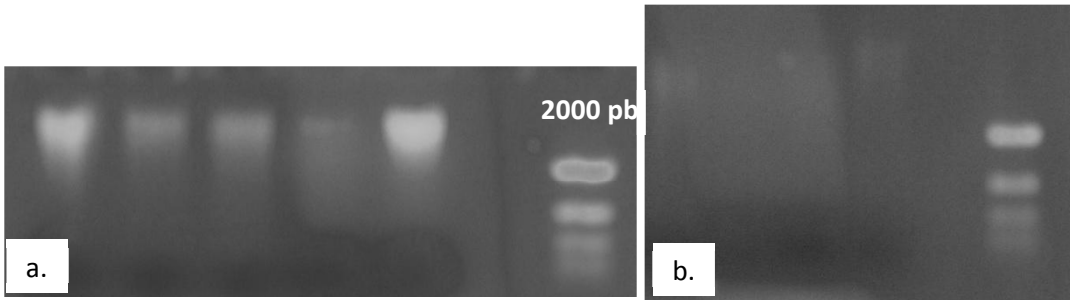


Fig. 4.2 Photographs of the gel electrophoresis of DNA extracted from questing adult ticks using the manual spin-column Qiagen Blood & Tissue Kit **a**. gDNA obtained from fresh ticks or from ticks frozen for a few weeks (-80°C); the compact bands with high molecular weight indicate good quality/quantity DNA. **b**. genomic DNA obtained from 3-4 months old ticks preserved at – 80°C; note that the gDNA is highly degraded.

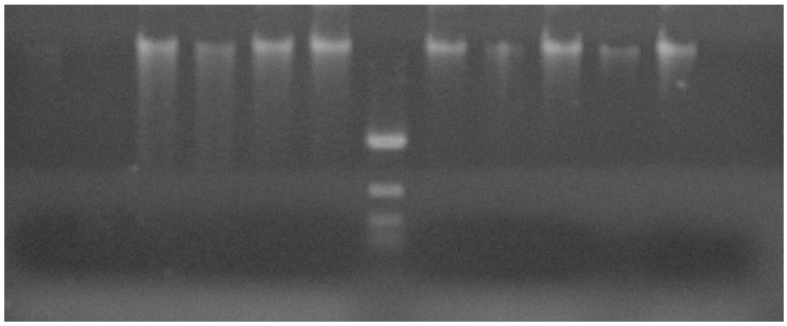


Fig. 4.3 Photographs of the gel electrophoresis of gDNA obtained from ticks preserved at – 80°C for 3-4 months using the KingFisher™ Cell and Tissue Kit with magnetic-beads.

Table 4.7 Comparison of gDNA extraction results from adult questing ticks (F= adult female; M= adult male) preserved at -80°C, with the two described methods. **p<0.05.

DNA extraction method	**Mean gDNA concentration (ng/µL ± S.E.)**	
	F	**M**
Spin-column, QIAgen Blood&Tissue	15.15 (±3.0)	4.9 (±1.7)
Magnetic-beads, KingFisher Cell and Tissue Kit	36.49 (±3.5)	12.02 (±1.2)
Mann-Whitney test	**	**

4.3 Protocol development for bloodmeal analysis in questing ticks

4.3.1 DNA sequencing: universal vertebrate *cytochrome b* primers

The method was applied to 33 questing nymphs with unknown bloodmeal source. As *Bos taurus* was amplified in the preliminary trials of this protocol, I excluded BSA from the reaction mix and *B. taurus* amplification no longer occurred. No potential host DNA was amplified in any sample. Human DNA was occasionally obtained. We attempted to design a new primer pair targeting a shorter fragment of *cytochrome b* that still allowed the amplification of a large number of vertebrate species. *Cytb* sequences available from GenBank and sequences we obtained during the optimization step from control samples, were aligned using Clustal X v. 2.0. A reference sequence of *Homo sapiens* was also added to avoid designing primers cross-reacting with contaminant human DNA. However, the high interspecific variability in this mtDNA region did not permit new primer design with the necessary features described above.

4.3.2 DNA sequencing: species-specific primers

Both primer pairs (*Apodemus* and *Capreolus*) proved to reliably amplify host DNA from control samples of target species and, therefore, the optimized protocols were applied to questing ticks (Table 4.8). Bloodmeal source was identified as *A. flavicollis* in 5 out of 115 ticks using the long amplicon primer set, and in 9 out of 109 using the short amplicon primer set. *C. capreolus* was amplified in 1 out of 97 questing ticks. There was no repeatability between amplifications obtained from the two *Apodemus* primer sets. In an attempt to improve repeatability of amplification results,

we used bloodmeal-positive questing tick DNA samples to test different settings in the thermal cycling and/or reaction mix. No improvements were achieved.

4.3.3 DNA sequencing: host group primers

As reported in Table 4.9, five group specific primer sets were designed; however, only use of degenerations allowed the primers to target all the selected species in *cytb* and *d-loop* mtDNA regions. Testing on control samples of target species showed that all primers, except Ruminants, resulted in reliable amplifications. Optimized protocols were tested on a large number (97 to 115) of questing ticks (mainly nymphs; Table 4.9). As primers were not always tested on the same tick, we could infer identification success rate only at primer set level and not globally for the 'host group' approach.

Table 4.8 Species-specific primer features; results of the testing on control samples (K+ampl) and testing on questing ticks.

	Primer name and sequence	Ta (°C)	length(bp)	Host species amplified	(n°) K+ ampl.	n° questing ticks	n° ticks with bloodmeal ID
Apodemus	*BM_cytb_Apodemus* (long amplicon) F_AATACACTATACATCAGACACA R_TACTGCGAATAGGAGAAT	53	214	*A. flavicollis; A. sylvaticus*	(4) good	115	5
	BM_cytb_Apodemus_S (short amplicon) F_AATACACTATACATCAGACACA R_s_GTCCTACGTGTAGAAATAAG	52	140	*A. flavicollis; A. sylvaticus*	(4) good	109	9
Capreolus	*BM_capreolus* (Garros et al. 2011) UNIV2_F_TGAGGACAAATATCATTYTGAGGRGC CAP_R_TTGTCCGCGTTTGATGGGATTCCTATC	56	240	*C. capreolus*	(3) good	97	1

Table 4.9 Host group primer features and target species; results of the testing on control samples (K+ampl) and testing on questing ticks.

Host group	Primer name and sequence	mtDNA target	Ta (°C)	length (bp)	Host species amplified	(n°) K+ ampl.	n° questing ticks	n° ticks with bloodmeal ID
Rodents	BM_cytb_Rodents F_GCTGTHATAGCMACWGCAT R_GTRGCTTTRTCWACTGAGAA	*cytb*	53	150	*A. flavicollis, A. sylvaticus, M. glareolus, S. vulgaris, M. avellanarius*	(2, -, 1, 1, 1) good	86	4
Soricomorpha	BM_d-loop_Inset F_GCRTATCAYCTCCAWTRGGTTAT R_GGGCGATTTTAGGTGAGAT	*d-loop*	55	173	*S. araneus, S. antinorii, C. russula, C. suaveolens, E. europaeus*	(-, 1, -, 1, 1) good	77	1
Ruminants	BM_d-loop_Rumin F_CCYCWTGCWTATAAGC R_GCAGGTSAWYAAGCTC	*d-loop*	53	144	*C. elaphus, R. rupicapra, C. capreolus*	(1, 1, 1) variable	70	1
Canidae	BM_cytb_Canidi F_CTGCCGAGACGTTAACTA R_CCAATRTTTCATGTTTCTATG	*cytb*		141	*C. l. domesticus, V. vulpes*	(2, 1) good	59	6
Passeriformes	BM_cytb_Passeriformi F_TACACAGCAGAYACBWCHCTAG R_GTTTCAGGTTTCTTTRTT	*cytb*	54	180	*T. merula, T. philomelus, E. rubecula, L. megarhyncos, P. major*	(1, 1, 1, 1, 1) good	83	0

4.3.4 Standardized semi-automated test for species-specific and host group primers

Complementary use of species-specific primers (for *Apodemus*, long and short amplicons) and host group primers (Rodents, Soricomorpha, Passeriformes) allowed bloodmeal source identification in 10 out of 94 analyzed nymphs (10.6%). Sequencing allowed species level host identification, as follows: three *C. lupus familiaris*, two *A. flavicollis*, and one each of *M. glareolus*, *M. musculus*, *C. suaveolens*, *B. taurus* and *L. europaeus*. However, we also report that: *C. l. familiaris* was amplified with the Rodents primers, *L. europaeus* with *Apodemus* (long amplicon) primers and *C. capreolus* with *Apodemus* (short amplicon). Additionally, only amplification of *L. europaeus* was obtained for each one of the three sample replicates. Considering the low specificity of these primers, we did not continue with this approach.

4.3.5 Real-time High Resolution Melting Analysis

4.3.5.1 Group-specific primers design and testing

Six group-specific primers, targeting the 20 chosen host species, were selected to allow identification of tick bloodmeal sources using HRMA (Table 4.10). For each of these primer pairs, conventional PCR resulted in amplification of the expected mtDNA target for all the control samples from both tissue and engorged ticks, as confirmed by BLASTn searches. None of the PCR negative controls or the human DNA samples were amplified. Primer dimers or short aspecific multiple amplicons were occasionally visible in the QIAxcel images (reported in Fig. 4.4 and Fig 4.5), as is typical of PCR reactions for which appropriate template is lacking. Since amplification of non-target species included in the testing of Muroidea, Soricidae, Passeriformes or Caprinae primers did not occur during conventional PCR (data not shown), only DNA control

samples from the target species were included in the testing of Real-time HRMA of each host group.

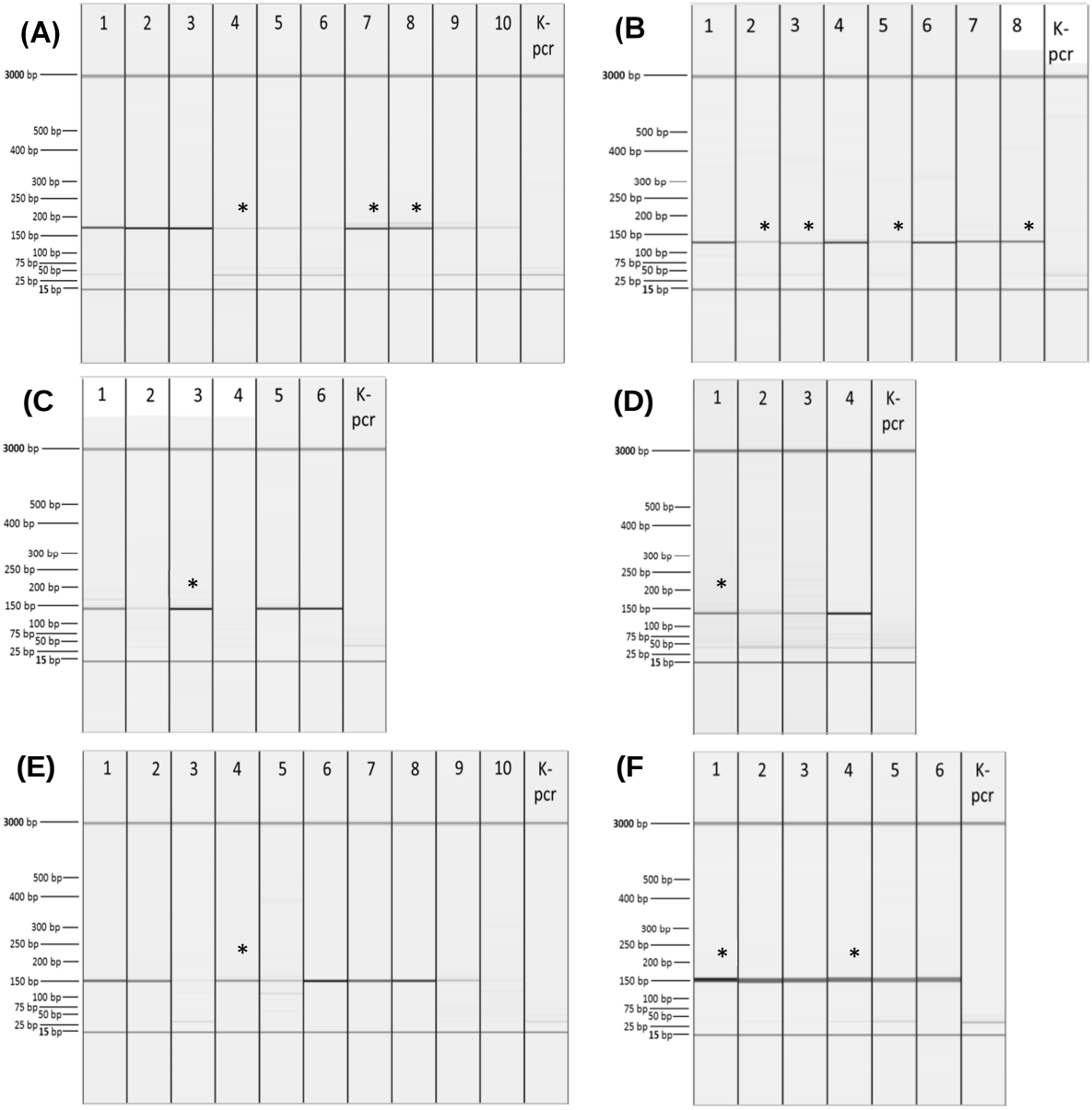


Fig. 4.4 QIAxcel capillary electrophoresis images of conventional PCR of a representative subset of DNA control samples obtained from host tissue or from engorged ticks directly collected from the host, and from human DNA, for each pair of HRMA group-specific primers. * sequenced PCR products. Size markers can be seen at 15 bp and 3000 bp. Bands appearing at about 40 bp are primer dimers. These bands disappeared when we increased the quantity of DNA template in the Real-time reaction. (A) Muroidea: *M. m. domesticus*: lane 1, tissue; *M. glareolus*: lanes 2-3, tissues; lanes 4-5, engorged *I. ricinus* larvae; *A. sylvaticus*: lane 6, 1:100 diluted tissue; lane 7, tissue; *A. flavicollis*: lane 8, tissue; lanes 9-10, engorged *I. ricinus* larvae; (B) Soricidae: *S. antinorii*: lane 1, tissue; lane 2, engorged *I. ricinus* larva; *S. minutus*: lane 3,

tissue; *C. leucodon*: lane 4, tissue; lane 5, engorged *I. ricinus* larva; *C. suaveolens*: lane 6, tissue; lanes 7-8, engorged *I. ricinus* larvae; (C) Passeriformes: *T. merula*: lane 1, tissue; lane 2, engorged *I. ricinus* nymphs; *T. philomelos*: lane 3, tissue; lane 4, engorged *I. ricinus* larva; *E. rubecula*: lane 5, tissue; lane 6, engorged *I. ricinus* larva; (D) Canidae: *C. l. familiaris*: lanes 1-3, engorged *I. ricinus* females; *V. vulpes*: lane 4, tissue; (E) Caprinae: *R. rupicapra*: lanes 1-2, tissues; lanes 3-5, engorged *I. ricinus* females; *C. hircus*: lanes 6-8, tissues; *O. aries*: lanes 9-10, engorged *I. ricinus* females; (F) Cervidae: *C. capreolus*: lanes 1-3, tissues; *C. elaphus*: lanes 4-6, tissues. K-pcr: PCR negative control.

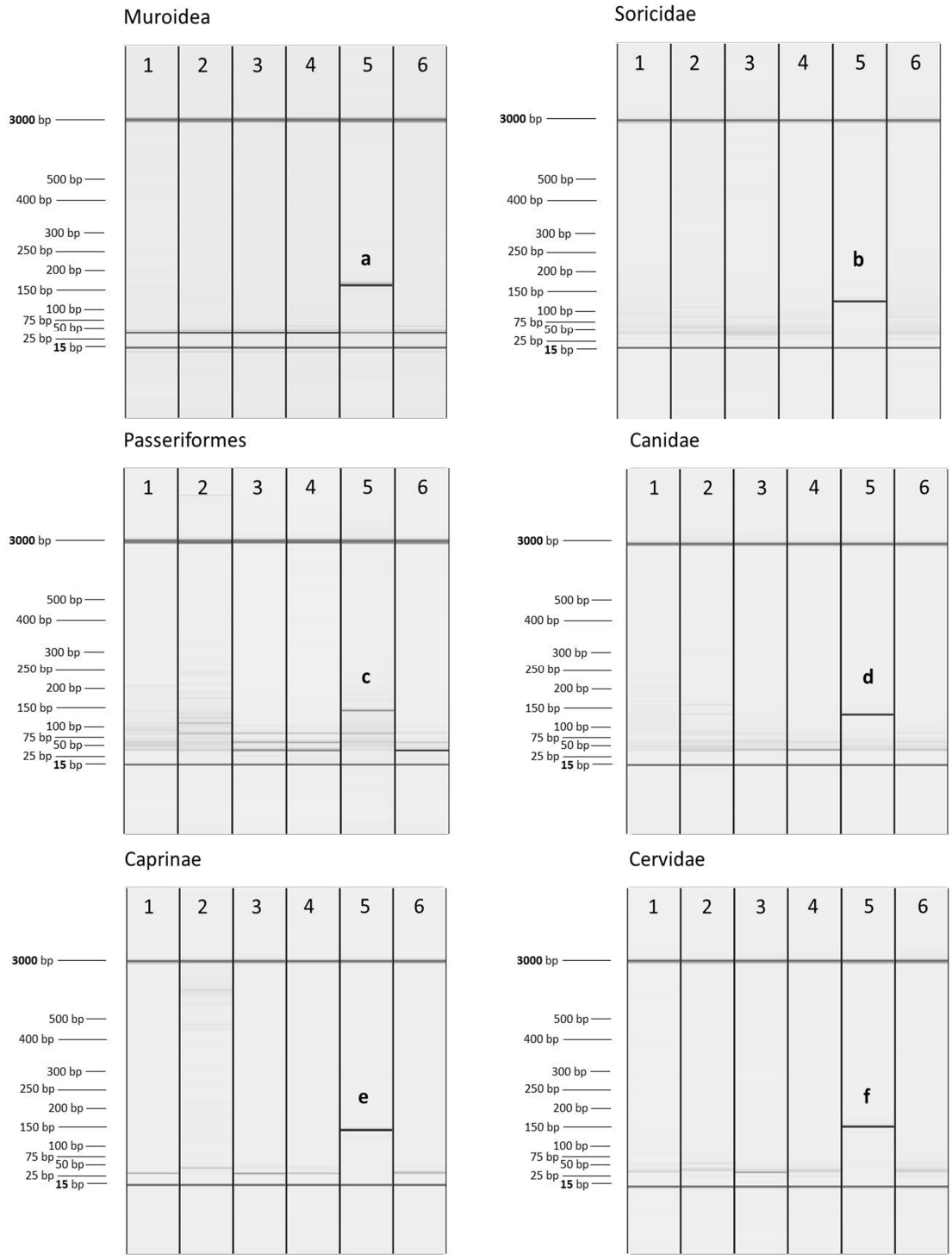


Fig. 4.5 (next page) QIAxcel capillary electrophoresis images of conventional PCR on human DNA for each HRMA group-specific primer set: lane 1, human DNA extracted from partially engorged *I. ricinus* nymph collected while feeding; lane 2, whole human blood; lane 3, human hair; lane 4, negative extraction control; lane 6, negative PCR control. Lane 5, target host species DNA control sample, as follows: a. *M. m. domesticus* (tissue); b. *C. suaveolens* (tissue); c. *T. merula* (engorged *I. ricinus* nymph); d. *V. vulpes* (tissue); e. *C. hircus* (tissue); f. *C. capreolus* (engorged *I. ricinus* female).

4.3.5.2 HRMA validation

Muroidea

Melting temperatures of *M. glareolus* (81.0-81.2 °C) and *M. m. domesticus* (80.7-80.9 °C) *d-loop* amplicons were different enough from each other and from the two *Apodemus sp*. to allow discrimination of these three genera; however, as shown by the normalized melting chart (Fig. 4.6A) and reported in Table 4.10, melting profiles of *A. sylvaticus* and *A. flavicollis* are fully overlapping having a T_m 81.3 °C and 81.4 °C, respectively, so discrimination of the two *Apodemus* species was not possible using these primers and HRMA. The amplified *d-loop* fragment produced a melting profile with a single peak for all four species tested (Fig. 4.6B).

Soricidae

Amplicons produced a melting profile with a single melting peak (Fig. 4.6D) with reliable results between replicates and, where tested, between samples of the same species. The melting temperature of *S. antinorii* (80.1-80.4 °C) and *S. minutus* (80.3-80.5 °C) amplicons were not sufficiently different from each other to be diagnostic for species identification (Fig. 4.6C and Tab. 4.10). However, the melting curves of *Sorex* species were well-separated from those of the *Crocidura* species tested. In addition, *C. suaveolens* (81.4-81.5 °C) can be distinguished from *C. leucodon* (82.1-82.3 °C).

Passeriformes

Melting profiles of the expected amplicons were consistent between replicates and samples of the same species. Furthermore, amplicons produced profiles with one melting peak with T_m diagnostic for the targeted species: *T. merula* 80.4-80.6 °C; *T. philomelos* 83.9-84.0 °C; *E. rubecula* 83.5-83.8 °C (Fig. 4.6E, F; Tab. 4.10). However, since only 0.2 °C separates the melting peaks of *T. philomelos* and *E. rubecula*, we

suggest amplicons be sequenced if unknown samples have melting peaks between the ranges of these two species.

Canidae

The melting profiles of these two species are easily discriminated by HRMA, both on the basis of T_m and by shape (Fig. 4.6G; Tab. 4.10). Amplicons from *C. l. familiaris* produced a profile with a single melting peak (80.3-81.3 °C), although some variability was recorded between samples. In fact, alignment of the sequences obtained from the two most differentiated *C. l. familiaris* HRM profiles (*a*, *b*) showed that sample *a* had one transversion (T->C) compared to sample *b* causing a + 0.5 °C shift in T_m. Instead amplicons of *V. vulpes* from the single DNA sample produced a profile with two melting peaks (81.3 °C and 83.5-83.6[e] °C) (Fig. 4.6H).

Caprinae

Melting profile analysis indicated that *C. hircus* and *R. rupicapra* can be discriminated thanks to their profile with two melting peaks (Fig. 4.6J): although the higher temperature peaks are completely overlapping (80.5 °C *C. hircus* and 80.3-80.6 °C *R. rupicapra*), the lower temperature peaks are separated by an average of 0.92 °C (i.e. T_m 76.5 °C for *C. hircus* and 75.3-75.7 °C for *R. rupicapra*). For *O. aries*, both melting temperature (74.5-75.0 °C and 81.7-82.0 °C) and melting profile shape easily permit identification (Fig. 4.6I and J).

Cervidae

The melting profile of the two species can be easily discriminated (Fig 4.6K and L; Tab. 4.10). Special feature of this *d-loop* amplicon is the presence of two melting regions, resulting in two peaks: 80.5-80.9 °C and 82.5-82.9 °C for *C. capreolus*, and 80.7-80.8

°C and 83.6-83.7 °C for *C. elaphus*. No intraspecific variability was observed between different samples of *C. elaphus*, while for *C. capreolus*, a clear shift of -0.3 °C in both melting peaks was observed for the sample from *C. capreolus* tissue compared to those obtained from engorged ticks (see Fig. 4.6K). To test if this shift could be attributed to variable DNA concentration, serial dilutions of the tissue DNA (starting concentration: 2.44 ng/µL) were subjected to HRMA. As shown in Fig. 4.7 (A, B), if the amplification curve rises early (C_T <25) above the threshold, there is a decrease in T_m (C_T 11: undiluted sample); conversely, the rising of amplification curves between 25 to 31 cycles (diluted samples) does not have a significant effect on melting temperature (Fig. 4.7). However, as Fig. 4.7C shows, even with this slight shift in melting temperature correlated with DNA content, all *C. capreolus* samples (diluted and undiluted) are easily differentiated from *C. elaphus*.

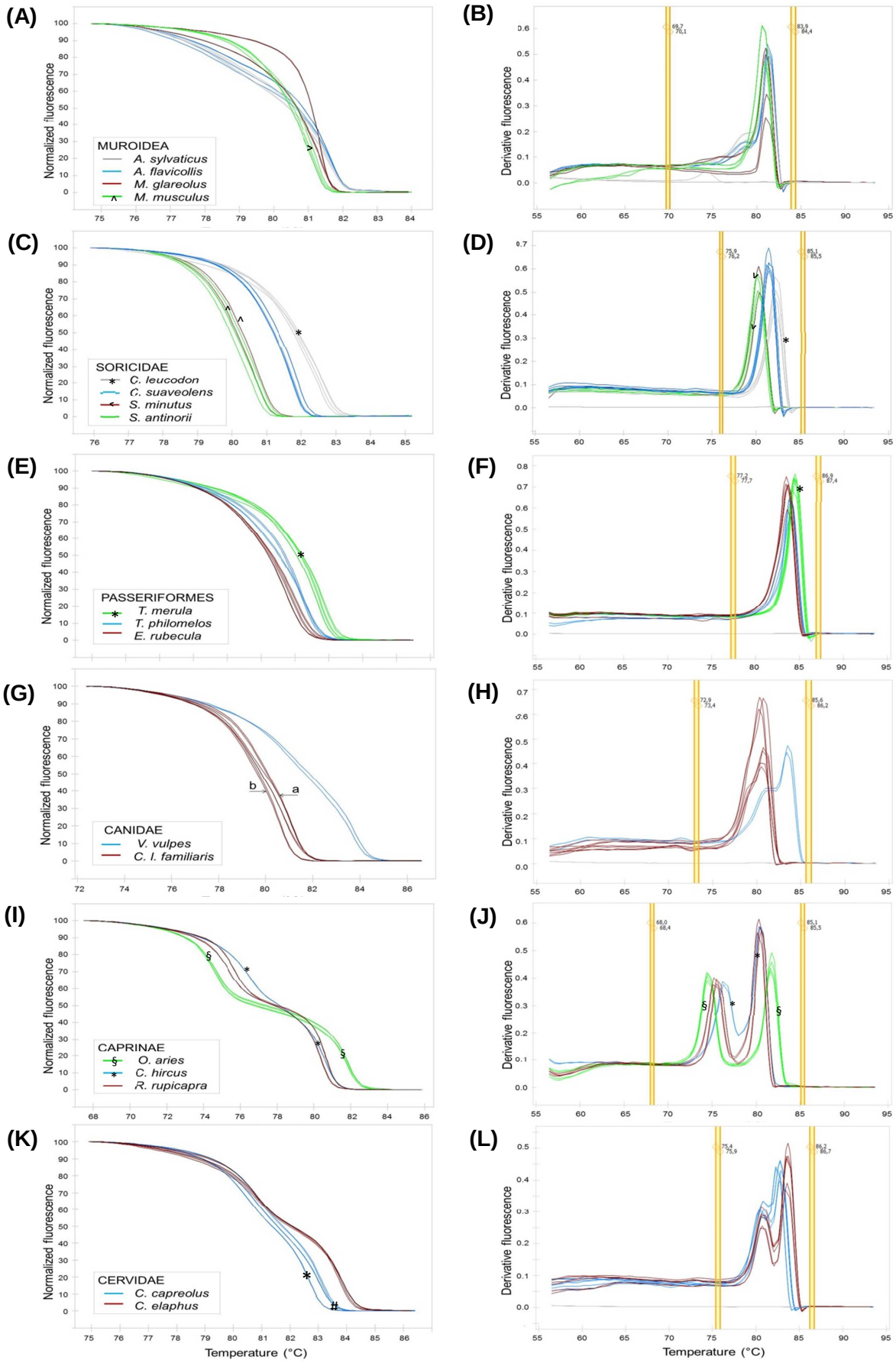
The results on control samples provide the proof-of-principle that the host group primers described here can reliably amplify host DNA and that selected amplicons permit to identify the targeted host species (or genera, in the case of *Sorex* and *Apodemus*) by HRMA.

Table 4.10 Target species within each host group, features of host group specific primers and amplicons and related HRMA parameters. All species within each host group can be distinguished from one another using the HRMA method described here unless otherwise indicated. T_a, annealing temperature; T_m sim., simulated melting temperature from uMELT^{TM} (Dwight et al., 2011); T_m1, melting temperature peak 1; T_m2, melting temperature peak 2 (if present); T_m obs, observed melting temperature expressed as *minimum-maximum* range; n.a., not available. Mean T_m obs was generally 2-6 °C lower than T_m sim. (Footnotes on the following page).

Targets and amplification parameters				**Amplicon features**				**HRMA**			
Host group	**Primer name mtDNA Target Primer (5' - 3')**	**T_a (°C)**	**Target species**	**Size (bp)**	**GC %**	**T_m sim (°C) T_m1**	**T_m2**	**Control samples[a]**	**T_m obs (°C) T_m1**	**T_m2**	**HRM[b] Fig. 4.6**
Muroidea	HRM_Rod *d-loop*	60	*A. flavicollis*	175	45	86.4	-	2E	81.4 [c]	-	A - B
	F_TCTGGTTCTTACTTCAGGGC		*A. sylvaticus*	175	44	86.1	-	2T	81.3 [c]	-	
	R_TTCATGCCTTGACGGCTATG		*M. glareolus*	176	44	85.9	-	2E	81.0-81.2	-	
			M. m. domesticus	175	43	85.5	-	2T	80.7-80.9	-	
Soricidae	HRM_Sor *d-loop*	62	*S. minutus*	137	44	84.2	-	1T	80.3-80.5[d-e]	-	C - D
	F_TCAGCCCATGCCGACACAT		*S. antinorii*	137	43	83.9	-	1T – 1E	80.1-80.4 [d]	-	
	R_GCCCCCATAGAGAATAAGCC		*S. araneus*	137	42	83.4	-	n.a.			
			C. leucodon	136	45	84.7	-	1T – 1E	82.1-82.3	-	
			C. suaveolens	137	47	86.0	-	1T – 2E	81.4-81.5	-	
			C. russula	136	46	85.0	-	n.a.			
Passeriformes	HRM_Pas *12S*	60	*T. merula*	155	52	90.2	-	1T – 3E	80.4-80.6	-	E - F
	F_ATCCACGATATTACCTGACCATT		*T. philomelos*	155	50	90.0	-	1T – 2E	83.9-84.0	-	
	R_TACCCCATTGCTTCCATTCC		*E. rubecula*	156	50	89.6	-	1T – 3E	83.5-83.8	-	
Canidae	HRM_Can *d-loop*	61	*C. l. familiaris*	147	44	82.7	85.9	4E	80.3-81.3[f]		G - H
	F_CCGCAACGGCACTAACTCTA		*V. vulpes*	146	49	84.5	88.6	1T	81.3	83.5-83.6[e]	
	R_CCATTGACTGAATAGCACCTTG										
Caprinae	HRM_Cap *12S*	57	*C. hircus*	158	37	80.1	86.3	1T	76.5	80.5	I - J
	F_TAAATCTCGTGCCAGCCA		*R. rupicapra*	158	37	79.6	86.6	2E	75.3-75.7	80.3-80.6	
	R_GTAGGGTTACTTTCGTCAT		*O. aries*	158	36	78.3	88.0	3E	74.5-75.0	81.7-82.0	
Cervidae	HRM_Cer *d-loop*	60	*C. capreolus*	168	45	84.5	88.6	1T – 2E	80.5-80.9	82.5-82.9	K - L
	F_CGATGGACTAATGACTAATCAG		*C. elaphus*	169	47	85.2	88.9	1T – 2E	80.7-80.8	83.6-83.7	
	R_TTATGGGGATGCTCAAGATG										

[a]Number of control DNA samples used in HRMA testing; T, extracted from host tissue; E, extracted from engorged ticks from different individuals.
[b]HRM normalized and derivative plots
[c]The melting temperature, as well as the melting profiles, for *A. sylvaticus* and *A. flavicollis d-loop* amplicons are fully overlapping, so discrimination of these two species is not possible using these primers.
[d]The melting temperature for *S. antinorii* and *S. minutus d-loop* amplicons are not sufficiently different from each other to be diagnostic for species identification.
[e]Reported range refers to the variation observed between the two replicates of the same single control sample.
[f]Wide range of T_m is related to mutations in the sequence of the used control samples (see Fig. 4.6G).

Fig. 4.6 (next page) HRMA of positive control samples of the species listed in the legends using group-specific primer sets. (A, B) Muroidea, (C, D) Soricidae, (E, F) Passeriformes, (G, H) Canidae, (I, J) Caprinae, (K, L) Cervidae; Figs. 1A, C, E, G, I and K are normalized melting plots, while Figs. 1B, D, F, H, J and L are derivative melting plots. Yellow bars delineate pre- and post- melting normalization regions. For Canidae (G, H), alignment of the sequences obtained from the two most differentiated *C. l. familiaris* HRMA profiles (*a*, *b*) showed that sample *a* had one transversion (T->C) compared to sample *b* causing a + 0.5 °C shift in T_m. In Fig.1K, L melting profiles from *C. capreolus* tissue (*) are notably different from melting profiles from engorged ticks (#), possibly related to a difference in DNA concentration.


(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
(I)
(J)
(K)
(L)
Normalized fluorescence
Derivative fluorescence
Temperature (°C)
MUROIDEA
A. sylvaticus
A. flavicollis
M. glareolus
M. musculus
SORICIDAE
C. leucodon
C. suaveolens
S. minutus
S. antinorii
PASSERIFORMES
T. merula
T. philomelos
E. rubecula
CANIDAE
V. vulpes
C. l. familiaris
CAPRINAE
O. aries
C. hircus
R. rupicapra
CERVIDAE
C. capreolus
C. elaphus

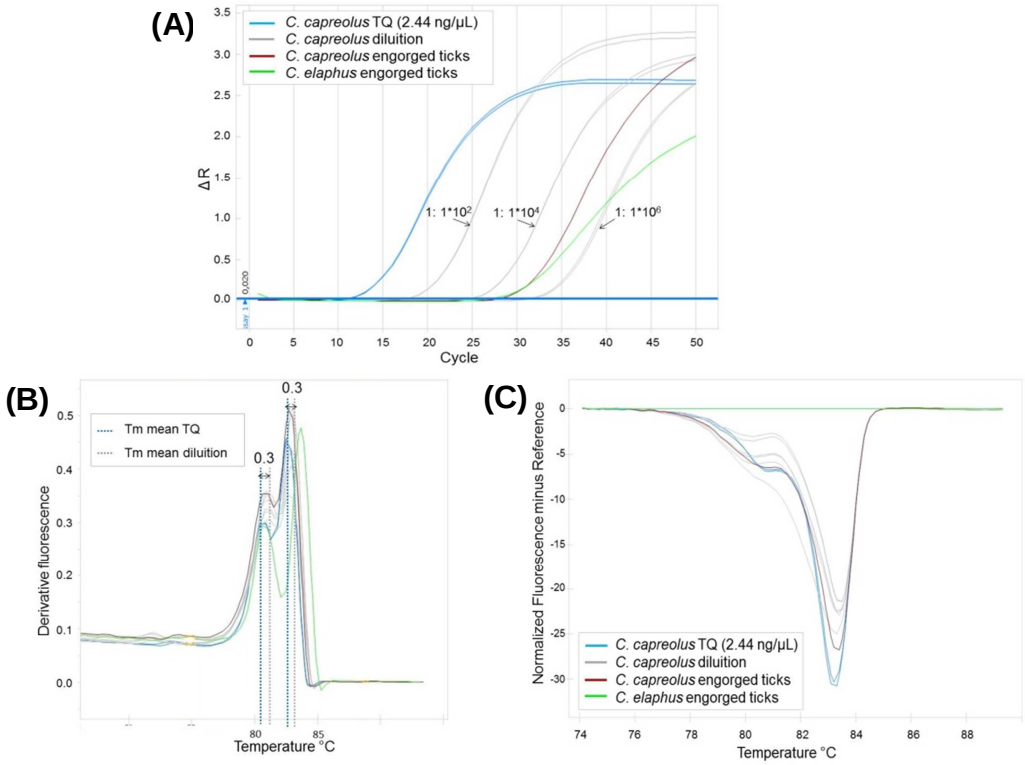


Fig. 4.7 Effect of DNA concentration on melting temperature of the targeted *d-loop* amplicon in Cervidae: (A) amplification graph of undiluted and diluted DNA from one tissue sample of *C. capreolus;* the start of amplification is directly correlated with the level of dilution; (B) derivative melting graph showing the shift in T_m (-0.3 °C) of both peaks in diluted compared to undiluted samples. Diluted samples, amplifying between 25 and 31 cycles all have comparable T_m; (C) difference graph showing the relative difference in melting profiles of all *C. capreolus* diluted and undiluted samples relative to the baseline (*C. elaphus* engorged tick). Despite the deviation in melting temperature of diluted *C. capreolus* samples, they can all be clearly differentiated from *C. elaphus*.

4.3.5.3 HRMA testing on questing ticks

Using our primers and the described HRMA protocol, bloodmeals were successfully identified in 34 out of 52 questing nymphs analyzed (i.e. sensitivity was 65.4 %; Table 4.11). However, as a result of mixed bloodmeals (see below) an additional eight amplicons were generated. Sequencing confirmed that HRMA allowed the correct identification of 35 out of 42 (83.3 %) bloodmeal sources to species level (including *Bos taurus*; see below), and five to genus level (*Sorex* sp. and *Apodemus* sp.; samples 14_CA and 13_PI: *S. antinorii*; samples 12_CO, 1_PI and 5_PI: *A. flavicollis*;

Appendix). Of the remaining two amplicons, sample 12_TR had an aspecific melting profile, within the target species range (see Appendix, Table A1) and was confirmed by sequencing as *Crocidura leucodon*. The last amplicon, 5_PI, was identified by HRMA as *M. glareolus*, whereas the BLASTn search of the sequenced amplicon suggested *A. flavicollis* (98% probability; see Appendix, Table A1). Alignment of sample 5_PI and the *A. flavicollis* control sample sequences revealed a 3 bp deletion (GTG) in sample 5_PI that caused a variation in melting temperature to match that of *M. glareolus.*

Identification of more than one host from the same tick (i.e. amplification by more than one host group primer set) occurred in 8 out of 34 (23.5 %) nymphs. *B. taurus* and *C. l. familiaris* were both found in 3 nymphs; for the other 5 nymphs, the pairs of hosts included: *Apodemus* sp. and *Crocidura suaveolens*; *Apodemus* sp. and *C. elaphus*; *C. leucodon* and *C. elaphus*; *C. leucodon* and *V. vulpes*; and *C. l. familiaris* and *C. elaphus.*

As reported in Table 4.11, amplicons from at least one questing tick tested positive for each of the targeted Canidae and Cervidae hosts; for Soricidae, *S. araneus* and *C. russula* were not found in any questing ticks, nor were *M. m. domesticus* and *M. glareolus* from Muroidea host group. No nymphs were positive for Caprinae or Passeriformes.

Table 4.11 Results of host identification with HRMA using field-collected questing nymphs.

Host DNA identification		**Site**[a]				
Host group	**Host genera/species**	**CO**	**TR**	**CA**	**PI**	**Total**
Muroidea	*Apodemus sp.*	1			2	3
	M. glareolus					0
	M. m. domesticus					0
Soricidae	*Sorex sp.*			1	1	2
	S. araneus					0
	C. leucodon		2	1	4	7
	C. suaveolens	1	1	1	2	5
	C. russula					0
Passeriformes	*T. merula*					0
	T. philomelos					0
	E. rubecula					0
Canidae	*C. l. familiaris*	6	3		1	10
	V. vulpes		2			2
'Caprinae'	*O. aries*					0
	R. rupicapra					0
	C. hircus					0
	B. taurus	3				3
Cervidae	*C. capreolus*	1		1		2
	C. elaphus	1	2	1	4	8
n ticks with identified bloodmeal/*n* ticks tested		9/13	8/12	5/14	12/13	34/52
% ticks with identified bloodmeal		69.2	66.7	35.7	92.3	65.4
n mixed bloodmeals		4	2	0	2	8
n different host species/genera identified		6	5	5	6	9

[a]CO, Condino; TR, Transacqua; CA, Cadine; PI, Pietramurata.

In most cases, identification of the bloodmeal was straightforward: i.e., the T_m and melting profile of questing tick (i.e. unknown) samples were clearly within the range of control samples (see example of Cervidae in Fig. 4.8).

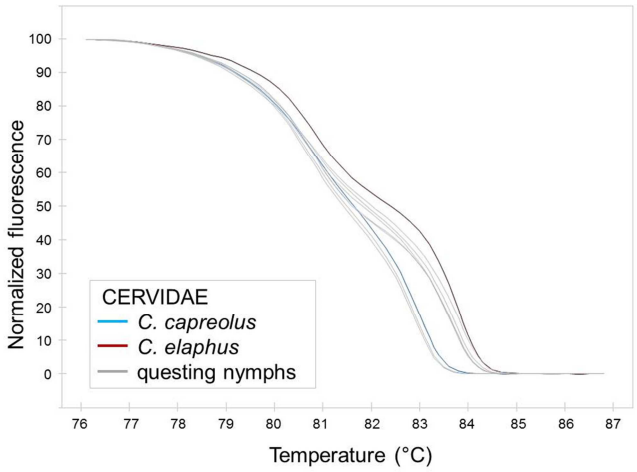


Fig. 4.8 Normalized melting plot of the Cervidae primer set showing the melting profile obtained from control samples and questing nymphs that provided amplification. Note that the melting curves of unknown samples are very similar to those of positive samples despite a slight deviation of T_m, ensuring correct species identification of unknown (i.e. questing tick) bloodmeals.

Very occasionally, amplicons gave T_m and/or melting curve profiles clearly divergent from those of control samples (see example in Fig. 4.9A). These amplicons were removed from the HRMA melting curve graphs to make examining the remaining curves easier (see example in Fig. 4.9B). The aspecific amplicons were then sequenced to verify their identity. A BLASTn search confirmed that these sequences were mainly derived from tick DNA or simply short primer dimer amplifications (see Appendix, Table A1). However, while testing the Caprinae primers on questing ticks, by sequencing three unusual amplicons, we confirmed that *Bos taurus* was also amplified (in samples 2_CO, 3_CO and 5_CO), and has an HRMA profile similar to but clearly distinguishable from those obtained for the tested Caprinae species, with double melting peaks and a T_m of 80.8-81.0 and 83.7-84.1 °C, respectively (Fig. 4.10).

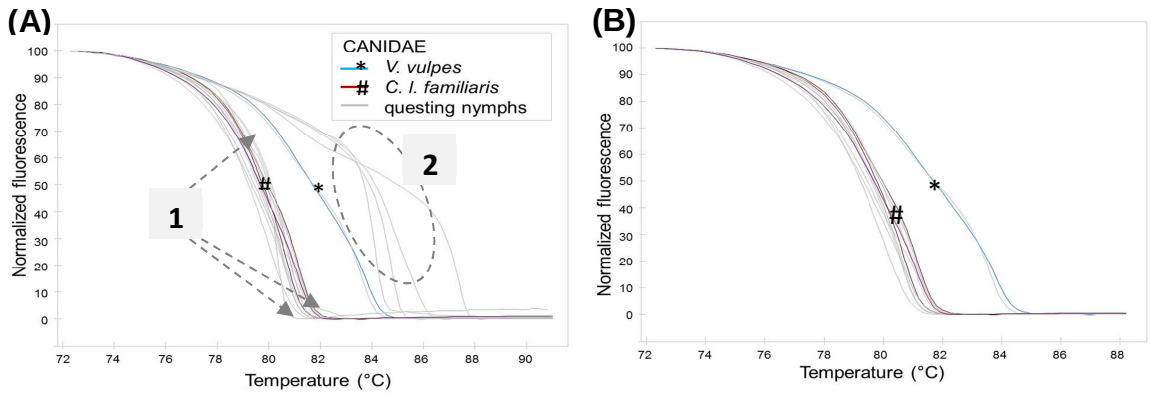


Fig. 4.9 Analysis of HRMA data by means of melting plots: an example using Canidae HRMA on questing nymphs. (A) Normalized melting graph of all amplified samples: note that the HRMA curves obtained for amplicons from questing ticks are very similar to (1) or clearly different from (2) control sample curves (in this case: *C. l. familiaris* and *V. vulpes*); (B) normalized melting graph reporting only those samples with melting profiles matching those of control samples.

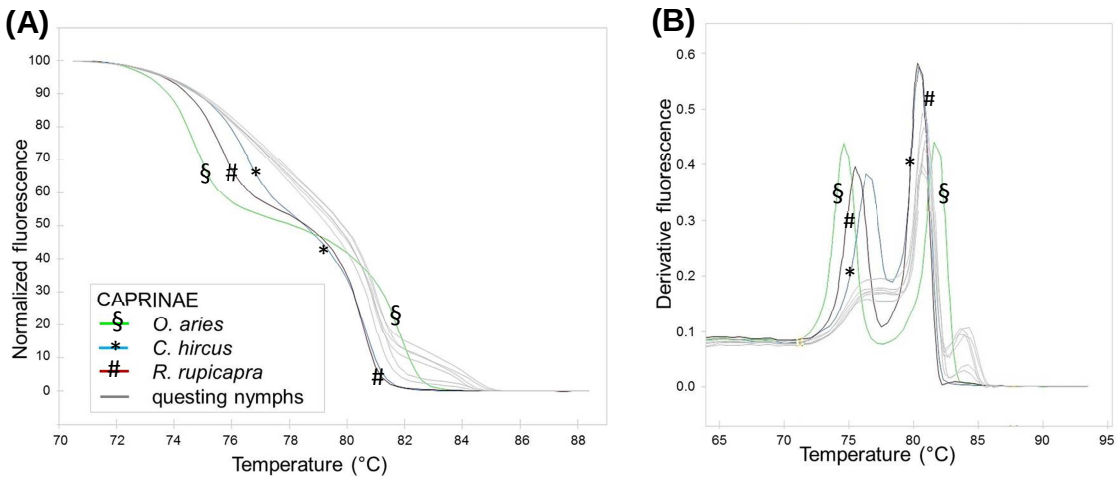


Fig. 4.10 HRMA plots for Caprinae. (A) Normalized; (B) derivative. Grey lines indicate unexpected HRMA profiles obtained from two replicates of samples 2_CO, 3_CO and 5_CO, later identified by sequencing as *Bos taurus*. Note that these curves are similar in shape to those of other hosts, and very different from the unusual curves in Fig. 4.9A..

4.3.6 Comprehensive Real-time HRMA bloodmeal analysis results for questing nymphs in Province of Trento

Results of the HRMA bloodmeal analysis of DNA extracted from questing both using magnetic-beads method (749) and manual spin-column method (99; of which 52 were also presented in the previous section, since they were part of the optimization procedure) are included so that about 30 nymphs for each of the 30 sampling sites were analysed (Table 4.12 for details).

Overall, larval bloodmeals were identified in 215 out of 848 nymphs (25.4% identification success; Table 4.13); DNA from multiple hosts (mixed bloodmeals) was recovered from 23 nymphs (10.7%). The linear model identified the DNA extraction method as the only factor that significantly affected the identification success ($p < 0.001$). Nymph DNA extraction (n=99) with QiaAmp® DNA Investigator resulted to be the most efficient, having a 55.1% identification success, compared to the 22.4% obtained from ticks extracted with KingFisher™ Cell and Tissue DNA kit (n=749) (Table 4.12). Identification success varied widely between sampling site (from 3.3% to 92.3%; Table 4.12), but no significant relationship between identification success and either sampling year, sampling month and or habitat type emerged from our data (Fig. 4.11). It should be noted that once DNA extracted with the applied to KingFisher™ Cell and Tissue DNA kit extracted nymphs, HRMA showed a constant consistent increase in the T_m of target species amplicons; sequencing gave proof of their identity and their melting temperatures were later used as reference. During this optimization step, a *Cricetus griseus* bloodmeal T_m (82.4 °C), amplified using HRM_Rod primers, was identified by sequencing and blasting (BLASTn identity score 99%); it possibly represent an alien individual deriving from escaped pets.

Table 4.12 DNA extraction methods, sampling time and number of analyzed nymphs for each site and relative identification success rate.

DNA extraction method	site	site code	sampling time	nymphs analyzed (n°)	identification success (%)
QiaAMP	Brentonico	BRE[a]	May-2012	15	46.7
	Transacqua	TRA	June-2012	12	66.7
	Grigno Valsugana	GRI[a]	April-2013	16	31.3
	Pietramurata	PIE	May-2012	13	92.3
	Cadine	CAD[a]	May-2012	14	35.7
	Giovo	GIO[a]	April-2013	16	43.8
	Condino	CON	April-2012	13	69.2
			Total	**99**	**55.1**
Thermo KingFisher	Caoria	CAO	April-2013	30	30.0
	Cavedine	CAV	April-2013	30	20.0
	Passo del Durone	PDU	May-2012	34	32.4
	Telve	TEL	June-2012	30	13.3
	Val Genova	VGE	May-2013	30	13.3
	Vervò	VER	May-2012	30	20.0
	Mezzocorona	MEZ	June-2012	30	16.7
	Molveno	MOL	May-2012	30	3.3
	Grigno Valsugana	GRI[a]	April-2013	16	43.8
	Laghi di Lamar	LAM	May-2012	31	9.7
	Tione di Trento	TIO	May-2012	30	10.0
	Volano	VOL	May-2012	30	50.0
	Lundo	LUN	May-2012	30	6.7
	Trento	TRE	May-2013	34	17.6
	Pinzolo	PIN	May-2012	30	20.0
	Cadine	CAD[a]	May-2012	8	25.0
	Tesino	TES	June-2012	30	20.0
	Pergine	PER	April-2012	30	20.0
	Revò	REV	May-2012	30	13.3
	Segonzano	SEG	May-2012	30	23.3
	Dimaro	DIM	May-2013	30	33.3
	Giovo	GIO[a]	April-2013	16	31.3
	Ala	ALA	May-2012	30	33.3
	Brentonico	BRE[a]	May-2012	17	29.4
	Levico	LEV	April-2012	30	26.7
	Cavalese	CVS	May-2012	23	21.7
	Ledro	LED	May-2012	30	20.0
			Total	**749**	**22.4**

[a]site having samples extracted either with QIAamp or Thermo King Fisher.

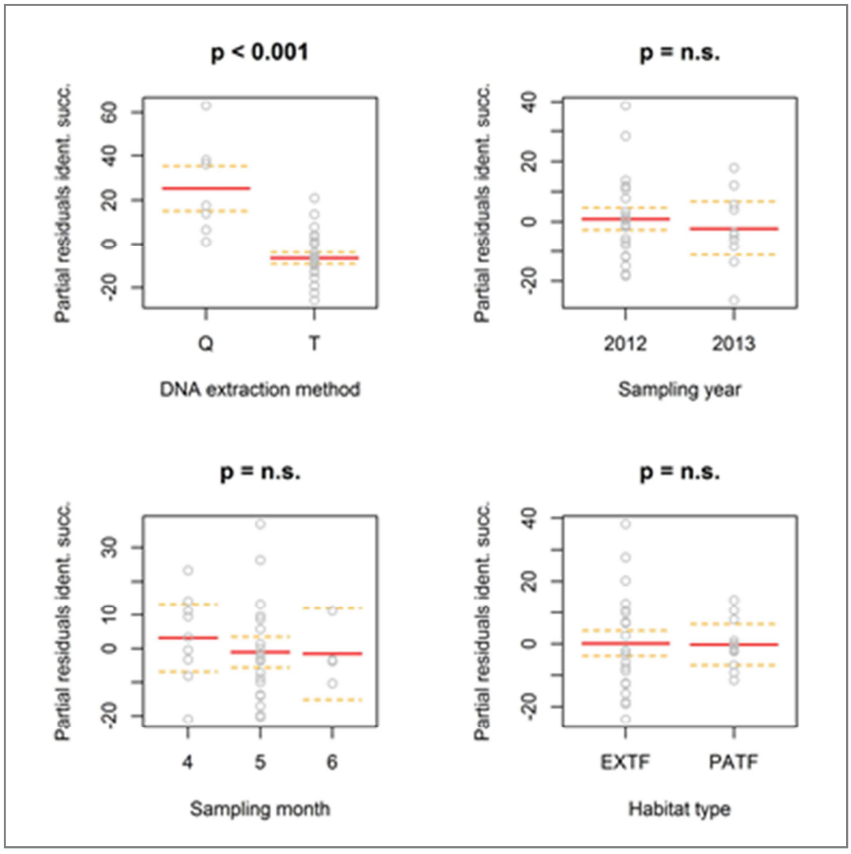


Fig. 4.11 Linear model, partial residuals for identification success in ticks (%) and explanatory variables: DNA extraction method (Q: QiaAMP® DNA Investigator; T: KingFisher™ Flex Magnetic Particle Processor), sampling year, sampling month (4: April; 5: May; 6: June) and habitat type (see text for detail). The only significant explanatory variable is DNA extraction method; Q is more efficient than T, as underlined by the residuals plot. n.s.= not significant.

Identification of bloodmeal source at species level was possible for 137 amplicons, while genus level was reached, as already described in the previous chapter, for *Apodemus* (N=67) and *Sorex* (N=9), and additionally for *Turdus* (N=19) and *Ovis* (N=7) (Table 4.13). In fact, first HRMA could not reliably discriminate between *T. philomelos* and *E. rubecula* control samples and, in addition, sequencing of amplicons having a slightly deviating T_m (84.10 °C, 84.14°C and 84.24 °C) from *T. philomelos/E. rubecula* (T_m of amplicons from questing nymphs 84.44-84.50°C) revealed amplifications of two *Turdus* spp. DNA, not recognizable at species level by BLASTn search, and also a *T. philomelos* (100% identity score by BLASTn); therefore amplicons

having a T_m in the range 84.00 to 84.50 °C were all classified as *Turdus* spp. / *E. rubecula* (Table 4.13). Moreover, the application of the protocol on a larger sample set showed that the Cervidae primers amplify a larger range of Cetartiodactyla hosts than predicted, because they also amplify some species targeted by Caprinae primers (HRM_Cap) (*Ovis* spp. and *R. rupicapra*), as well as tick hosts not previously considered, such as *Dama dama.* Because T_m of several species overlapped, identification by HRMA only became complex for Cervidae amplicons and therefore I had to resort to sequencing more often than expected in HRMA. Sequence analysis of *Ovis* amplicons obtained with HRM_Cer primers, both from an engorged tick collected while feeding on a mouflon and from questing nymphs, showed the inability of the chosen mitochondrial control region fragment to confidently discern domestic (*Ovis aries*) and wild (*Ovis aries musimon*) sheep. BLASTn reports 99-100% identity scores for *Ovis aries, O. a. musimon* and *O. orientalis* (Alignment and BLASTn results in Appendix 2); consequently, all sequences for which BLASTn reported various sheep species and subspecies at similar identity score, were classified here as *Ovis* spp. In five cases, HRMA led to misidentification of *Apodemus* spp bloodmeal as *M. glareolus*, because of intraspecific mutations (Appendix 3). The most common larval hosts were Rodentia (28.9%), mainly *Apodemus* spp (28.0%). The second most frequent host group was Carnivora (28.4%), with *C. l. familiaris* accounting for 21.3% and *V. vulpes* for 7.1%. Cetartiodactyla species fed 17.2% of larvae, *C. elaphus* and *C. capreolus* being the most common hosts (6.3% and 4.2%, respectively). 14.6% of the identified bloodmeals belongs to Passeriformes order and, lastly, 10.9% of bloodmeals were from Soricomorpha. Of the entire list of target hosts (Table 4.13) *C. russula*, *S. araneus* and *M. m. domesticus* did not appear to be larval bloodmeal sources in the study area.

Larval host identification success was similar in EXTF (130/506; 25.7%) and PATF (85/342; 24.9%) sites (Fig. 4.12; Table 4.13). The proportion of Soricomorpha bloodmeals was higher in EXTF than in PATF (15.2% and 4.3%; $p(\chi^2) < 0.05$), while the opposite was true for Passeriformes (PATF: 22.3% and EXTF: 9.6%; $p(\chi^2) < 0.05$) and Carnivora (PATF: 37.2% and EXTF: 22.8%, respectively; $p(\chi^2) = 0.06$). No significant differences were observed between the proportions of Rodentia (EXTF = 31.7%; PATF = 24.5%; $p(\chi^2) = 0.34$) or Cetartiodactyla (EXTF = 20.7%; PATF = 11.7%; $p(\chi^2) = 0.11$ acting as larval hosts in the two forest habitats (Fig. 4.12).

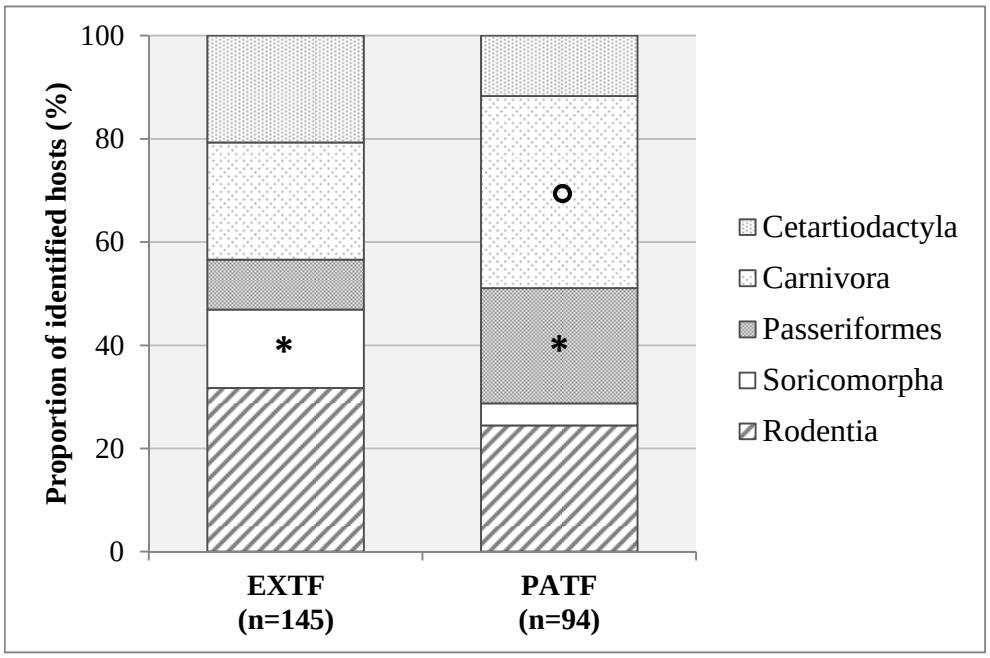


Fig. 4.12 Proportion of host identification in *I. ricinus* nymphs collected in the two forest habitat types: extensive forest (EXTF) and forest patches (PATF). * ($p<0.05$) and O ($p<0.10$) as indicated by the chi-square test. A significantly higher number of bloodmeals from Soricomorpha were identified in EXTF sites than in PATF, while Passeriformes were significantly more represented in PATF sites. Carnivores tend to be more abundant in PATF sites.

Table 4.13 Larval host identifications in questing nymphs collected in EXTF and PATF sites in Province of Trento

Target hosts groups (primer used)	**Tot EXTF[a] (%)**	**Tot PATF[a] (%)**	**TOTALS (%)**
Rodentia (HRM_Rod)	**46 (31.7)**	**23 (24.5)**	**69 (28.9)**
Apodemus spp.	44 (30.3)	23 (24.5)	67 (28.0)
M. glareolus	1 (0.69)	0	1 (0.4)
M. musculus	0	0	0
C. grigeus[b]	1 (0.69)	0	1 (0.4)
Soricomorpha (HRM_Sor)	**22 (15.2)**	**4 (4.3)**	**26 (10.9)**
Sorex spp.	9 (6.2)	0	9 (3.8)
S. araneus	0	0	0
C. leucodon	7 (4.8)	2 (2.1)	9 (3.8)
C. suaveolens	6 (4.1)	2 (2.1)	8 (3.3)
C. russula	0	0	0
Passeriformes (HRM_Pas)	**14 (9.6)**	**21 (22.3)**	**35 (14.6)**
T. merula	8 (5.5)	8 (8.5)	16 (6.7)
Turdus spp.[b] / *E. rubecula*[c]	6 (4.1)	13 (13.8)	19 (7.9)
Carnivora (HRM_Can)	**33 (22.7)**	**35 (37.2)**	**68 (28.4)**
C. l. familiaris	28 (19.3)	23 (24.5)	51 (21.3)
V. vulpes	5 (3.4)	12 (12.7)	17 (7.1)
Cetartiodactyla	**30 (20.7)**	**11 (11.7)**	**41 (17.2)**
Ovis spp[e, d]	5 (3.4)	2 (2.1)	7 (2.9)
R. rupicapra[d]	3 (2.1)	0	3 (1.3)
C. hircus[d]	2 (1.4)	0	2 (0.8)
B. taurus[d]	0	3 (3.2)	3 (1.3)
C. capreolus[e]	8 (5.5)	2 (2.1)	10 (4.2)
C. elaphus[e]	12 (8.3)	3 (3.2)	15 (6.3)
D. dama[b, e]	0	1 (1.1)	1 (0.4)
n° nymphs analyzed	506	342	848
n° nymphs with blood meal	130	85	215
n° total hosts	145	94	239
% identification success	25.7	24.9	25.4
n° mixed bloodmeal	14	9	23

[a] EXTF: extensive forest; PATF patchy forest (see text for details)

[b] host not originally considered as target for primer set, but identified after HRMA, by sequencing and BLASTing

[c] HRMA of Passeriformes amplification did not allowed reliable discrimination between *Turdus* spp. and *E. rubecula*

[d] host amplified with HRM_Cap (Caprinae primer set, HRM_Cap)

[e] host amplified with HRM_Cer (Cervidae primer set, HRM_Cer)

4.4 Population genetics

4.4.1 Microsatellites amplification

STR genotyping at the 10 selected loci proved to be unsatisfactory from the outset. As reported in Table 4.14, issues both molecular and technical in nature, such as mutations in repeat sequences other than repeats, lack of amplifications, and stuttering were observed.

PCR artifacts, like stuttering and incomplete terminal adenylation (+/-A'), increased the complexity of STR scoring, but could be easily solved by an accurate electropherogram check. More concerning was the presence of 'extra alleles' (Fig. 4.13), the lack of amplification and the high number of homozygotes, the presence of alleles with 'not-repeat' length (i.e. in dinucleotide, presence of both even and odd alleles; Fig. 4.14), and alleles outside of the expected range (Fig. 4.15). Sequencing of some of the most difficult STRs (IRN-4; IRN-12; IRN-17; IRN-37; IR-32 AND IR 39; Table 4.14) and their alignment against GenBank deposited reference sequences, provided evidence that mutations (in/del), other than expected variation in the number of repeats, were occurring, both in the region flanking the repeats and in the repeat itself. Null allele and unexpected allele lengths could be consequences of these mutations. 'Extra alleles' could be a consequence of locus duplication in the *I. ricinus* genome.

Testing of the 4 STR loci, developed for *I. scapularis* (Fagerberg et al., 2001), on *I. ricinus* ticks, did not give any positive PCR amplification.

For all of the above reasons, STR genotyping of *I. ricinus* was abandoned as unreliable, also because current statistical methods cannot deal with fragments of mixed mutation types and rates of mutation.

Table 4.14 Microsatellite (STR) loci amplification testing results and issues observed. (STR seq= locus sequencing; yes= done and sequence readable; not readable=presence of multiple peaks; no=not performed).

STR locus	n° samples	STR seq	Results (n° samples/n° samples tested)
IRN-4	17	yes	# some amplifications failed # high number of homozygotes (11/17) # mutation in the STR flanking region and in the STR region
IRN-7	41	not readable	# odd and even alleles # anomalous electrophoretic profile
IRN-8	25	no	# no amplification (5/25) # high number of homozygote (15/25) # stutters
IRN-12	38	yes	# odd and even alleles in heterozygotes # mutation in the STR flanking region # in/del in the STR repeat region
IRN-17	31	yes	# mutation in the STR flanking region # in/del in the STR repeat region
IRN-28	31	no readable	# stutters # more than 2 alleles/individual (contamination or aspecific DNA amplification)
IRN-30	38	no	# more than 2 alleles/individual (contamination or aspecific DNA amplification)
IRN-37	27	yes	# stutters # mutation in the STR flanking region # in/del in the STR repeat region
IR-32	37	yes	# mutation in the STR flanking region and in the STR region # no amplification (2/26) # high number of homozygote (11/26)
IR-39	19	yes	# no amplification (3/19) # high number of homozygote (11/19) # mutation in the STR flanking region and in the STR region

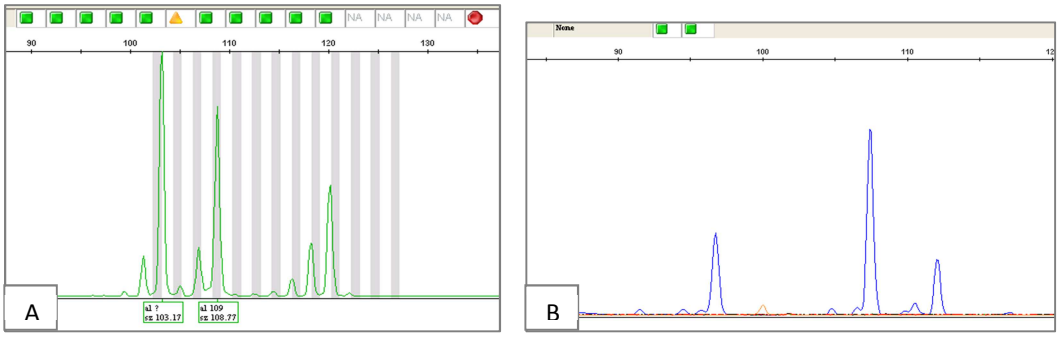


Fig. 4.13 GeneMapper screenshot of the perfect IRN-28 (A) and the compound IRN 30 (B) dinucleotide loci genotypes for two different ticks. Both presented an ‘extra allele’.

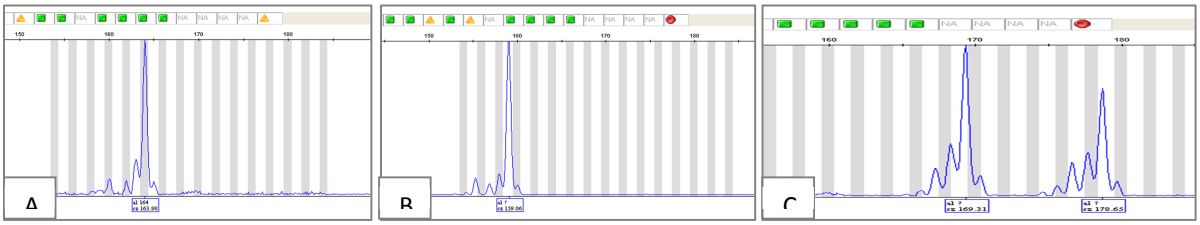


Fig. 4.14 GeneMapper screenshot of IRN-12 dinucleotide locus genotypes of three different individuals; A. homozygote for an odd allele (164); B. homozygote for an even allele (159), and C. heterozygote individual having an even (169) and an odd (178) allele.

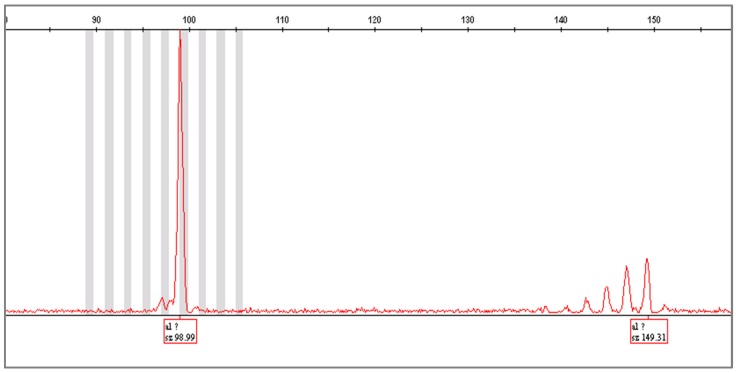


Fig. 4.15 GeneMapper screenshot of the perfect IRN-7 dinucleotide locus presenting one allele far outside the expected locus range (85-101 bp). Additionally, the first allele (the shorter) did not present stuttering, while the second did.

4.4.2 RAD-Sequencing

Illumina sequencing of the library created during protocol optimization gave 191,312,456 for both Reads 1 and Reads 2 sequences (46 Mb of data); the Quality Score, expressed in Phred 33 coding, was on average high for all sequence length (Fig. 4.16).

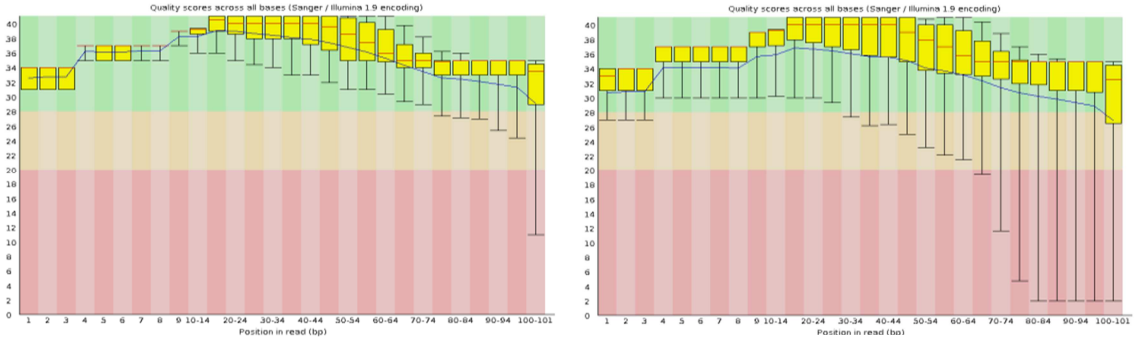


Fig. 4.16 Graphic representation of Quality Score (Phred 33) for bp position for the 2012 RAD-Seq library; Read1 left, Reads2 right.

After quality filtering, 76.3% of the total sequences (Reads1 and Reads2) were retained, 16.4% were discarded because of low quality; 15.7% of the raw Reads 1 were discarded because of ambiguous nucleotides in the RAD-Tag site and 7.7% because of ambiguous nucleotides in the barcode. Retained read numbers varied considerably between the methods used for gDNA extraction: phenol protocol gave a mean of only 586431 reads, while Qiagen spin-column and Thermo Scientific magnetic-beads methods gave, on average, 8115099 and 9004833 reads respectively, with Qiagen having an higher variation between samples (±S.D. 6636828 reads; Table 4.15). As a consequence, the *de novo* loci identification gave nearly zero results for phenol extracted samples, while Qiagen and Thermo both gave good results (Table 4.15). Therefore, the phenol protocol was subsequently abandoned, also given that

ThermoScientific magnetic-beads purification provides high quality DNA even from frozen ticks stored for more than a few weeks (see DNA extraction results).

Table 4.15 Quality filtering and *de novo* processing results for the first sequenced library (2012).

DNA extraction method	Retained reads		RAD-loci	
	mean	±S.D.	mean	±S.D.
Phenol protocol	586431	150498	70	63
Spin-columns, Qiagen	8115099	6636828	30971	11746
Magnetic-beads, ThermoScientific	9004833	3785041	40518	10698

Overall, Illumina sequencing of the 11 paired-end libraries yielded about 2 376 million 100 bp reads for 246 samples (Table 4.16 and Table 4.17). Here I report the genotyping results obtained from the different approaches and the relative results obtained from population structure analysis.

Table 4.16 Raw RAD-Seq paired-end sequencing results.

Sequencing lane	Raw paired-end sequencing results
Library 2012	382624912
Lane1-2014	458303582
Lane2-2014	430250288
Lane3-2014	341141432
Lane4-2014	411335750
Lane5-2014	352676018

Table 4.17 Final sample numbers and sex of RAD-Seq adults *I. ricinus* for each site are reported.

SAMPLES SUMMARY FOR RAD-SEQ			
	Female	Male	TOT
Ala	5	0	5
Brentonico	8	1	9
Cadine	7	1	8
Caoria	7	2	9
Cavedine	8	2	10
Cavalese	7	1	8
Condino	9	2	11
Dimaro	4	3	7
Finland	2	0	2
Giovo	7	2	9
Grigno	7	1	8
Lamar	8	2	9
Ledro	5	2	7
Levico	7	1	9
Lundo	4	3	7
Mezzocorona	7	1	8
Molveno	7	0	7
Passo Durone	8	2	10
Pergine	4	4	8
Pietramurata	7	1	8
Pinzolo	7	2	9
Revò	5	2	7
Segonzano	4	1	5
Telve	7	1	8
Tesino	7	1	8
Tione	7	1	8
Transacqua	7	3	10
Trento	7	1	8
Val Genova	3	2	5
Vervò	7	2	9
Volano	8	2	10
TOT	**197**	**49**	**246**

4.4.2.1 De novo *SNP calling without PCR duplicate removal*

Preliminary trimming of raw data to 80 bp and successive quality filtering with minimum quality score of 10 retained between 57.36 and 76.26% of sequences per lane (Table 4.18).

Preceeding analysis of the whole dataset, an investigative analysis regarding sequencing results was conducted. Examining the number of reads retained for unique barcode, each one been used 2 to 6 times in different lanes, showed for barcodes

ACACG (used 6 times) and AGGAC (used 5 times) a clear deficiency in providing sequencing results, having respectively a median number of retained reads of 1522477 and 878894 in comparison to a median of 6186485 (Fig. 4.17).

No bias related to sampling site could be identified, as in each site different numbers of retained reads were retrieved for each sample. PER is the site with the lowest mean number of retained reads (3128341), CON the one with the highest (9008863) (Fig. 4.18).

On the other hand, females ticks (N=197) gave a 30% higher median number of retained reads (6916706) than males (N=59; 4869346), but the variability inside the two samples is high (Fig. 4.19).

Table 4.18 Quality filtering of raw data results according to sequencing lane. * Nine individuals for which gDNA was extracted using a phenol protocol did not give any RAD-Seq results.

	LANE 2012	**LANE 1**	**LANE 2**	**LANE 3**	**LANE 4**	**LANE 5**
Number of individuals	42*	42	42	43	44	43
Ambiguous Barcodes	31407784	37240680	40775142	50475630	40 616592	50232018
% R1	16.42	16.25	18.95	29.59	19.75	28.49
Ambiguous RAD-Tag	30011675	39932747	55385298	67858032	49823692	60323208
% R1	15.69	17.43	25.75	39.78	24.23	34.21
Low Quality	29403378	46445918	45638096	27282401	33901437	26212172
%	7.68	10.13	10.61	8.00	8.24	7.43
Retained Reads (R1+R2)	291802075	334684237	288451752	195525369	286994029	215908620
%	76.26	73.03	67.04	57.32	69.77	61.22

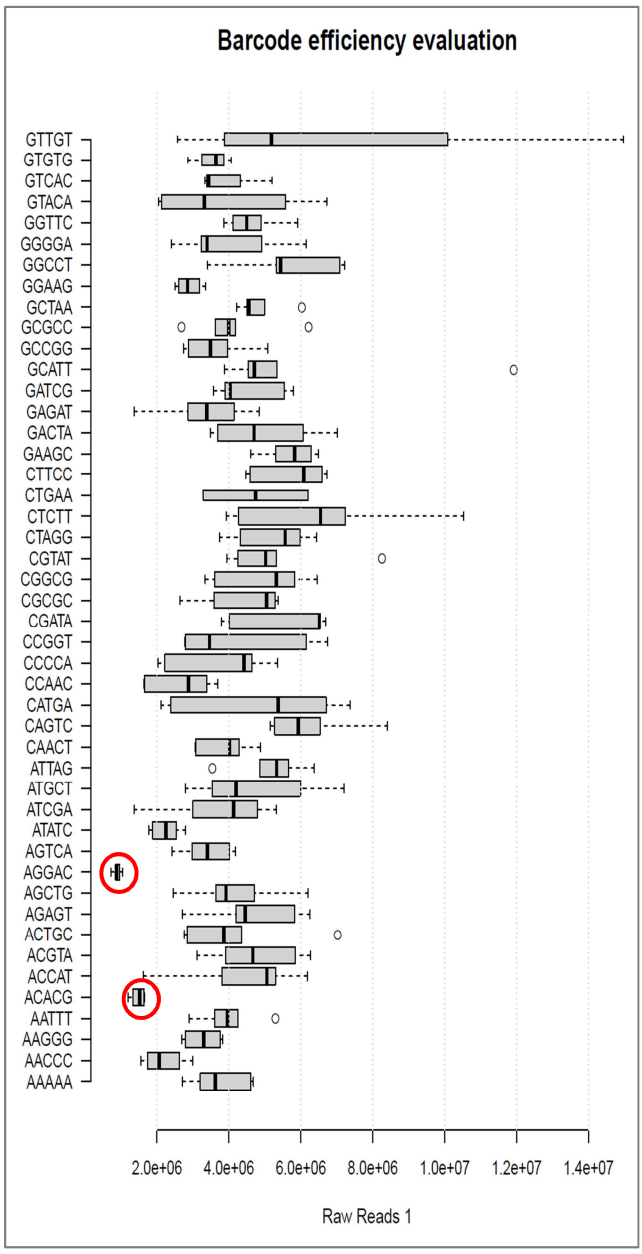


Fig. 4.17 Boxplot presenting median values of retained reads for each unique barcode. Note that barcodes AGGAC and ACACG having an extremely low median number of reads (red circles).

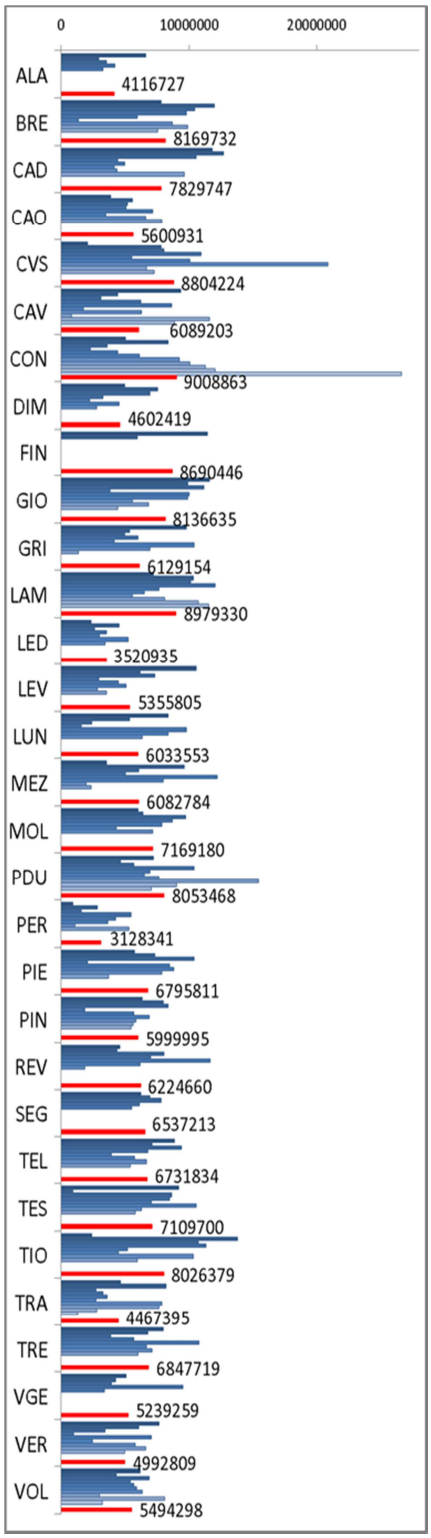


Fig. 4.18 Number of retained reads for each sample according to sampling site (blue bars) and mean values of retained reads per site (numbers and red bars).

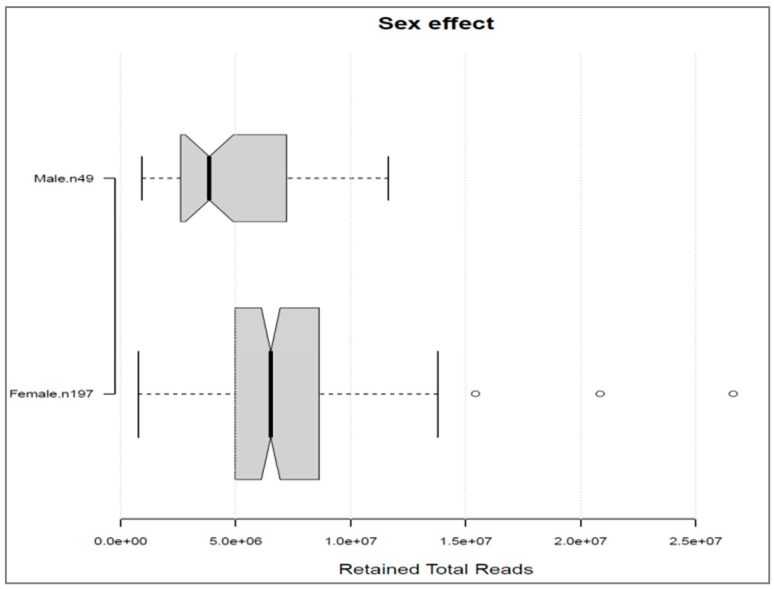


Fig. 4.19 Boxplot presenting median values of retained reads for males and females ticks.

Results of *de novo* loci identification and SNP calling were not those expected: about 45 000 loci (unique stack) were predicted using *radcounter v4* according to the estimate of genome size and GC content of *I. scapularis*, but the number of unique stacks (or loci) actually identified ranged from 357 to 56253 (mean 14265), and only 35 individuals had more than 30000 stacks. Mean merged coverage of identified loci ranged from 55 to 810 reads (mean 300). Analysis for the presence of PCR duplicates using *clone_filter* on raw sequences reported a mean number of clone reads of 75% (Lane2012 76.47%; Lane 1 –Lib1-12 72.19%; Lane 3 –Lib4-5 71.76%). The same analysis performed on two individual samples of Library 2012 and Lane 1, after demultiplexing and quality filtering, showed a clone reads for individuals ranged between 89.90 - 94.00% and 89.06 - 91.94%, respectively. The reason for which few loci for individual are identified and overrepresented, in terms of mean merged coverage, can most likely be attributed to an adapter concentration lower than the one declared by the supplier, leading to an inefficient ligation of the DNA fragment obtained

through the *SbfI* enzymatic digestion. However, the results could be also related to imprecise gDNA quantification and quality assessment or other errors in wet lab procedures or the specific feature of the investigated genome. The technical 'errors' meant that few adapter-ligated DNA fragments were then duplicated through PCR multiple times, increasing the % of PCR duplicates over the limit usually expected for RAD-Seq experiments. As a consequences of the few sequenced loci, final SNPs dataset is characterized by a high level of missing data and, consequently, statistical method had to be applied carefully to obtain robust results. By selecting only individuals with more than 30 000 identified loci and non-deleveraged loci (non-repetitive loci, according to algorithm identification process), with one or maximum two SNPs, removing loci for which at least one SNP position showed more than two alleles per individual, as they may represent paralogs, called in at least 50% of the selected individuals, a 'high coverage' dataset was produced and a *smart_pca* analysis was performed with default parameters and the *lsq project* algorithm enabled. Grouping individuals by site (Fig. 4.20) or geographical area (Fig. 4.21) did not suggest any particular genetic pattern between individuals.

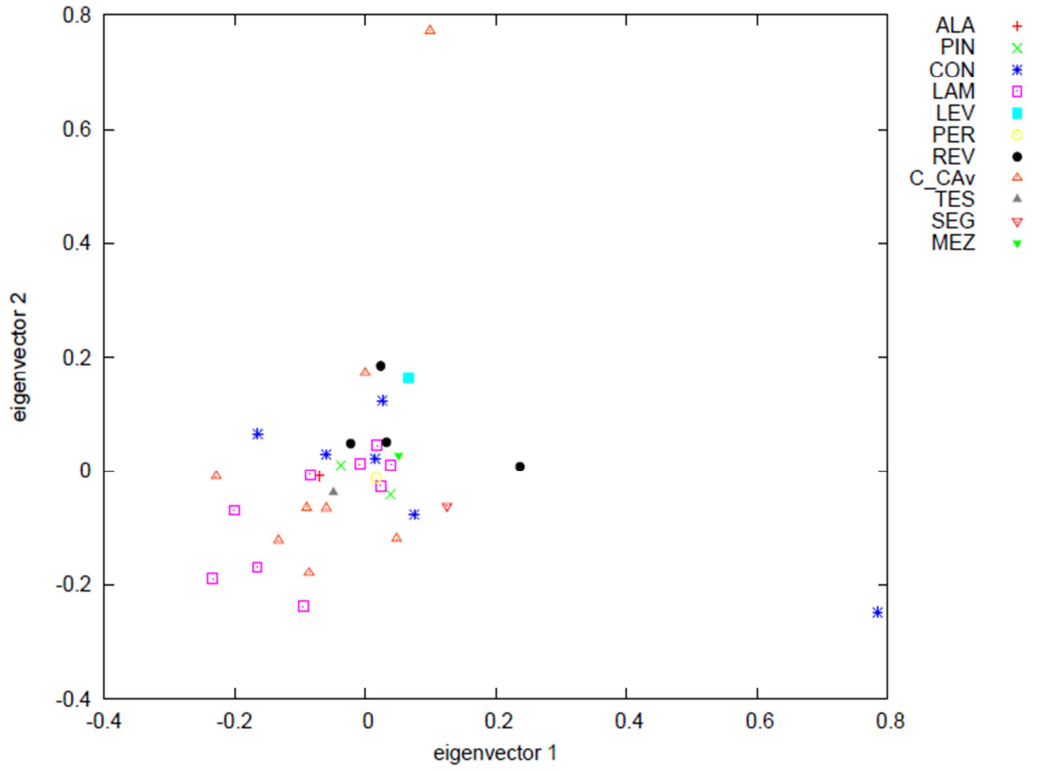


Fig. 4.20 *Smart_pca* plot of the 35 individuals presenting more than 30000 RAD loci; 1219 SNP were used in this analysis, being the ones with less than 50% missing rate; individuals are represented by means of the sampling site (C_CAv=CVS). Individuals coming from different sites are completely admixed.

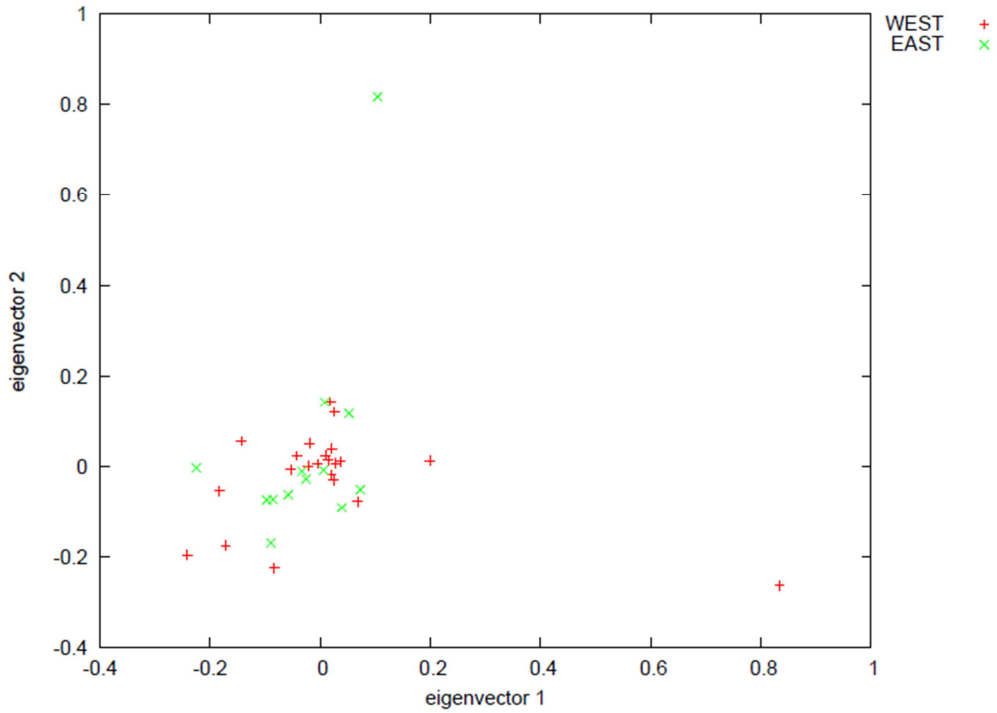


Fig. 4.21(previous page) *Smart_pca* plot of the 35 individuals presenting more than 30000 RAD loci; 1219 SNP were used in this analysis, being the ones with less than 50% missing rate; PAT I. ricinus individuals are classified by area (east or west of the Adige Valley). Some individuals lie out from the main central cluster; however no patterns of clustering could be identified.

The same *smart_pca* analysis was carried on a less conservative dataset. In this case, non-deleveraged loci with one to five SNPs and genotyped in at least 50 individuals were selected. Biallelic SNP, with a MAF (Minor Allele Frequency) higher than 1.5% (this is because a lower MAF could be a signature of SNPs derived by sequencing error) and missing call rate smaller than 80%, individuals with a missing call rate smaller than 99%, were retained producing a dataset of 228 individuals and 7967 SNPs (filtering performed with *vcftools*). Given specific algorithms applied by the *smart_pca* and the *lsqproject* algorithm, only 117 individuals and 6095 SNPs were analyzed. Again, no genetic structure can be identified among samples, as the PCA plot in Figure 4.22 shows.

Finally, a total of 214 109 biallelic SNPs were derived from the raw SNP dataset, selecting non deleveraged loci, having 1 to 5 SNPS each and retaining all individuals. This large SNP dataset was used to compute a MultiDimensional Scaling (MDS) analysis. All SNP loci and individual were retained without considering the missing rate. The resulting plot in Figure 4.23, showing individuals in a space of genetic distances, grouped by color according to sampling area and the two Finnish ticks in blue, displays a homogenous clouds of points, without any indication of spatial clustering.

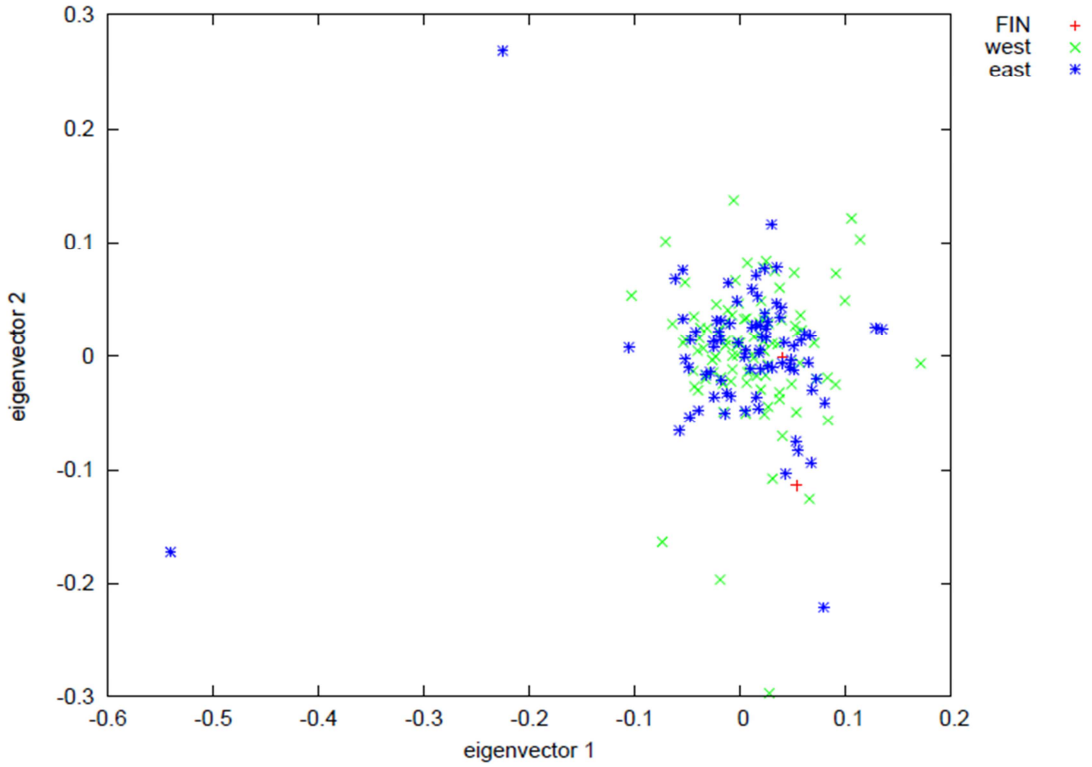


Fig. 4.22 *Smart_pca* plot obtained from the dataset of 7967 SNP (only SNP loci with <80% missing rate were retained) for 117 individuals; PAT individuals are classified by area (east or west of the Adige Valley). FIN: Finnish samples. Some individuals lie out from the main central cluster; however no patterns of clustering could be identified.

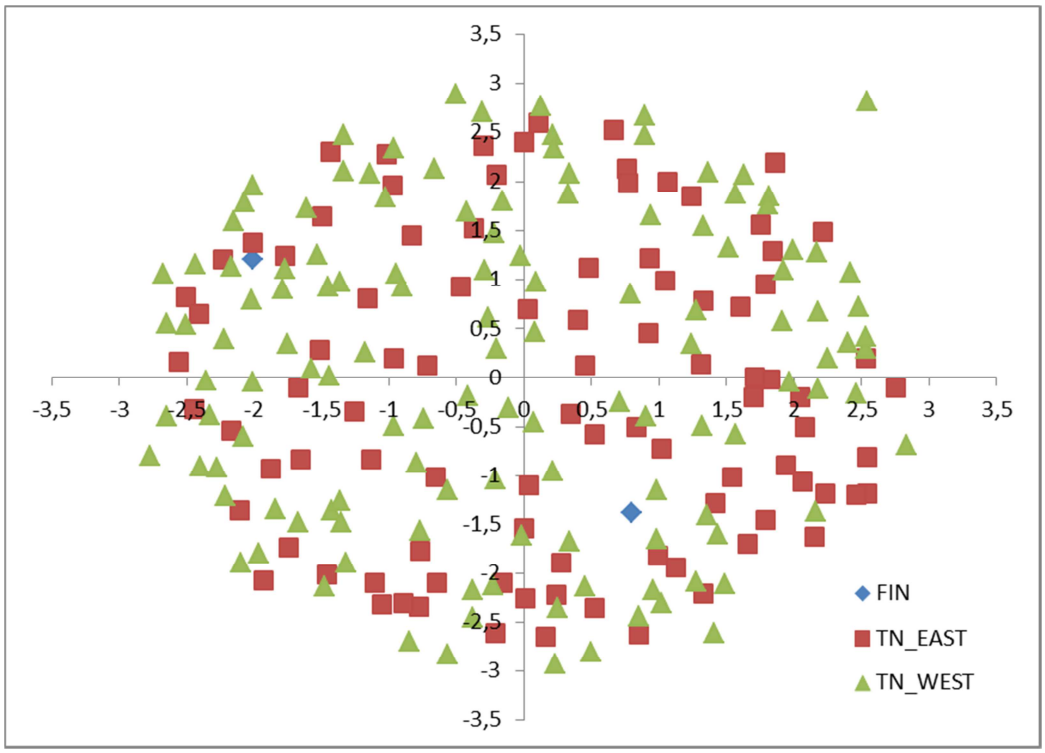


Fig. 4.23 Spatial distribution of 244 *I. ricinus* adults from PAT populations and two from Finland (FIN), based on MDS of genetic distances calculated from 214 109 biallelic SNPs. No genetic clustering emerges based on sampling area.

4.4.2.2 De novo *SNP calling with Stacks after PCR duplicate removal*

Trimming raw sequences to 90 bp resulted in a slightly lower sequence retention during the cleaning process, because of the generally lower Phred quality score in the final part of the sequences (Tab. 4.19).

After demultiplexing and quality filtering, sequences were cleaned from PCR duplicates, and consequently R1 sequences were reduced to a mean of 239153 per individual, (minimum 6168 to maximum 938819). According to that, minimum stack depth in *denovo_map.pl* was reduced to 2 (-m 2, see Materials and Method 3.3.6.3 *B.*).

Table 4.19 Quality filtering of raw data results according to sequencing lane.

	Lib2012	**Lib1-12**	**lib2-3**	**lib4-5**	**lib7-8**	**lib10-11**
Total Sequences	382624912	458303582	430250288	341141432	411335750	352676018
Ambiguous Barcodes	31407784	37240680	40775142	50475630	40616592	50232018
Low Quality	32073365	51204198	49496692	29098397	37013382	28177307
Ambiguous RAD-Tag	30011675	39932747	55385298	67858032	49823692	60323208
Retained Reads	289132088	329925957	284593156	193709373	283882084	213943485
% retained reads	**75.57**	**71.99**	**66.15**	**56.78**	**69.01**	**60.66**

De novo genotyping produced a minimum of 267 loci to a maximum of 57846 loci per sample, with a mean of 12792 loci per sample. Mean merged coverage per locus also varied per sample, ranging from 4X to 38X. A total of 219566 SNPs were identified, in non-deleveraged RAD loci with 1 to 5 SNPs each; missing call rate in individuals was high, varying between 72 to 99%. Before starting any population genetic structure analysis, all SNPs identified in positions higher than 79 bp were removed; in addition, according to results obtained from mini-contigs blasting (see 4.4.2.4), all SNPs identified in loci giving significant alignment with tick vectored pathogen (rickettsioses) and endoparasites (*C. m. mitochondrii*) were removed, as well as all loci for which at least one SNP position showed more than two alleles per individual, as they may represent paralogs. This left a dataset of 163444 SNPs. Using this dataset, analyses on genetic structuring were conducted on individual genotypes and on consensus genotypes at site level. *Smart_pca* and MDS, were used to analyse filtered SNPs datasets, but results of *smart_pca* clearly showed the bias caused by missing data for each individual, especially when used with default parameters (i.e. normalization of allele frequencies and out-group removal; see Fig. 4.24 for an example). Therefore, MDS was preferred to *smart_pca*, because it showed no bias related to sample missing rate.

MDS analysis did not suggest any obvious genetic clustering of the individuals; for example, Fig. 4.25 shows the MDS plot obtained from a dataset of 6824 SNPs, having a maximum missing call rate of 80%, for 215 individuals, as the 31 worst individuals (having more than 99% of missing genotypes) were removed. The same lack of clustering was obtained when MDS was applied to more conservative SNP dataset (1568 SNP for 215 individuals) or to only 'high quality samples' (Fig. 4.26). Even classification of samples by means of sampling site or sex (female vs males) did not reveal any genetic clustering.

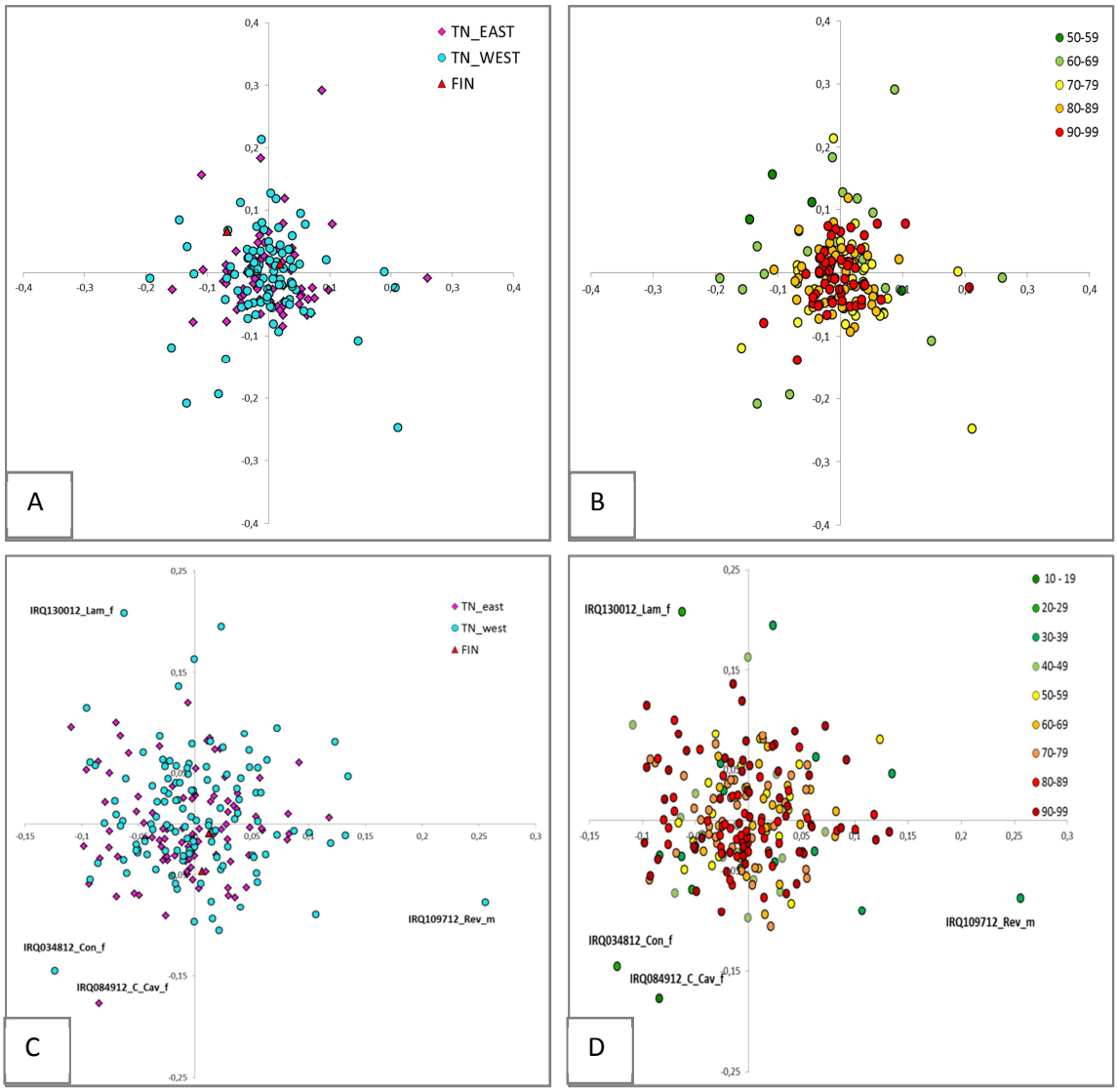


Fig. 4.24 (previous page) *Smart_pca* plots obtained by applying different parameters from a dataset of 6824 SNP for 215 individuals (only loci with <80% missing rate were selected). Left hand plots (A and C) present individuals pooled by sample area (east or west of the Adige River), while righthand plots (B and D) by means of their missing rate class (as defined in the legends). In A and B PCA was performed using default parameters. In C and D PCA was performed disabling normalization of allele frequencies and the outlier removal algorithm. In all cases, *lsq project* algorithm was enabled and no significant eigenvalues, by means of Tracy-Widom statistics, were identified. From A and B clearly emerge as the PCA analysis is biased by missing rate in the samples: high missing rate ones are concentrated in the center of the plot. Additionally, as emerge from C and D, removed outlier were the ones with the lowest rate of missing genotypes. Even disabling normalization, individual missing rate biased the output of the analysis.

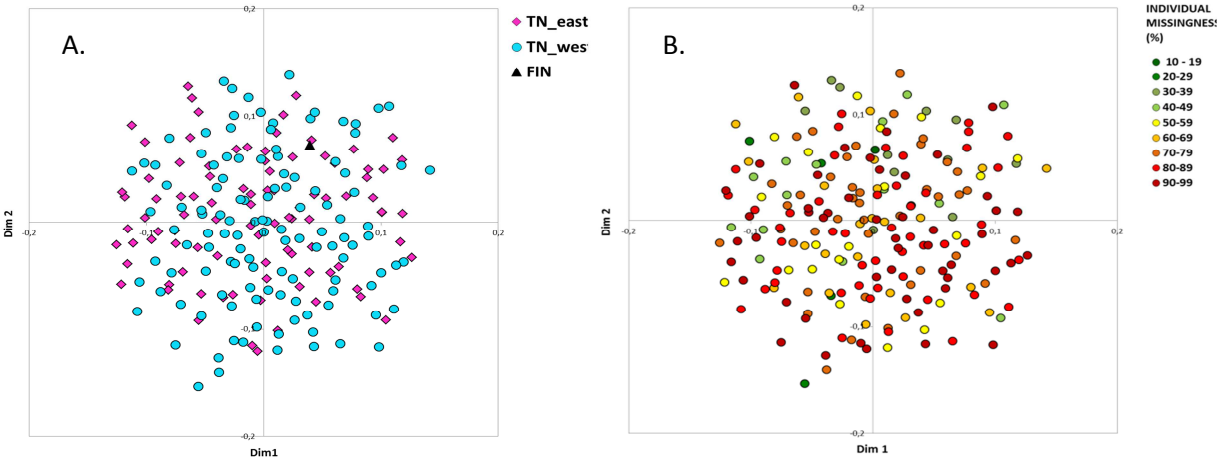


Fig. 4.25 MDS obtained from a dataset of 6824 SNPs for 215 individuals (only loci with <80% missing rate were selected). A. Individuals pooled by area of origin within PAT (east or west of the Adige River),; B. individual represented by means of their missingness class, as reported in the plot legend.

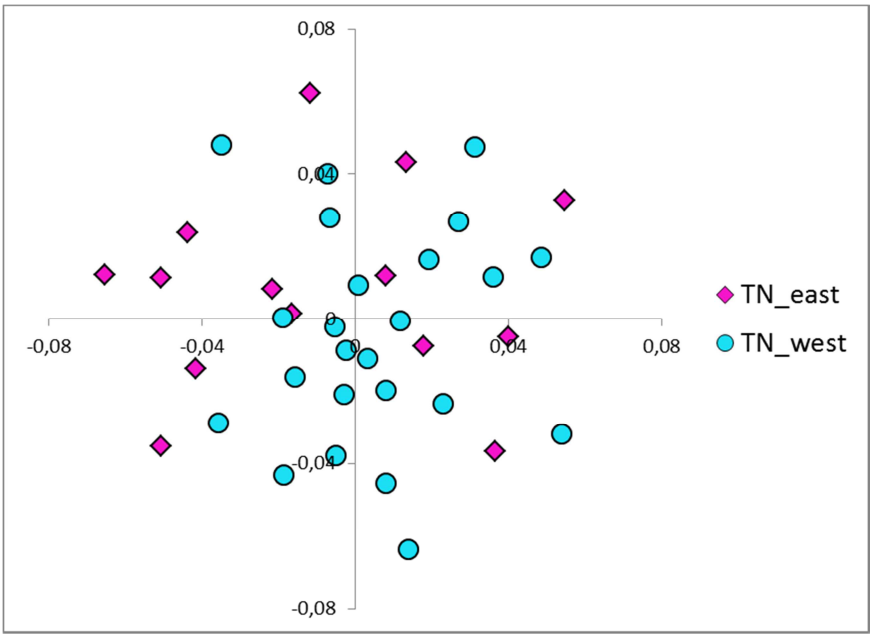


Fig. 4.26 MDS obtained from a dataset of 6977 SNPs, only loci with less than 50% of missing rate were allowed, for the 39 individuals for which > 25 000 stacks were identified.

In the same way, several SNP datasets, with varying stringencies in terms of locus/individual missingness, were used as input in *MDS* for site consensus genotypes, but in every case no genetic clustering was identified (example in Fig. 4.27).

The removal of PCR duplicates from the raw RAD-seq dataset meant that a *de novo* SNP calling and genotyping could be performed with higher confidence. All analyses confirm the absence of genetic clustering the sampled individuals, regardless of site origin. In contrast to population genetic studies of vertebrate species in PAT, no genetic pattern between east and west PAT could be detected.

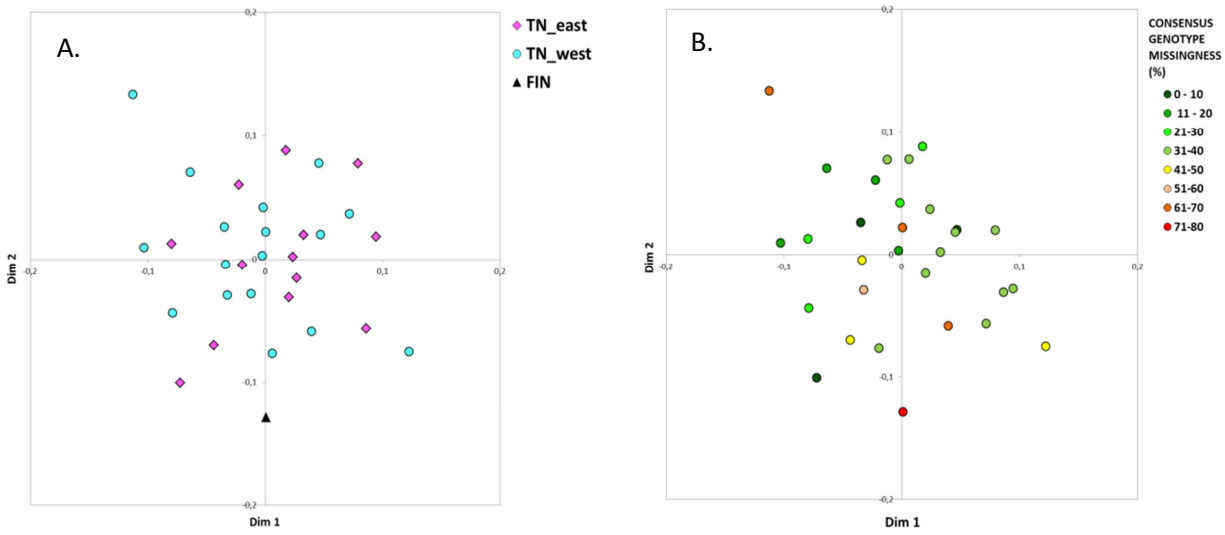


Fig. 4.27 MDS analysis of 22138 SNPs for 31 sites (consensus genotypes); loci with a missing rate less than 50% were retained. A. Individuals are pooled by area of origin within PAT (east or west of the Adige River),; B. individuals represented by missingness class, as reported in the legend.

4.4.2.3 SNP calling with use of a reference genome

Rather surprisingly, only 0.5% to 0.1% of *I. ricinus* individual paired-end sequences aligned to *I. scapularis* reduced reference genome and only 36 SNPs were obtained as a final output.

The PCA obtained from genotype probabilities (i.e. individual datasets pooled by PAT area), again showed an absence of structure, with a central cluster and three outliers individuals (Fig. 4.28). These three (IRQ084912_C_Cav, IRQ034812_Con and IRQ076112_Lam) are characterized by a high number of reads that aligned well with the reference genome. This result suggests that the *I. ricinus* population is well admixed and no genetic structure is present, but could be also a consequence of missing data, so that all individuals have the same genotype probabilities and therefore, cannot be distinguished one each other.

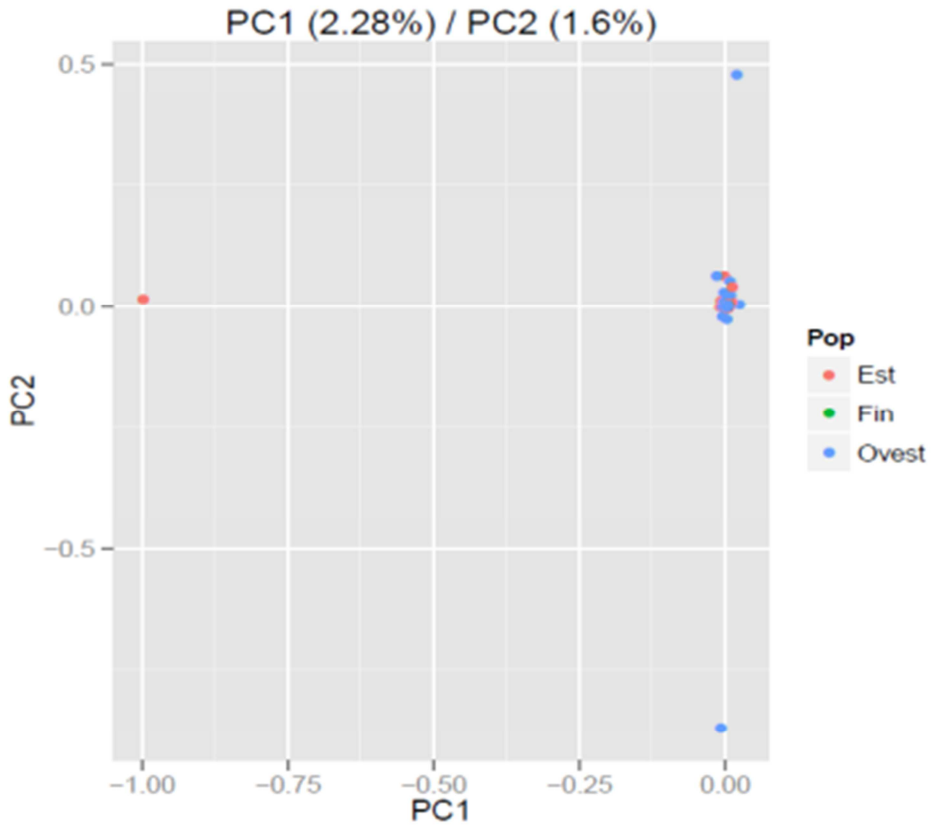


Fig. 4.28 PCA from genotype probabilities computed with ANGSD; individuals are represented by means of PAT area (Est or Ovest=East or West, as before used) and the two Finnish individuals (Fin) are in green.

4.4.2.4 Mini-contigs blasting results

A total of 71664 contigs, with a minimum length of 200 bp, were obtained. Of this, 12426 (17%) gave significant alignment with BLASTn. As reported in Fig. 4.29, most contigs (10762/12426; 86.6%) aligned with *I. scapularis*, 11 with *I. ricinus*, and 13 with *R. appendiculatus*. Interestingly, 13 hits aligned with sequences of the tick endosymbiont *Candidatus midichloria mitochondrii*, while 5 aligned with the Rickettsia spotted fever group.

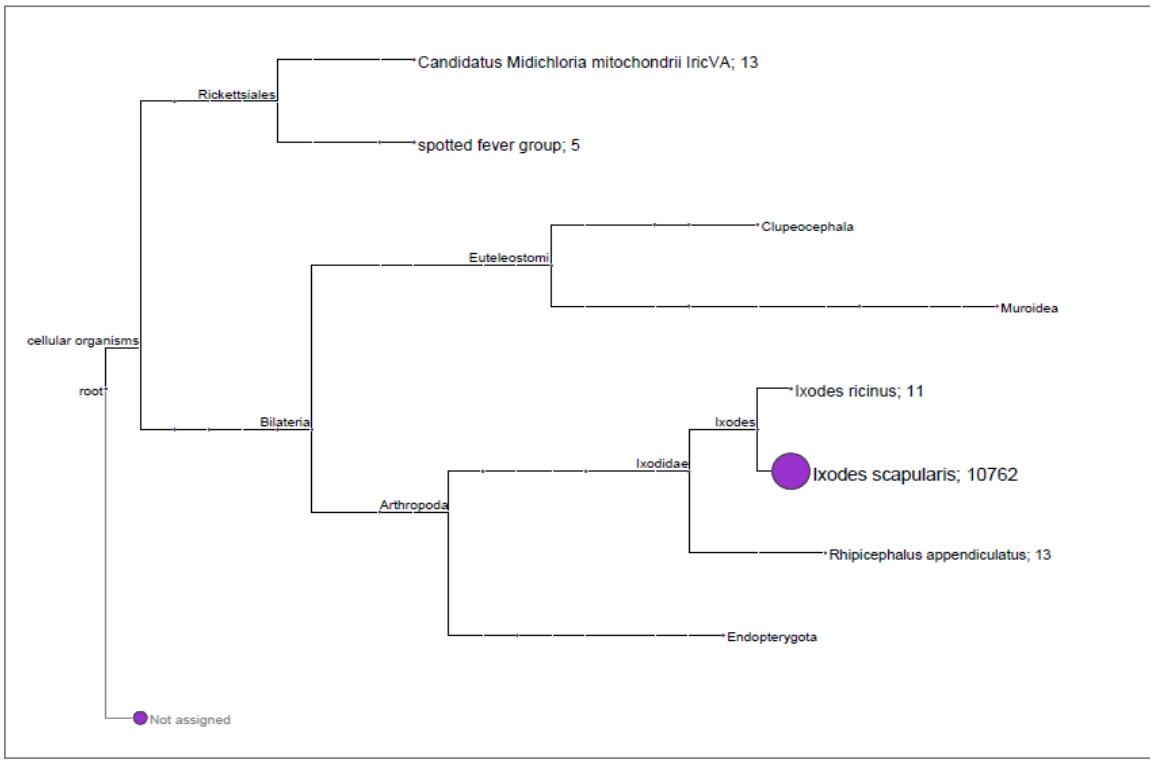


Fig. 4.29 MEGAN contigs BLASTn results visualization.

5. DISCUSSION

In TBD risk evaluation, it is essential to understand which factors are affecting vector population dynamics and the epidemiology of each specific tick-borne disease involved. In fact, in order to estimate the final pathogen transmission potential of the tick itself, it is necessary to consider the effect of both the biotic components of the interactions taking place between the vector, and a diversity of hosts and pathogens; intrinsic biological barriers (i.e. molecular, cellular, physiological and physical); and the abiotic component of the environment in which the TBD system is embedded (Pfäffle et al., 2013; Randolph, 2008; Fig. 5.1). These considerations are particularly important in the present context of rapid variations in climate, socio-demographic factors and land use, which not only strongly impact vector and hosts populations, but also modify the frequency of contacts of humans and domestic animals with infected ticks (which is directly correlated with disease risk).

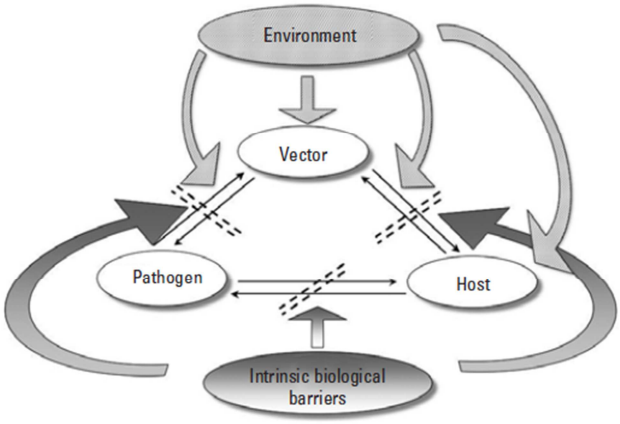


Fig. 5.1 The triangle of host- vector - pathogen interactions, showing the points of action of the intrinsic biological barriers to transmission and the extrinsic environmental factors (reprinted from Randolph et al., 2008).

In this study, I integrated two advanced molecular approaches, NGS population genomics and bloodmeal analysis by HRMA, to infer the host-use pattern of *I. ricinus* at a regional scale and the potential effect of this behavior on TBD epidemiology. First, I will discuss the novelty of the Real-time HRMA bloodmeal analysis protocol we developed and the future technical improvement that would made it more efficient; then, population genomics and bloodmeal analysis results will be discussed in the framework of reference literature.

5.1 A new tool for bloodmeal analysis in questing ticks

Molecular bloodmeal analysis is a milestone in the study of arthropod borne disease epidemiology, as underlined by the increasing body of research on this topic and its application to several hematophagous vectors (tsetse flies, *Anopheles* and *Culex* mosquitoes, reviewed in Kent, 2009; Triatominae bugs, Peña et al., 2012) including ticks (Estrada-Peña et al., 2005; Pichon et al., 2003, 2005, 2006; Humair et al., 2007; Morán Cadenas et al., 2007; Allan et al., 2010; Wodecka et al., 2014). However, although molecular methods can provide high specificity and sensitivity in bloodmeal analysis (Kent, 2009), their resolution is still limited by digestive process and mixed bloodmeals, as is the case of their application in ticks (Gómez-Dìaz and Figuerola, 2010). In fact, while there is urgent need for such a reliable method (Bolzoni et al., 2012; Estrada-Peña et al., 2013), the current molecular methods available for bloodmeal analysis in questing ticks are not considered sufficiently robust for application in field studies (Estrada-Peña et al., 2013), and questions have been raised concerning their susceptibility to contaminations (Collini et al., 2015a in press; Collini et al., 2015b).

Here, I present the novel application of Real-time HRMA as a new tool for the investigation of tick feeding ecology. Before testing the new technique, we explored a variety of DNA sequencing protocols, using universal vertebrate primers as well as species-specific and host group ones, since sequencing would be the most straightforward way to capture the wide host range of the sheep tick and/or to identify hosts to species level. In addition, reduced sequencing costs, as well as improved PCR reagents and instrumentation, were all factors favouring testing the sequencing approach by simply designing new primer sets. Although I did not obtain encouraging results, they provided key indications for the successive and successful design of the Real-time HRMA protocol. That is, the use of universal vertebrate *cytb* primers led to the amplification of contaminant human DNA, so consequently, I went on to design primers that did not cross-react with human DNA. In addition, the superior performance of primers amplifying shorter amplicons moved my primer design toward very short amplicons, and host group primers amplifying species out the range of the selected ones, led me to avoid degenerate primers.

The presented Real-time HRMA protocol, involving the amplification of host DNA by means of six newly designed host group primers, allowed the simultaneous screening and identification of bloodmeal sources in questing ticks for 17 of the most important European vertebrate tick host species (8 wild ungulates, rodents, shrews and birds, as well as the domestic dog, livestock species - goat, sheep and cattle -, and the commensal house mouse), and two genera (*Apodemus* and *Sorex*).

Our results confirmed the power of HRMA to identify the host species or genus from both control DNA samples and questing nymphs. Importantly, HRMA is a non-destructive post-PCR method, meaning that amplification products from questing ticks

can be sequenced to confirm species identity in certain cases (e.g. to distinguish *T. philomelos* and *E. rubecula*) and to reach species-level identification for cases in which HRMA can only clarify the genus (e.g. *Sorex* spp. and *Apodemus* spp.). In order to avoid unnecessary expense, I would recommend post-HRMA sequencing for only those amplicons with unusual melting profiles that fall within the T_m range of target species (e.g. sample 12_TR; Appendix-Table A1), or that have similar shape to target species but are outside the reported T_m range. These are worth sequencing as they may extend the T_m range of that species (by identifying intraspecific sequence variation), or even the list of target species for a particular primer set (as in the case reported here of *B. taurus*). However, as shown here, profiles that fall well beyond the range of target species or have a very unusual shape are unlikely to yield host DNA, but are more likely arise from contaminant DNA or primer dimers.

In the first application to questing ticks, we were able to identify bloodmeals in 65.4 % of questing nymphs. This sensitivity is higher than mean sensitivities published thus far for RLBH or RFLP for *I. ricinus* nymphs (24.5 % - 15.5%: Estrada-Peña et al., 2005; 49.4 %: Pichon et al., 2005; 33 %: Pichon et al., 2006; 38.2 %: Humair et al., 2007; 40.6 %: Morán Cadenas et al., 2007) and is similar to that of Wodecka et al. (2014; 62.8 %) and Allan et al. (2010; 62.8%). Our HRMA protocol also proved to have high identification success, correctly assigning host DNA to species or genera using HRMA alone in 40/42 (95.2 %) amplicons obtained from questing nymphs; of these, 35/42 (83.3%) were identified to species. This compares favourably to the 72 % and 62.3 % identification success to genera or species reported in Morán Cadenas et al. (2007) and in Humair et al. (2007), respectively, while all other above-mentioned authors were only able to identify host DNA to group, family or occasionally genus

level. Unfortunately, direct comparison of all three currently available methods, by testing the same nymphs with RLBH, RFLP and HRMA, is not possible at this time because of the limited quantity of eluted DNA available from each questing nymph. Only application of this new HRMA protocol to large collections of questing ticks will confirm its place among bloodmeal identification methods.

The lack of host identification in 18 out of 52 questing ticks may be a result of the time since the last bloodmeal, which we estimated as 9 to 14 months (Kirstein and Gray, 1996; Randolph et al., 2002), or specific individual developmental dynamics, heavily affected by site and climatic conditions (Morán Cadenas et al., 2007). However, it may be that some nymphs fed as larvae on species not included in our primer design process (e.g. *Podarcis muralis*, *Erinaceus europaeus*, *L. europaeus*, *S. vulgaris*, *Sus scrofa*, etc.), which are currently considered minor hosts in my study area. For this reason, additional primer sets need to be designed to expand host coverage (see 5.2).

My initial results showed HRMA host misidentification in only one individual, as a result of sequence variation at the intraspecific level, not predictable during amplicon selection and primer design. These errors derive from the relative scarcity of available GenBank mtDNA sequences for some of the selected host-species (e.g. *Apodemus* sp.) that are not currently the object of intensive genetic study, despite their importance in zoonotic disease cycles.

DNA from multiple hosts was detected in 23.5 % of tested nymphs. Even if this result is based on a relatively small number of samples, it is comparable to that obtained in 2007 by Morán Cadenas et al. (19.5 %) and Allan et al. (16.2%; 2010), although the efficacy of PCR-RLBH and PCR-RFLP protocols to detect mixed bloodmeals has not been thoroughly investigated to our knowledge (see Humair et al., 2007; Wodecka et

al., 2014). In addition, the presence of DNA from more than one host may increase the complexity of host identification with the above methods as well as those using direct sequencing (Alcaide et al., 2009; Kent, 2009). However, my HRMA approach allows unambiguous detection of multiple hosts, at least when these are species belonging to different host groups. The fact that no nymphs were initially found to have fed as larvae on targeted species of Passeriformes and Caprinae was probably not an indication of the suitability of the primer set, but simply a result of the small sample size; in fact, the bloodmeal screening of the much larger sample using this protocol also resulted in successful amplification of *Passeriformes* and *Caprinae* DNA from questing nymphs.

My results demonstrate that Real-Time HRMA is a reliable method for bloodmeal analysis in questing ticks. Although six different amplifications must be carried out on each tick, the single-step Real-time HRMA design described here, enabling reaction processing, screening and genotyping on the same instrument, still makes it simple and fast compared to other methods. In addition, the method is extremely useful for unambiguous identification of multiple host DNA (mixed bloodmeals). Our protocol also reduces errors common in multi-step molecular protocols and avoids amplification of both environmental and human contaminating DNA, a recurring problem in low quantity/quality DNA studies. Moreover, the lack of the sequencing step in all but the most dubious cases lowers the cost of analysis. In addition, Real-time HRMA reagents have costs comparable to those for conventional PCR, and are usually provided as a *supermix*, further reducing errors (Reed et al., 2007). Although the technique described here is optimized for nymphs, our protocol should also be easily applicable to adult questing ticks, given the larger bloodmeal of the nymphal stage compared to the larval one. This protocol could also be applied to other

species of ticks, and other hematophagous insects in general, if this were convenient, by expanding primer sets if necessary.

Use of the Real-time HRMA protocol developed here for the screening of questing nymphs collected from the remaining sampling sites of PAT gave us the possibility to test it on a large sample for a more reliable evaluation of its usefulness and to introduce a more automated configuration. At the same time, important results were obtained regarding the feeding biology of *I. ricinus* larvae in the study area, that would be discussed later.

As time and financial resources are important limiting factors in research, it is important to note that the more automated configuration enabled an increase in time and cost efficiency of the protocol: (i) automated DNA extraction allows the processing of 93 nymphs in one working day by two operators, as well as a two-thirds decrease in reagent costs (magnetic-beads automated Thermo 1.70 € +IVA *vs* manual spin-column Qiagen 4.96 €+IVA); (ii) robotic PCR reaction set-up decreased operator working time and reduced the errors and variability usually related to operator skills; (iii) the RotorGene instrument permitted to process 32 unknown samples at a time.

On the other hand, a notable decrease in identification success (25.4% overall), related to the introduction of the magnetic-beads automatized DNA extraction method, was observed. However, identification success also varied greatly between sampling sites (QIAamp: 31.3 – 92.3%; Thermo: 3.3 – 50.0%). Moràn-Cadenas et al. (2007) also showed that identification success varies according to site-specific climatic conditions (49.9% North facing slope, 41.0 % South facing slope). Since these conditions also direct affect the metabolic rate of ticks and their population dynamics, in the same sampling site, identification success also changed according to sampling time, so that

the North facing slope had higher values in spring and autumn (93% May and 73% October) and lower values in summer (20% July), while the South facing slope presented a low identification success from April to July (19 - 35%), with an increase during the summer and the highest peak in October (68%). Even if sampling time did not significantly explain the variation in identification success in our dataset, possibly because samples were all collected in spring, it could be that local micro-climatic variables did have an influence. Unfortunately, we do not have the relevant measurements at our sample sites, such as saturation deficit (the most important climatic variable in regulating tick activity and, therefore, metabolic rate; Tagliapietra et al., 2011), which would have allowed a better investigation of this effect.

As postulated soon after the development of the HRMA protocol, sequencing of amplicons having unusual melting patterns extended the list of target species for Cervidae (*D. dama*, *Ovis* spp. and *R. rupicapra*), Passeriformes (*Turdus* spp.) and Muroidea (*C. griseus*). These additional species are not distantly related to the target species and the mtDNA regions selected show relatively low levels of variability; consequently, in some cases, melting temperatures of these additional species partially overlap with the ones of primary target species, making the use of HRMA for species level identification not as straightforward as initial results suggested. On the other hand, analysis of a much larger dataset still showed that primers do not cross-react with distantly-related species compared to those for which they were designed; hence, sequencing of all amplicons could be avoided if host group or genus identification is considered detailed enough according to the scientific hypothesis of the study or the target of the bloodmeal analysis (i.e. identification of neglected host species; evaluation of a specific species as tick hosts). For example, as our results showed, the classification

of host according to Order still gave important insight into larval host distribution in the two major habitat types investigated.

5.2 Real-time HRMA: Technical problems to resolve

The Real-time HRMA protocol presented here gives a reliable indication of the blood feeding habit of ticks in a certain area, as the main host groups and, most importantly, hosts having a central role in the TBD epidemiological cycles in the Alps, are targeted.

Certainly, a higher identification success would help to improve confidence in the results since bias in the species detected (if it exists) could be ruled out. Such success would also give robustness and more credibility to the results of the statistical analyses used to infer hosts relative importance and model host-parasite systems. Manual QiaAMP® DNA Investigator was more efficient in amplifying host DNA, but may not be ideal for a large sample due to its higher costs and time needed to implement it. However, it should be noted that there is now a large variety of magnetic-beads reagent kits available for combined use with the KingFlex®Magnetic Particle Processor, some specifically designed for small samples sizes and forensic applications, such as QiaAMP® DNA Investigator, that could be tested and optimized.

Substantial improvement of the protocol could be made extending its host range coverage and enhancing species level identification by designing new primers for the current target species. Here, an in-deep evaluation of the availability of reference sequences suitable for HRMA from public databases for Reptiles (*Lacerta viridis*, *P. muralis*), other Carnivores (*Meles meles*, *Martes martes*, *Mustela nivalis*), and rodents (*Sciurus vulgaris*, *Glis glis*), or other species (i.e. *E. europaeus, Sus scrofa*) was done at

this time, showing, for some of these species, few sequences data available; genetic resources could be eventually obtained by *de novo* sequencing mtDNA region by means of universal primers. On the other hand, improving species level identification for the currently targeted species could be challenging, as available mtDNA genetic resources were already exhaustively examined to design the primers used here; others were tested and discarded (either by in-vitro by uMelt^{TM} and/or on control samples). However, the species level identification, could be improved, especially for the host group primers for Cervidae (multiple species having partially overlapping T_m), Muroidea (misidentification of *Apodemus* spp. because of intraspecific variation) and Passeriformes (*T. philomelos*, *Turdus* spp. and *E. rubecula* having overlapping T_m range), by the introduction of unlabeled probes (Reed et al., 2007) or by heteroduplex formation (Cheng et al., 2006).

In future, the development of multiplexed assays (Reed et al., 2007; Seipp et al., 2008; Gori et al., 2012) would further increase the time- and cost-saving properties of the devised method. Moreover, as less total tick DNA would be used in bloodmeal analysis with this method, a comparative analysis of current methods (i.e. RLBH) and the one presented here could be performed. More importantly with more DNA a parallel screening for TBD etiological agents (i.e. *Borrelia* spp., *Rickettsia* spp., *Anaplasma phagocytophylum, Babesia* spp.) could be performed on the same tick, as done in previously published bloodmeal studies (Pichon et al., 2003, 2005, 2006; Morán Cadenas et al., 2007; Allan et al., 2010; Wodecka et al., 2014). As presented in these articles, knowing both the larval host bloodmeal and the pathogens hosted by each tick would permit a more complete investigation of the importance of vertebrate species in tick population maintenance and their role in the epidemiological cycles of TBD, even

for those species rarely trapped or not currently considered important in TBD systems. However, it should be kept in mind that simultaneous identification of pathogen and host DNA does not imply reservoir status for the identified species, or vector status for the ticks, as Estrada-Peña et al. (2013) underlined, but additional laboratory studies must be performed before it can be concluded that a particular tick is a vector, or a host is a reservoir of a particular pathogen.

5.3 Tick biology and epidemiological implications

I. ricinus is traditionally depicted as a generalist parasite, able to exploit a wide range of mammals, birds and reptiles (Hoogstraal and Aeschlimann, 1982). The only constraint in host choice appears at the adult stage, when females preferentially feed on large mammals, in order to take the large bloodmeal needed for egg production (Tälleklint and Jaenson, 1994; Mejlon and Jaenson, 1997). In evolutionary terms, it would appear evident that adaptations allowing a parasite to track host responses to infections would make the parasite better equipped to exploit the host than a generalist; on the other hand, generalists might be more efficient at reacting to environmental variation influencing host diversity and availability. Given the intimacy of tick-host physical and physiological interactions taking place during the long bloodmeal (Brossard and Wikel, 2004), one may expect that a high level of specialization would be necessary to overcome the immune barriers of hosts combatting hematophagous parasitism, making exploitation of several host species difficult. Such specialization could lay the foundation for host-race formation, if not speciation, since a barrier to gene flow between tick populations exploiting different hosts could eventually evolve (reviewed in McCoy et al., 2013; Magalhães et al., 2007). Tick ‘races’ exploiting

different hosts would also present genetic structuring at different geographical scales, in relation to host vagility, since tick patterns of gene flow and dispersal appear to be largely determined by host movement during infestation (Porretta et al., 2013 and references therein and later shown and explained here). Host-specialization would have profound consequences on the current understanding of tick population dynamics and associated pathogens, since the reaction of each TBD system to climate and land use change will be different according to the host species involved.

Reviewing *I. ricinus* phylogenetics and population genetics literature, McCoy et al. (2013) came to the conclusion that different degrees of host association divergence may exist in local populations of the sheep tick, according to the time of tick population establishment as well as to the stability of the local host community (Fig. 5.2), as observed for other tick species (*I. uriae*, Kempf et al. 2009a; *Rhipicephalus micropilus*, De Meeûs et al., 2010). This conclusion, mainly based on results of microsatellite analysis of *I. ricinus* on a local scale (De Meeûs et al., 2002; Kempf et al., 2009b; Røed et al., 2006; Kempf et al., 2010; Kempf et al., 2011; De Meeûs et al., 2004), could be possibly rejected in light of the inappropriateness of microsatellites as a molecular markers for this species, as revealed by our analysis and highlighted by Quillery et al. (2013). Although the authors mentioned certain anomalies in the STR dataset (mostly null alleles) and recognized their importance in causing part of the heterozygote deficiency observed, instead of sequencing the markers as I did and discovering that their characteristics did not match those of true microsatellites, they suggested that local substructure and sex-biased genetic structure were the most possible biological explanations. Kempf et al. (2011), using the same microsatellites, concluded that host-associated populations seem to occur sympatrically, but in only some of the sites they

analyzed. Hence, we can conclude from the literature that no reliable evidence of tick 'races' has yet been published.

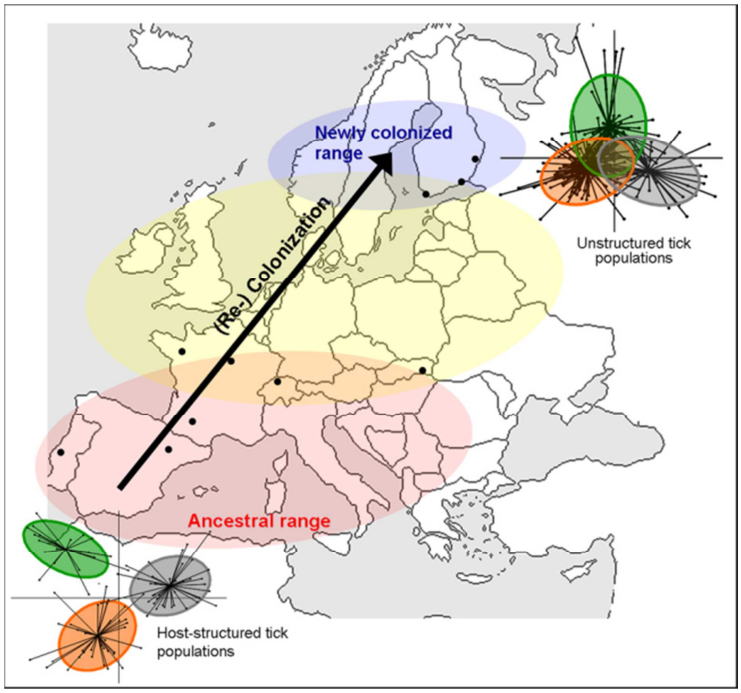


Fig. 5.2 Schematic representation of population expansion of *I. ricinus (arrow)* and hypothesized consequences for host-associated genetic structure. The red zone represents well-established *I. ricinus* populations that persisted in both Southern and Central Europe during the last glacial phase (Porretta et al., 2013). The blue area represents the recently colonized zone for *I. ricinus* and the yellow zone, a transition area where population age and history may be variable (Léger et al., 2013; Porretta et al., 2013). Under the hypothesis that the evolutionary age of a tick population may affect the evolution of host specialization, we would expect a strong pattern of specialization in the red zone, and no host specialization in the blue zone because ticks have only been exposed to local hosts for a few generations. In the yellow zone, patterns of host specialization may be more variable. On the figure, the degree of host-associated population structure of *I. ricinus* in the extreme zones are represented by a between-group analysis of neutral genetic variation, where each dot represents an individual tick and the color indicates different host-associated tick populations. Greater separation of groups indicates stronger genetic divergence. The relative evolutionary age of tick populations could be changed to some other habitat-based factor that may affect the evolution of host specialization. Reprinted from McCoy et al., 2013.

Here I present the first application of RAD-Seq to the study of tick population genetics. The varying sequencing success of individuals may be partially explainable by errors in library preparations; however, similar problems have been observed in other

studies using this NGS approach (Xu et al., 2014). The maximum number of RAD markers obtained in this study was significantly smaller than those expected, based on *I. scapularis* genome features (2.3 Gb and 45% GC); as reported in Reitzel et al. (2013 and reference therein), however, it is highly likely that the ratio of expected vs observed RAD-loci was biased by the presence of highly repetitive regions that make up a large fraction of the tick genome (see below) or, as the extremely unsuccessful alignment of *I. ricinus* sequences against the *I. scapularis* genome may suggest, the *I. scapularis* genome parameters used in the RAD-Seq experiment setting may not have been appropriate.

From the extensive analysis of genome wide SNPs retrieved by applying the RAD-Seq protocol on a large number of *I. ricinus* adults collected in the entire distributional range of the species in PAT, and using a variety of bioinformatics approaches, I can conclude that the population of sheep ticks in this area shows no genetic structuring. Individuals had a high degree of admixture and even the two samples from Finland did not cluster separately from the Alpine ones. The Adige River Valley, has previously been identified as a partial barrier to gene flow in red deer, roe deer and chamois populations (Pecchioli et al., 2006; Crestanello et al., 2009), as well as smaller organisms such as mountain hares (Pecchioli et al., 2006), the butterfly, *Erebia euryale* (Haubrich and Schmitt, 2007), and several alpine plants (Schönswetter et al., 2002; Albach et al., 2006). In fact, the area between Lake Garda and Innsbruck, including the Adige Valley, has been invoked as a zone of genetic discontinuity delimiting eastern and western Alpine populations of many other plant species (Thiel-Egenter 2007; Thiel-Egenter et al. 2009). However, the so-called ‘Brenner Line’ did not seem to affect genetic structuring in *I. ricinus* populations.

These findings are in agreement with studies based on mitochondrial (Casati et al., 2008) or on both mitochondrial and nuclear genes (Noureddine et al., 2011; Porretta et al., 2013), showing a lack of phylogeographic structure in the whole range of the mainland European *I. ricinus*, although the European population is clearly divergent from the north-African one (Noureddine et al., 2011). Divergence of the north-African sheep ticks is also supported by microsatellite analysis (De Meeûs et al., 2002) and is likely a consequence of genetic drift following the last Pleistocene glaciation and filling of the Mediterranean sea which now separates the two populations. In fact, seasonal activity and host-association differences have been recorded such that it has been postulated that the introduction of European ticks to Africa by migrating birds would no longer result in reproduction, gene flow and admixture with the native population (Noureddine et al., 2011). Since PAT was completely covered by the Alpine ice-sheet during the Last Glacial Maximum, the absence of geographical genetic structure in the local populations could reflect a rapid expansion of the species after the most recent Pleistocene glaciations, as found at the European level (Noureddine et al., 2011), resulting from a recolonization from the interconnected populations that persisted in both Southern and Central Europe during the Last Glacial Maximum (Porretta et al., 2013), where they presumably parasitized the mammal populations also found in these 'refugia' (Taberlet et al., 1998; Hewitt, 1999, 2000; Yannic et al., 2012 and references within). According to McCoy et al. (2013; Fig. 5.2), host-associated divergence is therefore unlikely. However, to explore this hypothesis, the genomic data generated here will be used by another PhD student, Alex Panziera (Univ. of Ferrara), to explore the demographic history of PAT tick populations after he develops software for the analysis of RAD-Seq data.

My results suggest that tick 'races' in this area are unlikely, in accordance with McCoy et al. (2013) that suggest a lack of host-association evolution in recently stabilized tick populations. In any case, it appears that the advantages of such specialization are outweighed by positive selection for plasticity for the exploitation of a wide range of hosts. This generalist behavior has had a fundamental role in the persistence of Pleistocene tick populations in suitable areas and in maintaining the interconnections between these populations at a level for which no sign of genetic divergence could be detected (Porretta et al., 2014), as well as in the admixture of present day tick populations (Noureddine et al., 2011; Casati et al., 2008). It will also allow ticks to continue to invade into new regions at higher altitude and latitude (Léger et al., 2013), and peri-urban and urban forest areas (Rizzoli et al., 2014), as a result of climate and land use changes.

In particular, by investigating the genetics of the adult stage, we are looking at the final result of the host-mediated dispersal that acted at the larval and nymphal stages. My results from feeding tick collection and bloodmeal analysis at a local scale both show that even the larval stage may exploit highly vagile wild species, like birds and deer, but also sheep, foxes and dogs. Finally, as suggested in other studies and species, the high presence of repetitive elements and transposable elements that characterize the large tick genomes (Geraci et al., 2007; Nene et al., 2009; Meyer et al., 2010), may provide the genetic variability generating the ability of ticks to exploit such a variety of different hosts (Mastretta-Yanes et al., 2014; Casacuberta and Gonzales, 2013; Sunter et al., 2008). However, in depth study on this topic has not yet been carried out, also because a definitive assembled genome is still lacking, paradoxically, for the very presence of these confounding repetitive elements themselves.

Bird migrations could also account for the recent large scale homogenization of sheep tick populations across mainland Europe, as several studies showed passerines infestation by ixodid ticks also during migration (Humair et al., 1993; Hasle et al., 2011; Comstedt et al., 2006; Waldenström et al., 2007; Lommano et al., 2014; Dubska et al., 2009). Dinnis et al. (2014), founding genetic discontinuity between tick populations in British Isles and Latvia, by using a multilocus sequences typing on mtDNA genes, discussed the effectivity of bird-mediated tick dispersal. As emerges from Figure 5.3 and Fig. 5.4, the British Isles are not part of the main south-west/north-east passerine migrations, which reduces the possibility of incoming birds carrying ticks from north-eastern Europe where Latvia lies. Italy, instead, is clearly part of the north-east, south-west post-breeding migration route and is recognized as a wintering locality for the north-central European breeding population of passerines (Busse, 2001; Spina and Volponi, 2008), and a breeding site for north-African wintering species. The genetic discontinuity found between the mainland and the British Isles, supports the hypothesis that birds play an important role in tick dispersals and could largely explain the lack of geographical structuring in *I. ricinus* ticks in mainland Europe, as well as in PAT, which also lies along the important migration routes described above. Climate change (i.e. increases in the mean temperature), inducing an extended questing period and reducing winter mortality, may additionally enhance tick dispersal by birds during the autumn migrations by increasing the tick-load per bird and also by promoting their survival in the new environment.

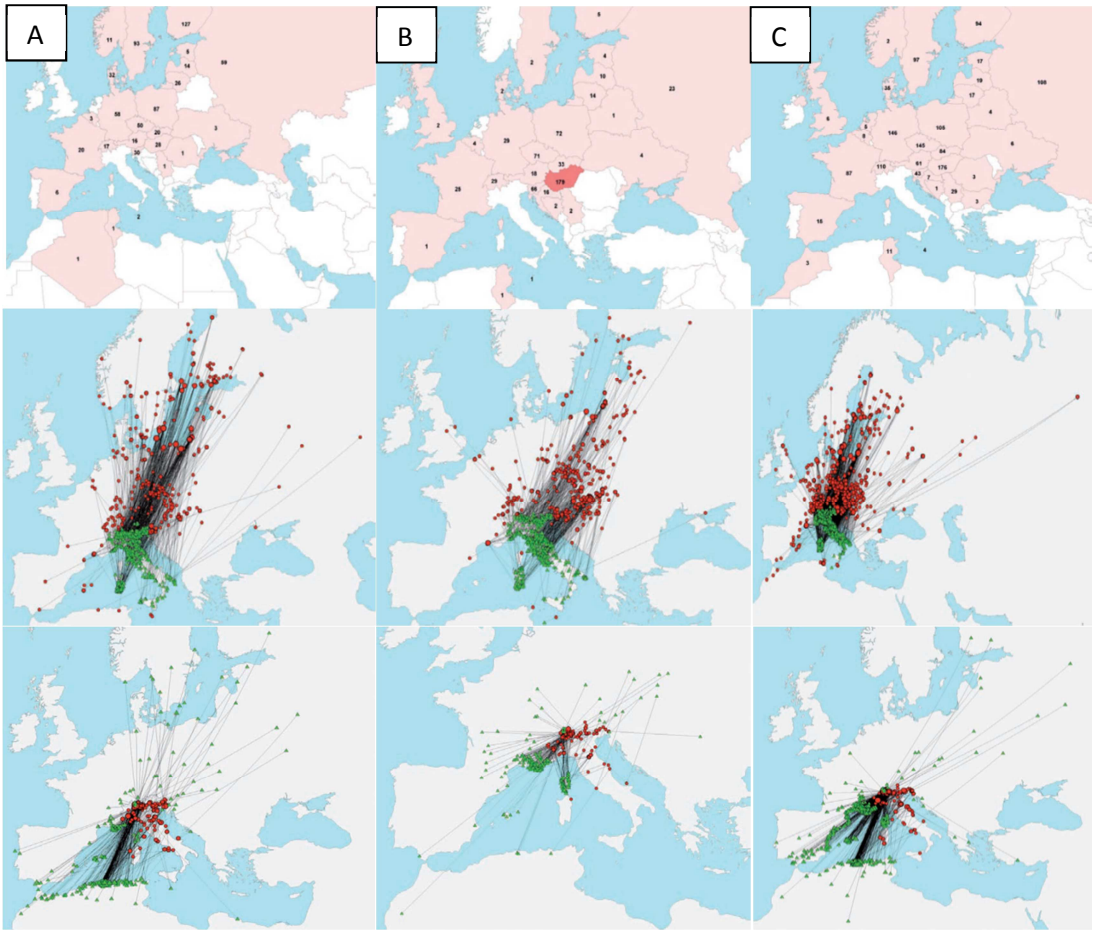


Fig. 5.3 Numbers of foreign ringed birds recovered in Italy for each Country of origin (above); movements of foreign ringed birds recovered in Italy (center), and Italian ringed birds recovered abroad (below). A. *E. rubecula*; B. *T. merula*; C. *T. philomelos* (Spina & Volponi, 2008; ISPRA)

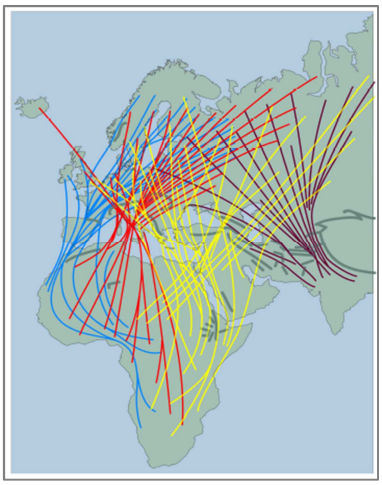


Fig. 5.4 Bird migratory routes at the Eurasian-Africa scale clearly showing Italy at the center of a south-west/north-east route (red), while the British Isles, seem to be part of a more western migration route (blue). Downloaded from: http://www.seen-net.eu/index.php?pg=mapflyways.

Deer (*C. elaphus* and *C. capreolus*) and foxes (*V. vulpes*) play an important role in regional scale admixture of tick population, because of the relevant number of all tick stages they can host and spread with their movement (Vor et al., 2010; Carpi et al., 2008; Dumitrache et al., 2014; Meyer-Kaiser et al., 2012). In PAT, it is well-documented that populations of these species are in a period of consistent growth and establishment in peri-urban and urban forest areas, where they can introduce and maintain tick populations, increasing concern for public health protection (Rizzoli et al., 2014; Mackenstedt et al., 2015). Specifically, adult roe deer (*C. capreolus)* show territorial behavior during spring and summer, but the habit of the dominant male to chase away subadults, could lead to a quick dispersal of parasitizing ticks over long distances. In additional, autumn migrations could cover variable distances, from the usual few thousand metres up to 100 km (Vor et al., 2010; Cagnacci et al., 2011). Unstable and size-variable home ranges and floating individuals often occur in red fox populations making them effective in ticks dispersal (Kolb, 1984; Cavallini, 1996).

In the Alps, including PAT, cattle and sheep (both recognized sheep tick hosts; Gray, 1998; also proven by this study) transhumance has been practiced for centuries; although recently much reduced, this practice still occurs. Cattle transhumance involves a vertical movement of the animals from the valley bottom up to the mountain pastures, while sheep flocks follow longer routes, from the Po Plain up to Alpine summer grazing (Bunce et al., 2004); in both cases, the influence on *I. ricinus* dispersal is almost certainly relevant, allowing ticks to cross geographical barriers rarely attempted by wild ungulates (i.e. large rivers; high mountain chains). The modern introduction of acaricide treatments will have reduced the role of domesticated animals in tick dispersal; however, recent studies have shown an upsurge of cattle pathogens vectored by *I.*

ricinus in wild populations of ungulates and in questing ticks, suggesting, on one hand, the continuing importance of cattle and sheep in tick and related diseases dispersal, and on the other, the presence of neglected wild reservoirs for domesticated animal pathogens ticks (Lòpez-Olvera et al., 2009; Rizzoli et al., 2014 and reference therein). At a global scale, the role of increased worldwide transportation of livestock in new tick species and tick-borne disease introductions in new areas is well-known (reviewed in Léger et al., 2013).

Human-mediated tick dispersal at a local scale can also be attributed to leisure activity accompanied by dogs (i.e. hiking and hunting). The importance of dogs in tick dispersal and tick population maintenance, supported for the first time by our bloodmeal analysis, is related to the potential ability of these animals to introduce infected ticks into the urban environment where a variety of urban-adapted hosts could maintain tick populations (such as foxes) and the epidemiological cycle of the consequently introduced pathogen (passerines birds, rodents and insectivores; Rizzoli et al., 2014).

From the findings of this study the complexity of the TBD system clearly emerges, even at the regional scale, and shows that *I. ricinus* ticks exploit a large variety of competent and non-competent vertebrate hosts, which then disperse the ticks over a wide range of distances according to the vagility of the host species itself. Therefore, the relationship between host biodiversity and zoonotic diseases need to be carefully evaluated according to the diseases and local vertebrate community of interest, in order to give better estimates of TBD prevalence and the contact rate for human populations (reviewed in Pfäffle et al., 2013; Bolzoni et al., 2012). Bloodmeal analysis in questing ticks represents an efficient way to perform such an investigation, as it gives estimates of the actual host use, including the association with vagile hosts that may not always be

resident in a certain area (i.e. field observations will not be effective), but that contribute to tick population persistence (maintenance hosts) or to specific TBD introductions and prevalence (reservoir/competent hosts).

The results obtained by the application of the newly developed protocol for bloodmeal analysis in PAT questing ticks, are an interesting example of the importance of this indirect way to monitor tick-host use and the possible epidemiological implications. In fact, our results from PAT were particularly interesting in the light of the recent review of Rizzoli et al. (2014) regarding TBD hazard in peri-urban and urban habitat. Large forest covers over the 50% of the Province and fauna includes most of the species recognized both as important competent reservoirs for *Borrelia burgdorferi* s.l., TBE virus and *Anaplasma phagocytophila*, and as tick maintenance hosts. Villages, as well as the main cities in PAT, are embedded in rural ecosystems where wild hosts could interact with domesticated animals and where employment (rangers, lumberjacks, farmers) and leisure activities (hunting, walking, fishing) may enhance the human contact rate with infected ticks. Therefore, it is epidemiologically relevant that rodents, the most important reservoir host group for both *B. burgdorferi* s.l. and TBEv, and deer, implicated in the tick population maintenance and amplification, are widely exploited as larval bloodmeal source in both EXTF and PATF habitats in PAT (31.7% and 24.5% respectively), establishing the basis for disease hazard as a result of infected tick presence in both habitats (Bolzoni et al., 2012). In PATF sites the presence of passerine ground-foraging birds is more pronounced; the role of this host group in the epidemiology of *B. burgdorferi* s.l. and in feeding TBEv infected ticks is widely recognized (Waldenström et al., 2007; Lommano et al., 2014; Humair et al., 1993; Dubska et al., 2009). Birds and deer may benefit from the presence of accessible field

crops and ornamental vegetation for their foraging and sheltering in the PATF habitat, while Soricomorpha, generally negatively affected by habitat fragmentation and agricultural activities (Canova and Fasola, 1991; Spinozzi et al., 2012), are more represented in EXTF. The percentage of bloodmeals obtained from dogs is particularly concerning, and is especially high in the forest patches near urban areas (24.5%). The role of dogs in the epidemiology of TBD is still not defined. They do not appear to be an amplification host for TBEv (Pfeffer and Dobler, 2011), or reservoir hosts for Lyme disease *Borrelia* spp. agents (Bhide et al., 2004); however, they can be heavily infested by ticks and thus, contribute to tick population maintenance. More importantly for human tick bite risk, they could also vector infected ticks, acquired during visits to natural forest, into peri-urban and urban parks and gardens where they may later drop off and parasitize humans (Trotta et al., 2012; Farkas et al., 2014; Rizzoli et al., 2014) or more competent hosts (i.e. passerine birds and rodents).

In PAT, as already pointed out, livestock transhumance is still practiced regularly. Interestingly, wild sheep or mouflon (*O. aries musimom*) populations are also present, often in the same grazing areas, and their distribution partially overlaps with tick current and potential habitat. Unfortunately, we are not currently able to discriminate between bloodmeals derived from wild (*O. aries musimon*) or the domestic sheep (*O. aries*), but it would particularly interesting to do so since it has recently been reported that mouflon could serve as wild reservoir host of *Anaplasma spp.*, as do deer, or other pathogens (various strains of *Babesia* spp.) from domesticated animals (Lòpez-Olvera et al., 2009; Rizzoli et al., 2014 and reference therein). Future research should aim at identifying a more suitable mtDNA molecular marker(s) for this purpose, even if other authors claims

this may not be possible, because of their close genetic relatedness (Lorenzini et al., 2011).

The occurrence of multiple bloodmeals in 10.7% of the nymphs screened was observed in previous bloodmeal studies (Morán Cadenas et al., 2007; Allan et al., 2010; Gray et al., 1999); moreover, Gray et al. (1999) observed the occasional collection of semi-engorged larvae by blanket dragging, thus supporting the reliability of mixed bloodmeals findings by molecular analysis in nymphs. The ability to have multiple bloodmeals, and its causes, deserves an in depth analysis. In fact, co-infections in questing ticks at both nymphal and adult stage, has been widely observed (Leutenegger et al., 1999; Kurtenbach et al., 2001; Pichon et., 2003, 2005, 2006; Swanson et al., 2006; Reis et al., 2011), as well as human cases deriving from multiple infection from a single tick bites proved to occur and the derived clinical pathology showed complex patterns that make diagnosis and prophylaxis challenging (Swanson et al., 2006; Mitchell et al., 1996; Nadelman et al., 1997). Multiple infections were explained up to now by transtadial/transovarial pathogen transmission and acquisition of additional pathogens by successive meals taken at different stages (Reis et al., 2011; Swanson et al., 2006); however a significant source of such co-infection could derive from repetitive bloodmeals at the same stage, resulting in the mixed bloodmeal identifications reported here, and elsewhere.

5.4 Future research

The applied methodologies developed and described here provide new knowledge regarding tick-host interactions and provide the basis for improved epidemiological models. The complex interactions taking place in TBD systems and the global changes

we are continuously facing stimulate new questions for further research. Specifically for the Real-time HRMA bloodmeal analysis protocol presented here, in addition to the technical improvements presented in section 5.2, further investigations are needed to determine whether mixed bloodmeals of species within the same host group are being overlooked (Albonico et al., 2013; McCarthy et al., 2013). As noted by Morán Cadenas et al. (2007) and in the discussion above, further testing should be also done to confirm whether the multiple host DNA is a result of voluntary drop off and secondary questing by the tick (true mixed bloodmeals), involuntary interrupted feeding, or unsuccessful attachment. It would be interesting to test the improved method with a large sample in urban parks tick populations to validate the growing public health concern that dogs play a significant role in increasing tick-human contact rate, and identify possible control strategies to decrease disease risk.

Analysis of the large genomic data produced for *I. ricinus* by RAD-Seq technology are still far from being concluded. For example, past demographic scenarios are currently being analysed as part of another PhD student at the University of Ferrara. As already pointed out, possible bias in the sequencing results may be related to the high presence of repetitive elements in the genome and/or, according to Van Zee et al. (2013), to the high density of SNPs along the genome that could have promoted allele drop-out because of mutation in the restriction site sequence (Gautier et al., 2012), as also pointed out by Quillery et al. (2013). The availability of a reference genome for *I. ricinus* would largely improve the confidence in SNP calling and the investigation of such genomic features that, as discussed above, may provide the genetic variation necessary to allow ticks to successfully interact with such a large variety of vertebrate species. Identification of SNPs in gene coding regions could be performed thanks to the

paired-end RAD-Seq format chosen, which allows the creation of contig sequences, and would permit allocation in a annotated genome and primer design; however, the effectiveness of alignment against annotated genomes of other arthropod species needs to be tested, as alignment against the *I. scapularis* genome has already proven to be unsatisfactory. And, least but not last, the identification of suitable markers for distinguishing male and female ticks, would be very useful for identifying sexes at all life cycle stages, useful for many epidemiological questions, such as sex-biased dispersal and assortative pairing in *I. ricinus*, as reported for microsatellites analysis (De Meeûs et al., 2002; Kempf et al., 2009b; Kempf et al., 2010).

6. CONCLUSIONS

Two molecular methods were optimized here for the first time for *I. ricinus*: bloodmeal analysis in questing nymphs by Real-time HRMA and RAD-Seq NGS for population genetic investigations. These two molecular methods represent innovations in the laboratory practice and analysis of the investigated vector and the results obtained thus far are proof-of-principle of their validity. However, there is still need of technical improvements for the bloodmeal method, and the analysis of the large set of generated RAD-Seq genomic data would benefit from the release of a *I. ricinus* reference genome.

My results from both approaches imply that *I. ricinus* is a medium- to long-distance disperser, mediated by the habit to feed on birds, deer, foxes, dogs and livestock, and it is confirmed by the fact that populations are genetically highly admixed. I also showed for the first time that domestic dogs are important tick hosts in some areas.

This knowledge could be combined with the other biotic and abiotic factors to model TBD incidence and emergence, as well the spread of the vector in new climatic suitable areas. The new bloodmeal analysis protocol could also be applied in other areas to identify important host communities and predict and control TBD dynamics; in fact, I have already had several requests in this regard. The epidemiological relevance of dogs as principal *I. ricinus* larval hosts deserves further specific attention in relation human tick bite risk and to the increasing reported cases of urban infected tick populations.

7. REFERENCES

Albach D.C., Schönswetter P., Tribsch A., 2006, Comparative phylogeography of the Veronica alpina complex in Europe and North America, *Molecular Ecology*, 15: 3269-3286

Albonico F., Loiacono M., Gioia G., Genchi C., Genchi M., Mortarino M., 2014, Rapid differentiation of *Dirofilaria immitis* and *Dirofilaria repens* in canine peripheral blood by real-time PCR coupled to high resolution melting analysis, *Veterinary Parasitology*, 200: 128-132

Alcaide M., Rico C., Ruiz S., Soriguer R., Muñoz J., Figuerola J., 2009, Disentangling vector-borne transmission networks: a universal DNA barcoding method to identify vertebrate hosts from arthropod bloodmeals, *PLoS ONE*, 4: e7092

Allan B.F., Goessling L.S., Storch G.A., Thack R.E., 2010, Blood meal analysis to identify reservoir hosts for *Amblyomma americanum* ticks, *Emerging Infectious Diseases*, 16: 433-440

Baird N.A., Etter P.D., Atwood T.S., Currey M.C., Shiver A.L., Lewis Z.A., Selker E.U., Cresko W.A., Johnson E., 2008, Rapid SNP Discovery and Genetic Mapping Using Sequenced RAD Markers. *PLoS ONE*, 3: e3376

Beaumont M.A., Bruford M., 1999, Microsatellites in conservation genetics. In: Goldstein D. B., Schlötterer (Ed), Microsatellites - Evolution and application, *Oxford University Press*, pp 165-182

Bhide M., Travnicek M., Curlik J., Stefancikova A., 2004, The importance of dogs in eco-epidemiology of Lyme borrelliosis: a review, *Veterinary Medicine*, 4: 135-142

Bolzoni L., Rosà R., Cagnacci F., Rizzoli A., 2012, Effect of deer density on tick infestation of rodents and the hazard of tick-borne encephalitis. II: Population and infection models, *International Journal for Parasitology*, 42: 373-381

Bown K.J., Lambin X., Ogden N.H., Begon M., Telford G., Woldehiwet Z., Birtles R.J., 2009, Delineating *Anaplasma phagocytophilum* ecotypes in coexisting, discrete enzoonotic cycles, Emerging Infectious Diseases, 15: 1948-1954

Bown K.J., Lambin X., Telford G., Heyder-Bruckner D., Ogden N.H., Birtles R.J., 2011, The common shrew (*Sorex araneus*): a neglected host of tick-borne infections? *Vector-Borne Zoonotic Diseases*, 11: 947-953

Brossard M., Wikel, S.K., 2004, Tick immunobiology, *Parasitology*, 129: S1: 61-76

Bunce R.G.H., Pérez-Soba M., Jongman R.H.G., Gómez Sal A., Herzog F., Austad I. (Eds.), 2004, Transhumance and biodiversity in European mountains, Report of the EU-FP5 project TRANSHUMOUNT (EVK2-CT-2002-80017), IALE

Busse P., 2001, European passerine migration system what is known and what is lacking. *Ring*, 23: 3-36

Cagnacci F., Bolzoni L., Rosà R., Carpi G., Hauffe H.C., Valent M., Tagliapietra V., Kazimivora M., Koci J., Stanko M., Lukan M., Henttonen H., Rizzoli A., 2012, Effect of deer density on tick infestation of rodents and the hazard of tick-borne encephalitis. I: Empirical assessment, *International Journal for Parasitology*, 42: 365-372

Cagnacci F., Focardi S., Heurich M., Stache A., Hewison M. A. J., Morellet N., Kjellander P., Linnell J. D. C., Mysterud A., Neteler M., Delucchi L., Ossi F. and Urbano F., 2011, Partial migration in roe deer: migratory and resident tactics are end points of a behavioural gradient determined by ecological factors, *Oikos*, 120: 1790–1802

Canova L., Fasola M., 1991, Communities of small mammals in six biotopes of northern Italy, *Acta Theriologica*, 36: 73-86

Carpi G., Cagnacci F., Neteler M., Rizzoli A., 2008, Tick infestation on roe deer in relation to geographic and remotely sensed climatic variables in a tick-borne encephalitis endemic area, *Epidemiology and Infection*, 136: 1416-1424

Casacuberta E., Gonzalez J., 2013, The impact of transposable elements in environmental adaptation, *Molecular Ecology*, 22: 1503–1517

Casati S., Bernasconi M.V., Gern L., Piffaretti J.-C., 2008, Assessment of intraspecific mtDNA variability of European *Ixodes ricinus* sensu stricto (Acari: Ixodidae), *Genetics and Evolution*, 8: 152-158

Catchen M.J., Amores A., Hohenlohe P., Cresko W., Postlethwait J.H., 2011, Stacks: Building and Genotyping Loci De Novo From Short-Read Sequences, *G3 Genes Genome Genetics*, 1: 171-182

Catchen M.J., Hohenlohe P., Bassham S., Amores A., Cresko W., 2013, Stacks: an analysis tool set for population genomics, *Molecular Ecology*, 22: 3124-3140

Cavallini P., 1996, Variation in the social system of the red fox, *Ethology Ecology and Evolution*, 8: 323-342

Cheng J.-C., Huang C.-L., Lin C.-C., Chen C.-C., Chang Y.-C., Chang S.-S., Tseng C.-P., 2006, Rapid detection and identification of clinically important bacteria by high-resolution melting analysis after broad-range ribosomal RNA real-time PCR, *Clinical Chemistry*, 52: 1997-2004

Collini M., Albonico F., Hauffe H.C., Mortarino M., 2015a (in press), Identifying the last bloodmeal of questing sheep tick nymphs (*Ixodes ricinus* L.) using high resolution melting analysis, *Veterinary Parasitology,* DOI: 10.1016/j.vetpar.2015.04.007

Collini M., Hauffe H.C., Masséglia S., Albonico F., Arnoldi D., Bailly X., Bard E., Galan M., Rossi C., Tagliapietra V., Vourc'h G., Rizzoli A., Mortarino M., 2015b, Identifying the last bloodmeal of questing wood tick nymphs (*Ixodes ricinus* L.) by DNA amplification: three approaches tested, Heraklion, Crete, Greece, "Genes, Ecosystems and Risk of Infections", 21-23rd April 2015

Comstedt P., Bergström S., Olsen B., Garpmo U., Marjavaara L., Mejlon H., Barbour A.G., Bunikis J., 2006, Migratory passerine birds as reservoirs of Lyme Borreliosis in Europe, *Emerging Infectious Diseases*, 12: 1087-1095

Crestanello B., Pecchioli E., Vernesi C., Mona S., Martìnková N., Janiga M., Hauffe H.C., Bertorelle G., 2009, The genetic impact of traslocations and habitat fragmentation in chamois (*Rupicapra*) spp., *Journal of Heredity,* 100: 691-708

Cringoli G., Iori A., Rinaldi L., Veneziano V., Genchi C., 2005, Zecche. Mappe Parassitologiche. Rolando Editore, Napoli

Danecek P., Auton A., Abecasis G., Albers C.A., Banks E., DePristo M.A., Handsaker R.E., Lunter G., Marth G.T., Sherry S.T., McVean G., Durbin R., 1000 Genomes Project Analysis Group, 2011, The variant call format and VCFtools, *Bioinformatics*, 27: 2156–2158

Davey J.W., Blaxter M.L., 2010, RADSeq: next-generation population genetics, *Briefings in Functional Genomics*, 9: 416-423

De la Fuente J., Estrada-Peña A., Venzal J.M., Kocan K.M., Sonenshine D.E., 2008, Overview: ticks as vectors of pathogen that cause disease in humans and animals, *Frontiers in Bioscience*, 13: 6938-6946

De Meeûs T., Beati L., Delaye C., Aeschlimann A., Renaud F., 2002, Sex biased dispersal in the vector of Lyme disease, *Ixodes ricinus*, *Evolution*, 59: 1802-1807

De Meeûs T., Koffi B.B., Barré N., De Garine-Wichatitsky M., Chevillon C., 2010, Swift sympatric adaptation of a species of cattle tick to a new deer host in New Caledonia, *Infection, Genetics and Evolution,* 10: 970–983

De Meeûs T., Lorimier Y., Renaud F., 2004, Lyme borreliosis agents and the genetics and sex of their vector, *Ixodes ricinus*. *Microbes and Infection,* 6: 299-304

De Meeûs T., McCoy K., Prugnolle F., Chevillon C., Durand P., Hurtrez-Boussès R.F., 2007, Population genetics and molecular epidemiology or how to "dèbusquer la bête", *Infection, Genetics and Evolution,* 7: 308-332

Delaye C., Aeschlimann A., Renaud F., Rosenthal B., De Meeûs T., 1998, Isolation and characterization of microsatellite markers in *Ixodes ricinus* complex (Acari: Ixodidae), *Molecular Ecology*, 7: 360-361

Delaye C., Beati L., Aeschlimann A., Renaud F., De Meeûs T., 1997, Population genetic structure of Ixodes ricinus in Switzerland from allozymic data: no evidence of divergence between nearby sites, *International Journal for Parasitology*, 27: 796-778

Dinnis R.E., Seeling F., Bormane A., Donaghy M., Vollmer S.A., Feil E.J., Kurtenbach K., Margos G., 2014, Multilocus sequence typing using mitochondrial genes (mtMLST) reveals geographic population structure of Ixodes ricinus ticks, *Ticks and Tick-borne Diseases*, 5: 152-160

Do H., Krypuy M., Mitchell P.L., Fox S.B., Dobrovic A., 2008, High resolution melting analysis for rapid and sensitive *EGFR* and *KRAS* mutation detection in formalin fixed embedded biopsies. *BMC Cancer*, 8: 142-156

Dubska L., Literak I., Kocianova E., Taragelova V., Sychra O., 2009, Differential role of passerine birds in distribution of *Borrelia* spirochaetes, based on data from ticks collected from birds during the postbreeding migration period in Central Europe, *Applied and Environmental Microbiology*, 75: 596-602

Dumitrache M.O., D'Amico G., Matei I.A., 2014, Ixodid ticks in red foxes (*Vulpes vulpes*) from Romania, *Parasites and Vectors*, 7: 1

Dunlop J.A., Selden P.A., 2009, Calibrating the chelicerate clock: a paleontological reply to Jeyaprakash and Hoy, *Experimental and Applied Acarology*, 48: 183–197

Dwight Z., Palais R., Wittwer C.T., 2011, uMELT: prediction of high-resolution melting curves and dynamic melting profiles of PCR products in a rich web application, *Bioinformatics Applications Note*, 27: 1019-1020

Emerson K.J., Merz J.M., Catchen J.M., Hohenlohe P.A., Cresko W.A., Bradshaw W.E., Holzpfel C.M., 2010, Resolving postglacial phylogeography using high-throughput sequencing, *Proceeding of the National Academy of Science*, 107: 16196-16200

Erali M., Wittwer C.T., 2010, High resolution melting analysis for gene scanning, *Methods*, 50: 250-261

Estrada-Peña A., 2009, Tick-borne pathogens, transmission rates and climate change, *Frontiers in Bioscience*, 14: 2674-2687

Estrada-Peña A., Bouattour A., Camicas J.-L., Walker A.R., 2004. Ticks of domestic animals in the Mediterranean region: a guide to identification of species, University of Zaragoza, Spain

Estrada-Peña A., Gray J.S., Kahl O., Lane R.S., Nijhof A.M., 2013, Research on the ecology of ticks and tick-borne pathogens – methodological principles and caveats, *Frontiers in Cellular and Infection Microbiology*, 3: 29

Estrada-Peña A., Osàcar J.J., Pichon B., Gray J.S., 2005, Host and pathogen detection for immature stages of *Ixodes ricinus* (Acari: Ixodidae) in North-Central Spain, *Experimental and Applied Acarology*, 37: 257-268

Estrada-Peña A., Venzal J.M., Sànchez Acedo C., 2006, The tick *Ixodes ricinus*: distribution and climate preferences in the western Paleartic, *Medical and Veterinary Entomology*, 20: 189-197

Etter P.D., Bassham S., Hohenlohe P.A., Johnson E., Cresko W.A., 2011, SNP Discovery and Genotyping for Evolutionary Genetics Using RAD Sequencing, Virginie Orgogozo and Matthew V. Rockman (eds.), *Molecular Methods for Evolutionary Genetics, Methods in Molecular Biology*, 772, DOI 10.1007/978-1-61779-228-1_9

Fagerberg A.J., Fulton R.E., Black W.C., 2001, Microsatellite loci are not abundant in all arthropod genomes: analyses in the hard tick, *Ixodes scapularis* and the yellow fever mosquito, *Aedes aegypti*, *Insect Molecular Biology*, 10:225:236

Falco R.C., Fish D., 1991, Horizontal movement of adult *Ixodes Dammini* (Acari, Ixodidae) attracted to CO2- baited traps, *Journal of Medical Entomology*, 28: 726-729

Farkas R., Gyurkovszky M., Lukás Z., Aladics B., Solymosi N., 2014, Seroprevalence of some vector-borne infections of dogs in Hungary, *Vector-borne and Zoonotic Diseases*, 14: 256-260

Garros C., Gardès L., Allène X., Rakotoarivony I., Viennet E., Rossi S., Balenghien T., 2011, Adaptation of a species-specific multiplex PCR assay for the identification of blood meal source in *Culicoides* (Ceratopogonidae:Diptera): applications on Palaearctic biting midge species, vectors of Orbiviruses, *Infection, Genetics and Evolution*, 11: 1103-1110

Gautier M., Gharbi K., Cezard T., Foucaud J., Kerdelhuè C., Pudlo P., Cornuet J.-M., Estoup A., 2012, The effect of RAD allele drop-out on the estimation of genetic variation within and between populations, *Molecular Ecology*, 22: 3165–3178

Geraci N.S., Johnston J.S., Robinson J.P., Wikel S.K., Hill C.A., 2007, Variation in genome size of argasid and ixodid ticks, *Insect Biochemistry and Molecular Biology*, 37: 399-408

Gilbert L., 2010, Altitudinal patterns of tick and host abundance: a potential role for climate change in regulating tick-borne diseases?, *Oecologia*, 162: 217–225

Gómez-Díaz E., Figuerola J., 2010, New perspectives in tracing vector-borne interaction networks, *Trends in Parasitology*, 26: 470-476

Gori A., Cerboneschi M., Tegli S., 2012, High-resolution melting analysis as a powerful tool to discriminate and genotype *Pseudomonas savastanoi* pathovars and strains, *PLOSone*, 7: e30199

Gray J.S., 1998, The ecology of ticks transmitting Lyme borreliosis, *Experimental & Applied Acarology*, 22:249-258

Gray J.S., Dautel H., Estrada-Peña A., Kahl O., Lindgren E., 2009, Effects of climate change on ticks and tick-borne diseases in Europe, *Interdisciplinary Perspective on Infectious Diseases*, 2009: 593232

Gray J.S., Kirstein F., Robertson J.N., Stein J., Kahl O., 1999, *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks and rodents in a recreational park in south-western Irelend, *Experimental and Applied Acarology*, 23: 717-729

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

Gundry C.M., Vandersteen J.G., Reed G.H., Pryor R.J., Chen J., Wittwer C.T., 2003, Amplicon melting analysis with labelled primers: a closed-tube method for differentiating homozygotes and heterozygotes, *Clinical Chemistry*, 49: 396-406

Halos L., Jamal T., Vial L., Maillard R., Suau A., Le Menach A., Boulouis H.-J. and Vayssier-Taussat M., 2004, Determination of an efficient and reliable method for DNA extraction from ticks, *Veterinary Research*, 35: 709-713

Hasle G., Bjune G.A., Midthjell L., Røed K.H., Leinaas H.P., 2011, Transport of *Ixodes ricinus* infected with *Borrelia* species to Norway by northward-migrating passerine birds, *Ticks and Tick-borne Diseases*, 2: 37-43

Haubrich K., Schmitt T., 2007, Cryptic differentiation in alpine-endemic, high-altitude butterflies reveals down-slope glacial refugia, *Molecular Ecology*, 16: 3643-3658

Helyar S.J., Hemmer-Hansen J., Bekkevold D., Taylor M.I., Ogden R., Limborg M.T., Cariani A., Maes G.E., Diopere E., Carvalho G.R., Nielsen E.E., 2011, Application of SNPs for population genetics of nonmodel organisms: new opportunities and challenges, *Molecular Ecology Resources*, 11: 123-136

Hewitt G., 2000, The genetic legacy of the Quaternary ice ages, *Nature*, 405: 907-913

Hewitt G.M., 1999, Post-glacial re-colonization of European biota, *Biological Journal of the Linnean Society*, 68: 87-112

Heyman P., Cochez C., Hofhuis A., Van der Giessen J., Sprong H., Porter S.R., Losson B., Saegerman C., Donoso-Mantke O., Niedrig M., Papa A., 2010, A clear and present danger: tick-borne diseases in Europe, *Expert Review of Anti-infective Therapy*, 8:33-50

Hill C.A., Gutierrez J.A., 2003, A method for extraction and analysis of high quality genomic DNA from ixodid ticks, *Medical and Veterinary Entomology*, 17: 224-227

Hohenlohe P.A., Bassham S., Etter P.D., Stiffler N., Johnson E.A., Cresko W.A., 2010, Population Genomics of Parallel Adaptation in Threespine Stickleback using Sequenced RAD Tags. *PLoS Genetic*, 6: e1000862

Hoogstraal H., Aeschlimann A., 1982, Tick-Host Specificity, *Bulletin de la Société Entomologique Suisse*, 55: 5-32

Hubbard M.J., Cann K.J., Wright D.J.M, 1995, Validation and rapid extraction of nucleic acid from alcohol-preserved ticks, *Experimental & Applied Acarology*, 19: 473-478

Hudson P.J., Rizzoli A., Rosà R., Chemini C., Jones L.D., Gould E.A., 2001, Tick-borne encephalitis virus in northern Italy: molecular analysis, relationships with density and seasonal dynamics of *Ixodes ricinus*, *Medical and Veterinary Entomology*, 15: 304-313

Humair P.F., Douet V., Cadenas F.M., Schouls L.M., Van De Pol I. and Gern, L., 2007, Molecular identification of bloodmeal source in *Ixodes ricinus* ticks using 12S rDNA as a genetic marker, *Journal of Medical Entomology*, 44: 869-880

Humair P.F., Turrian N., Aeschlimann A., Gern L., 1993, *Ixodes ricinus* immatures on birds in a focus of Lyme borrelliosis, *Folia Parasitologica*, 40: 237-242

Jeyaprakash A., Hoy M., 2009, First divergence time estimates of spiders, scorpions, mites and ticks (subphylum: Chelicerata) inferred from mitochondrial phylogeny, *Experimental and Applied Acarology*, 47: 1-18

Johnson P.T., Thieltges D.W., 2013, Diversity, decoys and the dilution effect: how ecological communities affect disease risk, *Journal of Experimental Biology*, 213: 961-70

Kang D., Sim C., 2013, Identification of *Culex* complex species using SNP markers based on high-resolution melting analysis, *Molecular Ecology Resources*, 13: 369-376

Kempf F., Boulinier T., De Meeûs T., Arnathau C., McCoy K. D., 2009a, Recent evolution of host associated divergence in the seabird tick *Ixodes uriae*, *Molecular Ecology*, 18: 4450-4462

Kempf F., De Meeûs T., Arnsthau C., Degeilh B., McCoy K., 2009b, Assortative pairing in *Ixodes ricinus* (Acari: Ixodidae), the European vector of Lyme Borreliosis, *Journal of Medical Entomology*, 46: 471-474

Kempf F., De Meeûs T., McCoy K., 2010, Wahlund effects and sex-biased dispersal in *Ixodes ricinus*, the European vector of Lyme borreliosis: new tools for hold data, *Infection, Genetics and Evolution*, 10: 989-997

Kempf F., De Meeûs T., Vaumourin E., Noel V., Taragel'ovà V., Plantard O., Heylen D., Eraud C., Chevillon C., McCoy K., 2011, Host races in *Ixodes ricinus*, the European vector of Lyme borreliosis, *Infection, Genetics and Evolution*, 11: 2043-2048

Kent R.J., 2009, Molecular methods for arthropod bloodmeal identification and applications to ecological and vector-borne disease studies, *Molecular Ecology Resources*, 9: 4-18

Kirstein F., Gray J.S., 1996, A molecular marker for the identification of the zoonotic reservoirs of Lyme borreliosis by analysis of the blood meal in its European vector *Ixodes ricinus*, *Applied and Environmental Microbiology*, 62: 4060-4065

Kocher T.D., Thomas W.K., Meyer A., Edwards S.V., Pääbo S., Villablanca F.X., 1989, Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers, *Proceding of the National Academy of Science USA*, 86: 6196-6200

Kolb H.H., 1984, Factors affecting the movements of dog foxes in Edinburgh, *Journal of Applied Ecology*, 21: 161-173

Korneliussen T.S., Albrechtsen A., Nielsen R., 2014, ANGSD: Analysis of Next Generation Sequencing Data, *BMC Bioinformatics*, 15: 356

Krasnov B.R., Stanko M., Morand S., 2007, Host community structure and infestation by Ixodid ticks: repeatability, diluition effect and ecological specialization, *Oecologia*, 154: 185-194

Kurtenbach K., De Michelis S., Sewel H.-S., Etti S., Schäfer S.M., Hails R., Collares-Pereira M., Santos-Reis M., Haninçova K., Labuda M., Bormane A., Donaghy M., 2001, Distinct combinations of *Borrelia burgdorferi* sensu lato genospecies found in individual questing ticks from Europe, *Applied and Environmental Microbiology*, 67: 4926-4929

Lao O., van Duijn K., Kersbergen P., de Knijff P., Kayser M., 2006, Proportioning whole-genome single-nucleotide–polymorphism diversity for thei of geographic population structure and genetic ancestry, *The American Journal of Human Genetics*, 78: 680-690

Laskay U., Burg J., Kaleta E.J., Vilcins I.-M. E., Telford III S.R., Barbour A.G., Wysocki V.H., 2012, Development of a host blood meal database: de novo sequencing of hemoglobin from nine small mammals using mass spectrometry, *Biological Chemistry*, 393: 195-201

Léger E., Vourc'h G., Vial L., Chevillon C., McCoy K.D., 2013, Changing distributions of ticks: causes and consequences, *Experimental and Applied Acarology*, 59: 219-244

Leutenegger C.M., Pusterla N., Mislin C.N., Weber R., Lutz H., 1999, Molecular evidence of coinfection of ticks with *Borrelia burgdorferi* sensu lato and the human granulocytic ehrlichiosis agent in Switzerland, *Journal of Clinical Microbiology*, 37: 3390–3391

Li H., Handsaker B., Wysoker A., Fennell T., Ruan J., Homer N., Marth G., Abecasis G., Durbin R.,1000 Genome Project Data Processing Subgroup, 2009, The Sequence Alignment/Map format and SAMtools, *Bioinformatics*, 25: 2078-2079

Lin J.-H., Tseng C-P., Chen Y.-J., Lin C-Y., Chang S.-S., Wu H.-S., Cheng J.-C., 2008, Rapid differentiation of Influenza A Virus subtypes and genetic screening for virus variants by High-Resolution Melting Analysis, *Journal of Clinical Microbiology*, 46: 1090-1097

Lindgren E., Jaenson T.G.T., 2006, Lyme borreliosis in Europe: influences of climate and climate change, epidemiology, ecology and adaptations measures, Copenhagen, Denmark: World Health Organization Regional Office for Europe

Lischer H.E.L., Excoffier L., 2012, PGDSpider: an automated data conversion tool for connecting population genetics and genomics programs, *Bioinformatics*, 28: 298-299

LoGiudice K., Ostfeld R.S., Schmidt K.A., Keesing F., 2003, The ecology of infectious disease: effects of biodiversity and community composition on Lyme disease risk, *Proceeding of the National Academy of Science*, 100: 567-571

Lommano E., Dvořák C., Vallotton L., Jenni L., Gern L., 2014, Tick-borne pathogens in ticks collected from breeding and migratory birds in Switzerland, *Ticks and Tick-borne Diseases*, 5: 871-882

López-Olvera J.R., Vidal D., Vicente J., Pérez M., Luján L., Gortázar C., 2009, Serological survey of selected infectious diseases in mouflon (*Ovis aries musimon*) from south-central Spain, *European Journal of Wildlife Research*, 55: 75-79

Lorenzini R., Cabras P., Fanelli R., Carboni G.L., 2011, Wildlife molecular forensics: Identification of the Sardinian mouflon using STR profiling and the Bayesian assignment test, *Forensic Science International: Genetics*, 5: 345-349

Lunter G., Goodson M., 2010, Stampy: A statistical algorithm for sensitive and fast mapping of Illumina sequence reads, *Genome Resources*, 21: 936-939

Mackenstedt U., Jenkins D., Romig T., 2015, The role of wildlife in the transmission of parasitic zoonoses in peri-urban and urban areas, *International Journal for Parasitology: Parasites and Wildlife*, 4: 71-9

Madhav N.K., Brownstein J.S., Tsao J.I., Fish D., 2004, A dispersal model for the range expansion of the blacklegged tick (Acari: Ixodidae), *Journal of Medical Entomology*, 41: 842-852

Magalhães S., Forbes M.R., Skoracka A., Osakabe M., Chevillon C., McCoy K.D. 2007, Host race formation in the Acari. *Experimental and Applied Acarology*, 42: 225-238

Mans B.J., de Klerk D., Pienaar R., Latif A., 2011, Nutalliella Namaqua: a living fossil and closest relative to the ancestral tick lineage: implications for the evolution of blood-feeding in ticks, *PLoS ONE*, 6: e23675

Mantelli B., Pecchioli E., Hauffe H.C., Rosà R., Rizzoli A., 2006, Prevalence of *Borrelia burgdorferi* s.l. and *Anaplasma phagocytophilum* in the wood tick Ixodes ricinus in Trentino, Italy, *European Journal of Clinical Microbiology and Infectious Diseases*, 25: 737-739

Mantke O.D., Escadafal C., Niedrig M., Pfeffer M., on behalf of the Working group foe Tick-borne encephalitis virus, 2011, Tick-borne encephalitis in Europe, 2007 to 2009, *Euro Survelliance*, 16: 19976

Marsot M., Henry P.-Y., Vourc'h G., Gasqui P., Ferquel E., Laignel J., Grysan M., Cahpuis J.L., 2012, Which forest bird species are the main hosts of the tick, *Ixodes ricinus*, the vector of *Borrelia burgdorferi* sensu lato, during the breeding season? *International Journal for Parasitology*, 42: 781-788

Mastretta-Yanes A., Zamudio S., Jorgensen T.H., Arrigo N., Alvarez N., Piñero D., Emerson B.C., 2014, Gene duplication, population genomics, and species-level differentiation within a tropical mountain shrub, *Genome, Biology and Evolution*, 6: 2611-2624

McCarthy J.K., Didham R.K., Brockerhoff E.G., Van Bysterveldt K.A., Varsani A., 2013, High-resolution DNA melt-curve analysis for cost-effective mass screening of pairwise species interactions, *Molecular Ecology Resources*, 13: 908-917

McCoy K.D., Boulinier T., Tirard C., Michalakis Y., 2003, Host-dependent genetic structure of parasite populations: differential dispersal of sea-bird tick host races, *Evolution*, 57: 288-296

McCoy K.D., Lèger E., Dietrich M., 2013, Host specialization in ticks and transmission of tick-borne diseases: a review, *Frontiers in Cellular and Infection Microbiology*, 3: 57

McCoy K.D., Tirard C., 2000, Isolation and characterization of microsatellites in the seabird ectoparasite *Ixodes uriae*, *Molecular Ecology*, 9: 2212-2213

McKenna A., Hanna M., Banks E., Sivachenko A., Cibulskis K., Kernytsky A., Garimella K., Altshuler D., Gabriel S., Daly M., DePristo M.A., 2010, The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data, *Genome Research*, 20: 1297–1303

Medlock J.M., Hansford K.M., Bormane A., Derdakova M., Estrada-Peña A., George J.-C., Golovljova I., Jaenson T.G.T., Jensen J.-K., Jensen P.M., Kazimivora M., Oteo J.A., Papa A., Pfister K., Plantard O., Randolph S.E., Rizzoli A., Santos-Silva M.M., Sprong H., Vial L., Hendrickx G., Zeller H., Van Bortel W., 2013, Driving forces for changes in geographical distribution of *Ixodes ricinus* ticks in Europe, *Parasites and Vectors*, 6: 1

Mejlon H.A., Jaenson T.G.T, 1997, Questing behavior of *Ixodes ricinus* ticks (Acari: Ixodidae), *Experimental and Applied Acarology*, 21: 747-754

Meyer J.M., Kurtti T.J., VanZee J.P., Hill C.A., 2010, Genome organization of major tandem repeats in the hard tick, *Ixodes scapularis*, *Chromosome Research*, 18: 357–370

Meyer-Kayser E., Hoffman L., Silaghi C., Pfister K., Mahling M., Passos L.M., 2012, Dynamics of tick infestations in foxes in Thuringia, Germany, *Ticks and Tick-borne Diseases*, 3: 232-239

Michaux J.R, Kinet S., Filippucci M.-G., Libois R., Besnard A., Catzeflis F., 2001, Molecular identification of three sympatric species of wood mice (*Apodemus sylvaticus*, *A. flavicollis*, *A. alpicola*) in western Europe (Muridae: Rodentia), *Molecular Ecology Notes*, 1: 260-263

Miller M.R., Duham J.P., Amores A., Cresko W.A., Johnson E.A., 2007, Rapid and cost-effective polymorphism identification and genotyping using restriction site associated DNA (RAD) markers, *Genome Resources*, 17: 240-248

Mitchell P.D., Reed K.D., Hofkes J.M., 1996, Immunoserologic evidence of coinfection with *Borrelia burgdorferi*, *Babesia microti*, and human granulocytic *Ehrlichia* species in residents of Wisconsin and Minnesota, *Journal of Clinical Microbiology*, 34: 724-727

Morán Cadenas F., Rais O., Humair P.F., Douet V., Moret J., Gern L., 2007, Identification of host bloodmeal source and *Borrelia burgdoferi* sensu lato in field-collected *Ixodes ricinus* ticks in Chaumont (Switzerland), *Journal of Medical Entomology*, 44: 1109-1117

Mukabana W.R., Takken W., Knols B.G.J., 2002, Analysis of arthropod bloodmeals using molecular genetic markers, *TRENDS in Parasitology*, 18: 505-509

Nadelman R.B., Horowitz H.W., Hsieh T.C., Wu J.M., Agueri-Rosenfeld M.E., Schwartz I., Nowakowski J., Varde S., Wormser G.P., 1997, Simultaneous human granulocytic ehrlichiosis and Lyme borrelliosis, *The New England Journal of Medicine*, 337: 27-30

Nava S., Guglielmone A.A., Mangold A.J., 2009, An overview of systematics and evolution of ticks, *Frontiers in Bioscience*, 14: 2857-2877

Nene V., 2009, Tick genomics - coming of age, *Frontiers in Bioscience*, 14: 2666–2673

Noureddine R., Chauvin A., Plantard O., 2011, Lack of genetic structure among Eurasian population of the tick *Ixodes ricinus* contrast with marked divergence from north-African populations, *International Journal for Parasitology*, 41:183-192

Önder Ö., Shao W., Kemps B.D., Lam H., Brisson, 2013, Identifying sources of tick blood meals using unidentified tandem mass spectral libraries, *Nature Communications*, 4: 1746

Ostfeld R., Keesing F., 2000, The function of biodiversity in the ecology of vector-borne zoonotic diseases, *Canadian Journal of Zoology*, 78: 2061–2078

Pääbo S., Poinar H., Serre D., Jaenicke-Després V., Hebler J., Rohland N., Kuch M., Krause J., Vigilant L., Hofreiter M., 2004, Genetic analyses from ancient DNA, *Annual Review of Genetics*, 38: 645-679

Parola P., Raoult D., 2001, Ticks and tickborne bacterial diseases in humans: an emerging infectious threat, *Clinical Infectious Diseases*, 32:897-928

Paschou P., Ziv E., Burchard E.G., Choudhry S., Rodriguez-Cintron W., Mahoney M.W., Drineas P., 2007, PCA-correlated SNPs for structure identification in worldwide human populations, *PLoS Genetics*, 3: e160

Patterson N., Reich D., 2006, Population structure and Eigenanalysis, *PLoS Genetics*, 2: e190

Pecchioli E., Hauffe H.C., Tagliapietra V., Bandi C., Chemini C., Genchi C., Rizzoli A., 2007, *Borrelia* genospecies isolated from the tick *Ixodes ricinus* in the Autonomous Province of Trento, Italy, *International Journal of Medical Microbiology*, 297: 53-59

Pecchioli E., Vernesi C., Crestanello B., Davoli F., Caramelli D., Bertorelle G., Hauffe H.C., 2006, Progetto FAUNAGEN: Conservazione e gestione della fauna: un approccio genetico. Report n. 35 of the Centro di Ecologia Alpina. 86 pp.

Peña V.H., Fernàndez G.J., Gòmez-Palacio A.M., Mejìa-Jaramillo A.M., Cantillo O., 2012, High-Resolution Melting (HRM) of the Cytochrome B Gene: A Powerful Approach to Identify Blood-Meal Sources in Chagas Disease Vectors, *PLoS Neglected Tropical Diseases*, 6: e1530

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

Pfeffer M., Dobler G., 2011, Tick-borne encephalitis virus in dogs – is this an issue? *Parasites and Vectors*, 4: 59

Pichon B., Egan D., Rogers M., Gray J., 2003, Detection and identification of pathogens and host DNA in unfed host-seeking *Ixodes ricinus* L. (Acari: Ixodidae), *Journal of Medical Entomology*, 40: 723-731

Pichon B., Kahl O., Hammer B., Gray J.S., 2006, Pathogens and host DNA in *Ixodes ricinus* nymphal ticks from a German forest, *Vector-Borne and Zoonotic Diseases*, 6: 382-387

Pichon B., Rogers M., Egan D., Gray J., 2005, Blood-meal analysis for the identification of reservoir hosts of tick-borne pathogens in Ireland, *Vector-Borne and Zoonotic Diseases*, 5: 172-180

Porretta D., Mastrantonio V., Mona S., Epis S., Montagna M., Sassera D., Bandi C., Urbanelli S., 2013, The integration of multiple independent data reveals an unusual response to Pleistocene climatic changes in the hard tick *Ixodes ricinus*, *Molecular Ecology*, 22: 1666-1682

Quillery E., Quenez O., Peterlongo P., Plantard O., 2013, Development of genomic resources for the tick *Ixodes ricinus*: isolation and characterization of single nucleotide polymorphisms, *Molecular Ecology Resources*, 14: 393-400

Randolph S.E., 2004, Tick ecology: processes and patterns behind the epidemiological risk posed by ixodid ticks as vectors, *Parasitology*, 129:S37-S65

Randolph S.E., 2008, Tick-borne disease systems, *Scientific and Technical Review of the Office International des Epizooties*, 27: 1-15

Randolph S.E., 2009, Tick-borne disease systems emerge from the shadows: the beauty lies in molecular detail, the message in epidemiology, *Parasitology*, 136: 1403-1413

Randolph S.E., Gern L., Nuttall P.A., 1997, Co-feeding ticks: epidemiological significance for tick-borne pathogen transmission, *Parasitology Today*, 12: 472-479

Randolph S.E., Green R.M., Hoodledd A.N., Peacey M.F., 2002, An empirical quantitative framework for the seasonal population dynamics of the tick *Ixodes ricinus*, *International Journal for Parasitol*ogy, 32: 979-989

Rasgon J.L., Stable isotope analysis can potentially identify completely-digested bloodmeals in mosquitoes, *PLoS ONE,* 3: e2198

Reed G.H., Kent J.O., Wittwer C.T., 2007, High-resolution DNA melting analysis for simple and efficient molecular diagnostics, *Pharmacogenomics*, 8: 597-608

Reis C., Cote M., Paul R.E.L., Bonnet S., 2011, Questing ticks in suburban forest are infected by at least six tick-borne pathogens, *Vector-Borne and Zoonotic Diseases*, 11: 907-916

Reitzel A.M., Herrera S., Layden M.J., Martindale M.Q., Shank T.M., 2013, Going where traditional markers have not gone before: utility of and promise for RAD sequencing in marine invertebrate phylogeography and population genomics, *Molecular Ecology*, 22: 2953-2970

Ririe K.M., Rasmussen R.P., Wittwer C.T., 1997, Product differentiation by analysis of DNA melting curves during the polymerase Chain reaction, *Analytical Biochemistry*, 245: 154-160

Rizzoli A., Hauffe H.C., Carpi G., Vourc'h G.I., Neteler M., Rosà R., 2011, Lyme borreliosis in Europe, *Euro Surveillance,* 16: e19906

Rizzoli A., Heidi C.H., Tagliapietra V., Neteler M., Rosà R., 2009, Forest structure and roe deer abundance predict tick-borne encephalitis risk in Italy, *PLoS ONE,* 4: e4336

Rizzoli A., Neteler M., Rosà R., Versini W., Cristofolini A., Bregoli M., Buckley A., Gould E.A., 2007, Early detection of tick-borne encephalitis virus spatial distribution and activity in the province of Trento, northern Italy, *Geospatial Health*, 2: 169-176

Rizzoli A., Rosà R., Mantelli B., Pecchioli E., Hauffe H., Tagliapietra V., Beninati T., Neteler M. and Genchi C., 2004, *Ixodes ricinus*, malattie trasmesse e reservoirs, *Parassitologia*, 46: 119-122

Rizzoli A., Silaghi C., Obiegala A, Rudolf I., Hubálek Z., Földvári G., Plantard O., Vayssier-Taussat M., Bonnet S., Špitalská E. and Kazimírová M., 2014, *Ixodes ricinus* and its transmitted pathogens in urban and peri-urban areas in Europe: new hazards and relevance for public health, *Frontiers in Public Health*, 2: 251

Røed K.H., Hasle G., Midthjell V., Skretting G., Leinaas H.P., 2006, Identification of 17 microsatellite primer for the tick, in *Ixodes ricinus*, using enriched genomic libraries, *Molecular Ecology Notes*, 6: 1165-1167

Rosà R., Pugliese A., 2007, Effects of tick population dynamics and host densities an the persistence of tick-borne infections, *Mathematical Biosciences*, 208: 216-240

Rowe H.C., Renaut S., Guggisberg A., 2011, RAD in the realm of next-generation sequencing technologies, *Molecular Ecology*, 20: 3499-3502

SantaLucia J.Jr., 1998, A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics, *Proceedings of the National Academy of Sciences USA*, 95: 1460-1465

Schlötterer C., 2004, The evolution of molecular markers- just a matter of fashion?, Nature Reviews Genetics, 5: 63-69

Schmidt K.A., Ostfeld R., 2001, Biodiversity and the dilution effect in disease ecology, *Ecology*, 82: 609-619

Schönswetter P., Tribsch A., Barfuss M., Niklfeld H., 2002, Several Pleistocene refugia detected in the high alpine plant *Phyteuma globulariifolium* Sternb. & Hoppe (Campanulaceaea) in the European Alps. *Molecular Ecology*, 11: 2637-2647

Seeb J.E., Carvalho G., Hauser L., Naish K., Roberts S., Seeb L.W., 2011, Single-nucleotide poluìymorphism (SNP) discovery and applications of SNPs genotyping in nonmodel organisms, *Molecular Ecology Resources*, 11: 1-8

Seipp M.T., Pattison D., Durtschi J.D., Jama M., Voelkeerdin K.V., Wittwer C., 2008, Quadruplex genotyping of *F5*, *F2*, and *MTHFR* variants in a single closed tube by high-resolution amplicon melting, *Clinical Chemistry*, 54: 108-115

Semenza J.C., Menne B., 2009, Climate change and infectious diseases in Europe, *Lancet Infectious Diseases*, 9: 365-375

Sojka D., Franta Z., Horn M., Caffrey C.R., Mareš M., Kopáček P., 2013, New insight into the machinery of blood digestion by ticks, *Trends in Parasitology*, 29: 276-285

Sonenshine, D.E., 1991. Biology of Ticks, volume 1. Oxford University Press, Inc., New York.

Sonenshine, D.E., 1993, Biology of Ticks. Oxford: Oxford University Press.

Spina F., Volponi S., 2008, Atlante della Migrazione degli Uccelli in Italia. 2. Passeriformi. Ministero dell'Ambiente e della Tutela del Territorio e del Mare, Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA). Tipografia SCR-Roma

Spinozzi F., Battisti C., Bologna M.A., 2012, Habitat fragmentation sensitivity in mammals: a target selection for landscape planning comparing two different approaches (bibliographic review and expert based), *Rendiconti Lincei: Scienze fisiche e Naturali*, 23: 365-373

Sunter J.D., Patel S.P., Skilton R.A., Githaka N., Knowles D.P., Scoles G.A., Vishvanath N., de Villiers E., Bishop R.P., 2008, A novel SINE family occurs frequently in both genomic DNA and transcribed sequences in ixodid ticks of the arthropod subphylum Chelicerata, *Gene*, 415: 13-22.

Swanson S.J., Neitzel D., Reed K.D., Belongia E.A., 2006, Coinfections acquired from *Ixodes* ticks, *Clinical Microbiology Reviews*, 19: 708-727

Taberlet P., Fumagalli L., Wust-Saucy A.-G., Cosson J.-F., 1998, Comparative phylogeography and postglacial colonization routes in Europe, *Molecular Ecology*, 7: 453-464

Tagliapietra V., Rosà R., Arnoldi D., Cagnacci F., Capelli G., Montarsi F., Hauffe H.C., Rizzoli A., 2011, Saturation deficit and deer density affect questing activity and local abundance of *Ixodes ricinus* (Acari, Ixodidae) in Italy, *Veterinary Parasitology*, 183: 114-124

Tälleklint L., Jaenson T.G.T., 1994, Transmission of *Borrelia burgdorferi* s.l. from mammal reservoirs to the primary vector of Lyme borreliosis, *Ixodes ricinus* (Acari: Ixodidae), in Sweden, *Journal of Medical Entomology*, 31: 880–886.

Thiel-Egenter C., 2007, Inter- and Intraspecific Differentiation and Genetic Diversity in Alpine Plants: When Phylogeography Meets Biogeography, PhD Thesis. University of Zurich, Zurich, Switzerland

Thiel-Egenter C., Holderegger R., Brodbeck S., IntraBioDiv-Consortium, Gugerli F., 2009, Concordant genetic breaks, identified by combining clustering tessellation methods, in two co-distributed alpine plant species. *Molecular Ecology*, 18: 4495-4507

Trotta M., Nicetto M., Fogliazza A., Montarsi F., Caldin M., Furlanello T., Solano-Gallego L., 2012, Detection of *Leishmania infantum*, *Babesia canis*, and rickettsiae in ticks removed from dogs living in Italy. *Ticks and Tick-Borne Diseases*, 3:294–297

Van Zee J., Black IV W.C., Levin M., Goddard J., Smith J, Piesman J., 2013, High SNP density in the blacklegged tick, *Ixodes scapularis*, the principal vector of Lyme disease spirochetes, *Ticks and Tick-borne Diseases*, 4: 63-71

Vor T., Kiffner C., Hagedorn P., Niedrig M., Rühe F., 2010, Tick burden on European roe deer (*Capreolus capreolus*), *Experimental and Applied Acarology*, 51: 405-417

Waldenström J., Lundkvist A., Falk K.I., Bergström S., Lindgren G., Sjösted A., Mejlon H., Fransson T., Haeming P.D., Olsen B., 2007, Migrating birds and tickborne encephalitis virus, *Emerging Infectious Diseases*, 13: 1215-1218

Wickramasekara S., Bunikis J., Wysocki V., Barbout A.G., 2008, Identification of residual blood proteins in ticks by mass spectrometry proteomics, Emerging Infectious Diseases, 14: 1273-1275

Winder L., Phillips C., Richards N., Ochoa-Corona F., Hardwick S., Vink C.J., Goldson S., 2011. Evaluation of DNA melting analysis as a tool for species identification. *Methods in Ecology and Evolution*, 2: 312-320

Wittwer C.T., 2009, High-Resolution DNA Melting Analysis: advancements and limitations, *Human Mutation*, 30: 857–859

Wittwer C.T., Reed G.H., Gundry C.N., Vandersteen J.G., Pryor R.J., 2003, High-resolution genotyping by amplicon melting analysis using LCGreen, *Clinical Chemistry*, 49: 853–860

Wodecka B., Rymaszewska A., Skotarczak B., 2014, Host and pathogen identification in blood meals of nymphal *Ixodes ricinus* ticks from forest parks and rural forest of Poland, *Experimental and Applied Acarology*, 62: 543-555

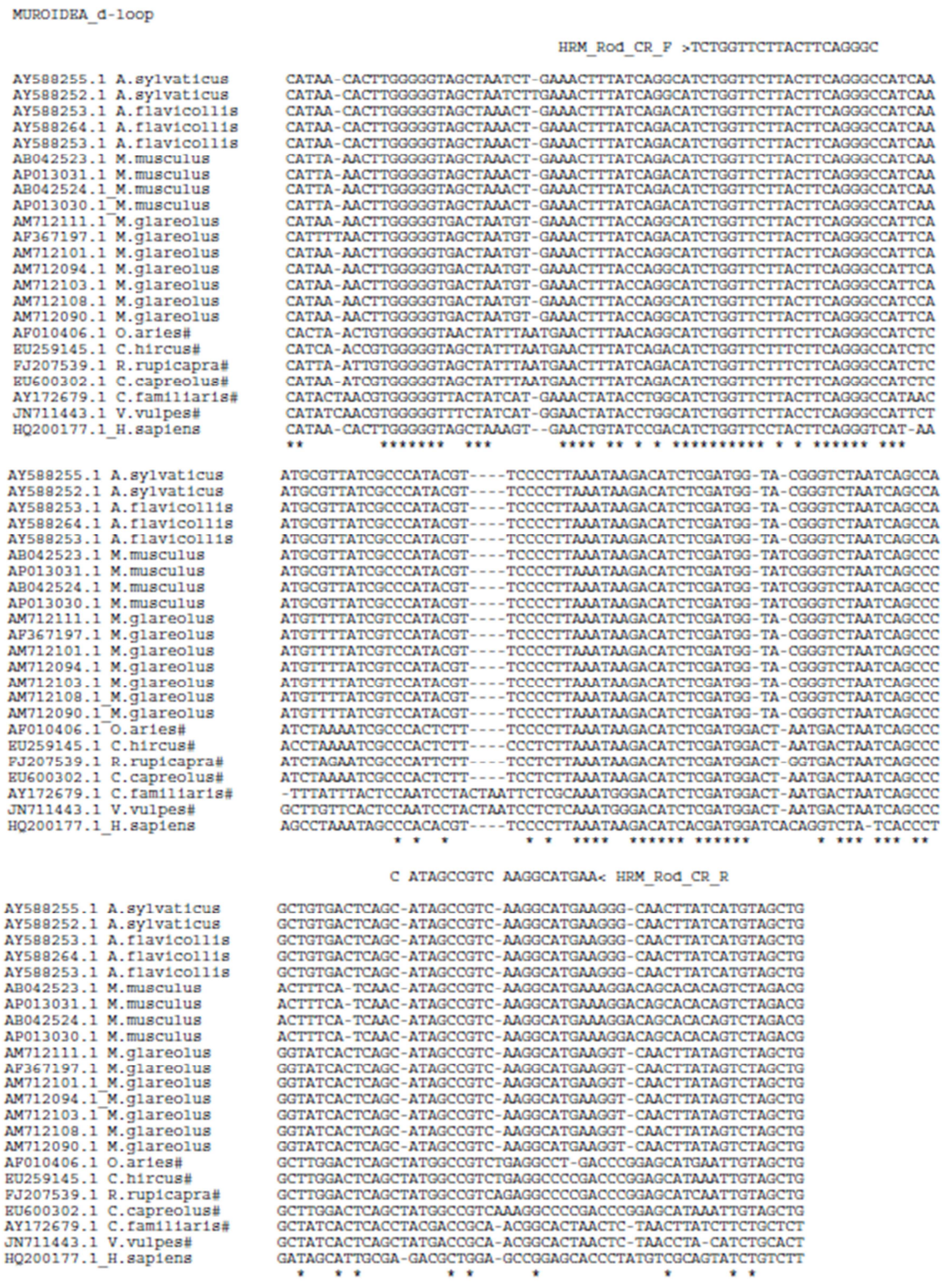
Xu G., Fang Q.Q., Keirans J.E., Durden L.A., 2003, Molecular phylogenetics analyses indicate that the *Ixodes ricinus* complex is a paraphyletic group, *Journal of Parasitology*, 89: 452-447

Xu P., Xu S., Wu X., Tao Y., Wang B., Qin D., Lu Z., Li G., 2014, Population genomic analyses from low-coverage RAD-Seq data: a case study on the non-model cucurbit bottle gourd, *The Plant Journal*, 77: 430-442

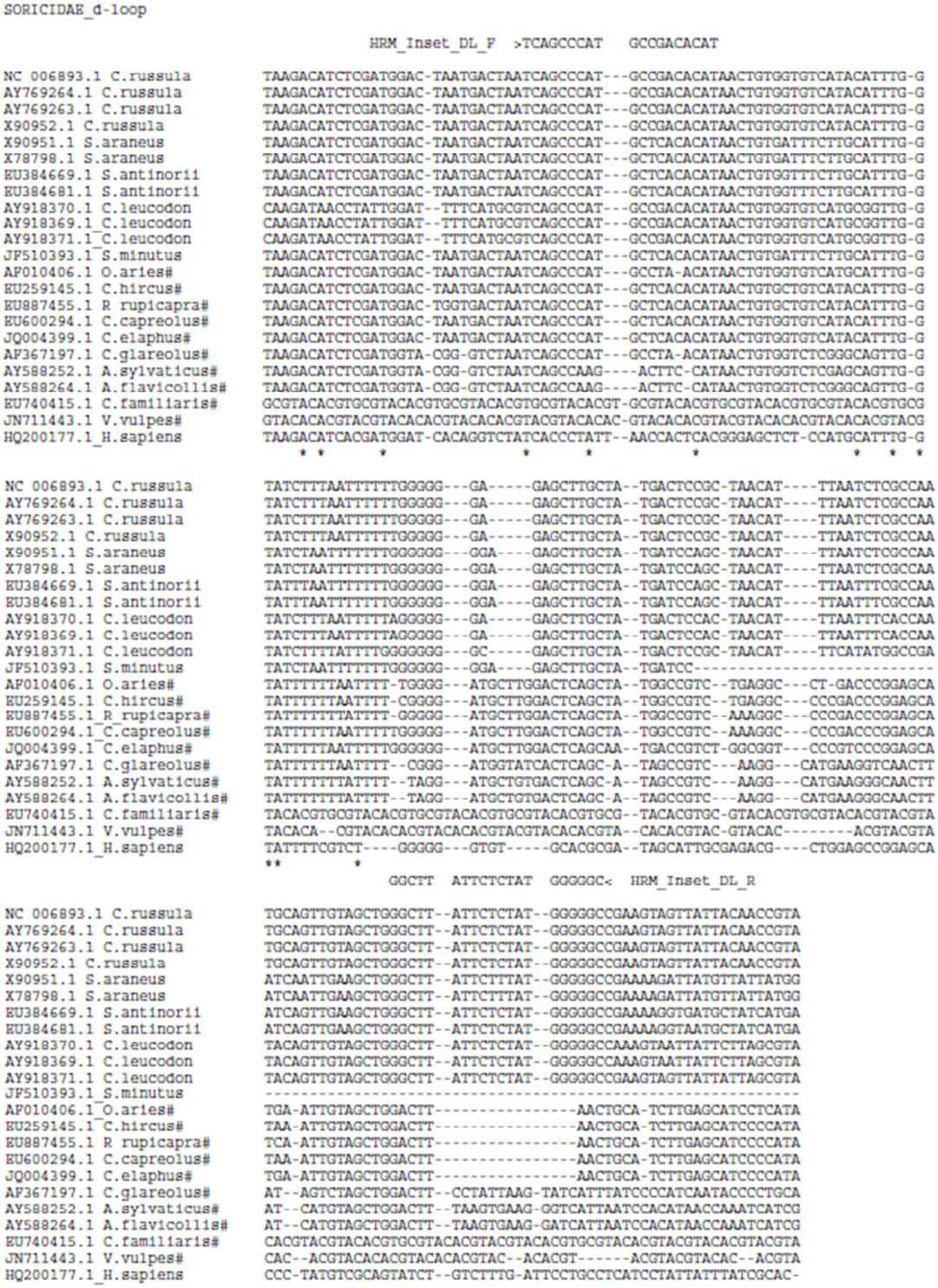
Yannic G., Pellissier L., Dubey S., Vega R., Basset P., Mazzotti S., Pecchioli E., Vernesi C., Hauffe H.C., Searle J.B., Hausser J., 2012, Multiple refugia and barriers explain the phylogeography of the Valais shrew, *Sorex antinorii*, *Biological Journal of the Linnean Society*, 105: 864-880

8. APPENDIX

Appendix 1 Part of the host group alignments of previously published GenBank mtDNA sequences created using Clustal X v. 2.0. Details regarding primer positioning.



SORICIDAE_d-loop



PASSERIPORMES_12S

HRM_Pass_12SF2 >ATCCACGATATTACCTGACCATT

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KJ676688 E.rubecula           ACCCACCTAGAGGAGCCTGTTCTGTAATCGATGATCCACGATATTACCTGACCATTTCTTGCCCAAAACAGCC
KJ676686 T.merula             ACCCACCTAGAGGAGCCTGTTCTGTAATCGATGATCCACGATATTACCTGACCATTCCTTGCACGAAACAGCC
AP484935.1 T.philomelos       ACCCACCTANAGGAGCCTGTTCTGTAATCGATGATCCACGATATTACCTGACCATTTCTTGCACGAAACAGCC
AM902522.1 T.philomelos       ACCCACCTAGAGGAGCCTGTTCTGTAATCGATGATCCACGATATTACCTGACCATTTCTTGCACGAAACAGCC
KJ676687 T.philomelos         ACCCACCTAGAGGAGCCTGTTCTGTAATCGATGATCCACGATATTACCTGACCATTTCTTGCACGAAACAGCC
FN675614.1 T.philomelos       ACCCACCTAGAGGAGCCTGTTCTGTAATCGATGATCCACGATATTACCTGACCATTCCTTGCACGAAACAGCC
AY012102 S.araneus#           ATCCCTCTAGAGGAGCCTGTTCTGTA-TCGATAAACCCCCGATAAACCTCACCACCCCTTGCTAA-TTCAGCT
EU740412.1 C.l.familiaris#    ATCCCTCTAGAGGAGCCTGTTCTATAATCGATAAACCCC-GATAAACCTCACCACCTTTCGCTAA-TTCAGTC
JN711443.1 V.vulpes#          ATCCCTCTAGAGGAGCCTGTTCTATAATCGATAAACCCC-GATAAACCTCACCATCCCTTGCTAA-TACAGTC
AY121990.1 C.capreolus#       ACCCTTCTAGAGGAGCCTGTTCTATAATCGATAAACCCC-GATAGACCTCACCACCCCTTGCTAA-TACAGTC
AY184430.1 C.elaphus#         ACCCTTCTAGAGGAGCCTGTTCTATAATCGATAAACCCC-GATAAACCTCACCATTCCTTGCTAC-TACAGTC
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NC 001941.1 O.aries#          ACCCTTCTAGAGGAGCCTGTTCTATAATCGATAAACCCC-GATAAACCTCACCAATCCTTGCTAA-TACAGTC
AY670666.1 R.rupicapra#       ACCCTTCTAGAGGAGCCTGTTCTATAATCGATAAACCCC-GATAAACCTCACCAATCCTTGCTAA-TACAGTC
AF441243.1 C.russula#         ATCCATCTAGAGGAGCCTGTTCTATAATCGATACACCCC-GATCAACCTCACCACTTCTTGCTAA-TTCCGCC
AJ311131.1 A.sylvaticus#      ATCCATCTAGAGGAGCCTGTTCTATAATCGATACACCCC-GATCTACCTCACCATCTCTTGCCAAATTCAGCC
AJ311164.1 A.flavicollis#     ATCCATCTAGAGGAGCCTGTTCTATAATCGATACACCCC-GATCTACCTCACCATCTCTTGCCAAATTCAGCC
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JN989561.1 H.sapiens          ATCCCTCTAGAGGAGCCTGTTCTGTAATCGATAAACCCC-GATCAACCTCACCACCTCTTGCT-----CAGCC
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AY121990.1 C.capreolus#       TATATACCGCCATCTTCAGCAAACCCTA-AAAA-GGAATAAAAGTAAGCACAACCATC---ATACATAAAAACG
AY184430.1 C.elaphus#         TATATACCGCCATCTTCAGCAAACCCTA-AAAA-GGTACAAAAGTAAGCACAATCATA---ATACATAAAAACG
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AY670666.1 R.rupicapra#       TATATACCGCCATCTTCAGCAAACCCTA-AAAA-GGAACAAAAGTAAGCTCAATCATA---GCACATAAAGACG
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AJ311131.1 A.sylvaticus#      TTAGGTCAAGGTGTAGCCAATGAGGTGGGAAGAAATGGGCTACATTTTCTTTAC-AAAGAACA----TTA
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CANIDAE d-loop

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AM181037.1 V.vulpes                 ACTTGCTATCACTCAGCTATGACC-------GCA--ACGGCACTAACTC-TAACCTA-CATCTGCACTCAG
AY177656.1 C.familiaris             ATCTGCTATCACTCACCTACGACC-------GCA--ACGGCACTAACTC-TAACTTATCTTCTGCTCTCAG
AY177644.1 C.familiaris             ATCTGCTATCACTCACCTACGACC-------GCA--ACGGCACTAACTC-TAACTTATCTTCTGCTCTCAG
AY177652.1 C.familiaris             ATCTGCTATCACTCACCTACGACC-------GCA--ACGGCACTAACTC-TAACTTATCTTCTGCTCTCAG
AY177660.1 C.familiaris             ATCTGCTATCACTCACCTACGACC-------GCA--ACGGCACTAACTC-TAACTTATCTTCTGCTCTCAG
AY172678.1 C.familiaris             ATCTGCTATCACTCACCTACGACC-------GCA--ACGGCACTAACTC-TAACTTATCTTCTGCTCTCAG
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NC 002008.4 C.familiaris            ATCTGCTATCACTCACCTACGACC-------GCA--ACGGCACTAACTC-TAACTTATCTTCTGCTCTCAG
EU740415.1 C.familiaris             ATCTGCTATCACTCACCTACGACC-------GCA--ACGGCACTAACTC-TAACTTATCTTCTGCTCTCAG
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AM181037.1 V.vulpes                 TATCA---ACTCCG---TGCACAAT-------TCAAGGTGCTATTC---AGTCAATGGTTTCAGGACATAA
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X78798.1 S.araneus#                 CATAAATTGACAAA---ACTGGTGTAAGTACAGGAGAAATGGATTCAG-GTAAAAATAATCAATGGTCACA
HQ200179.1_H.sapiens                GA-------ACATA---CTTACTA------------AAGTGTGTT--------AATTAATTAATGCTTGTA
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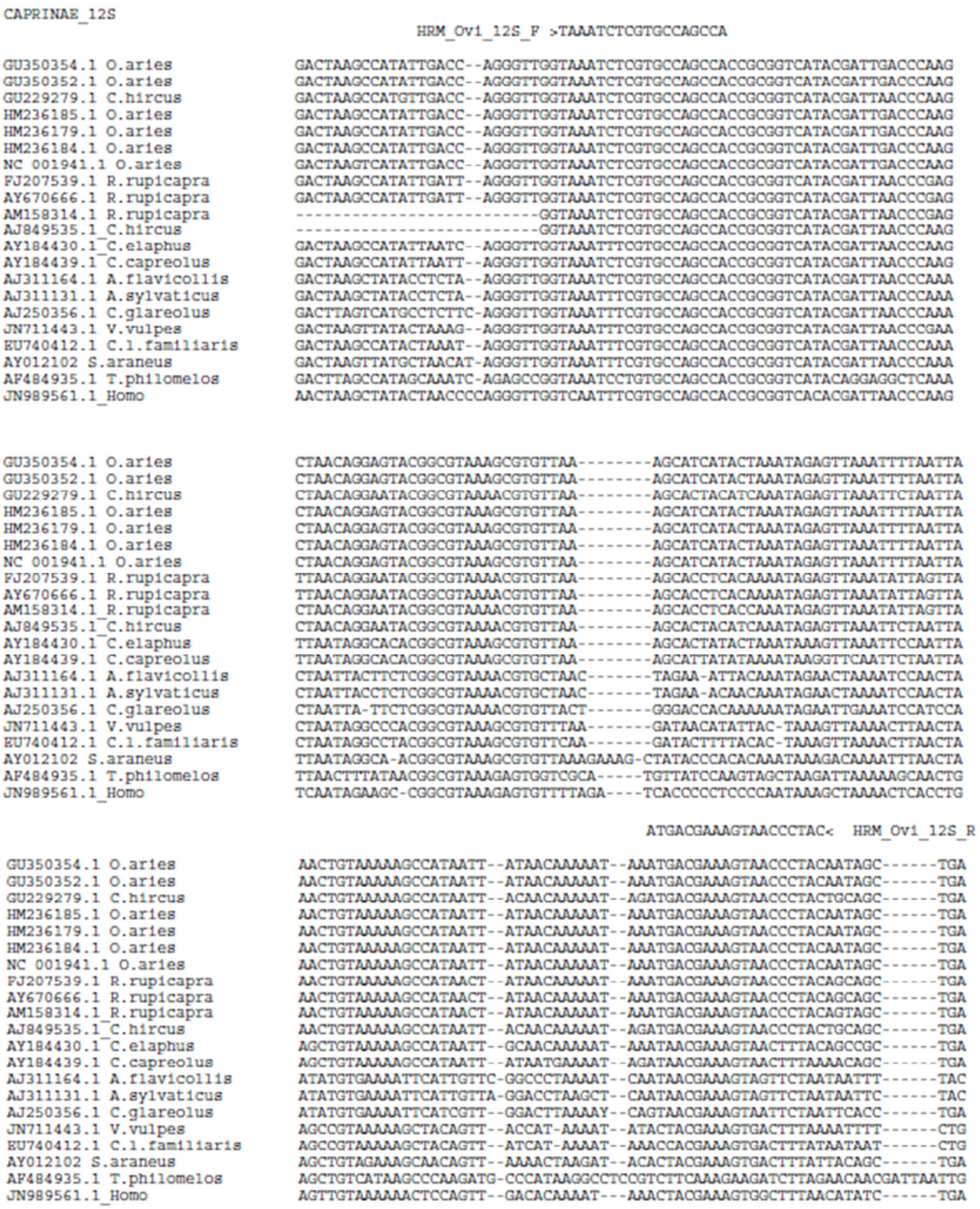
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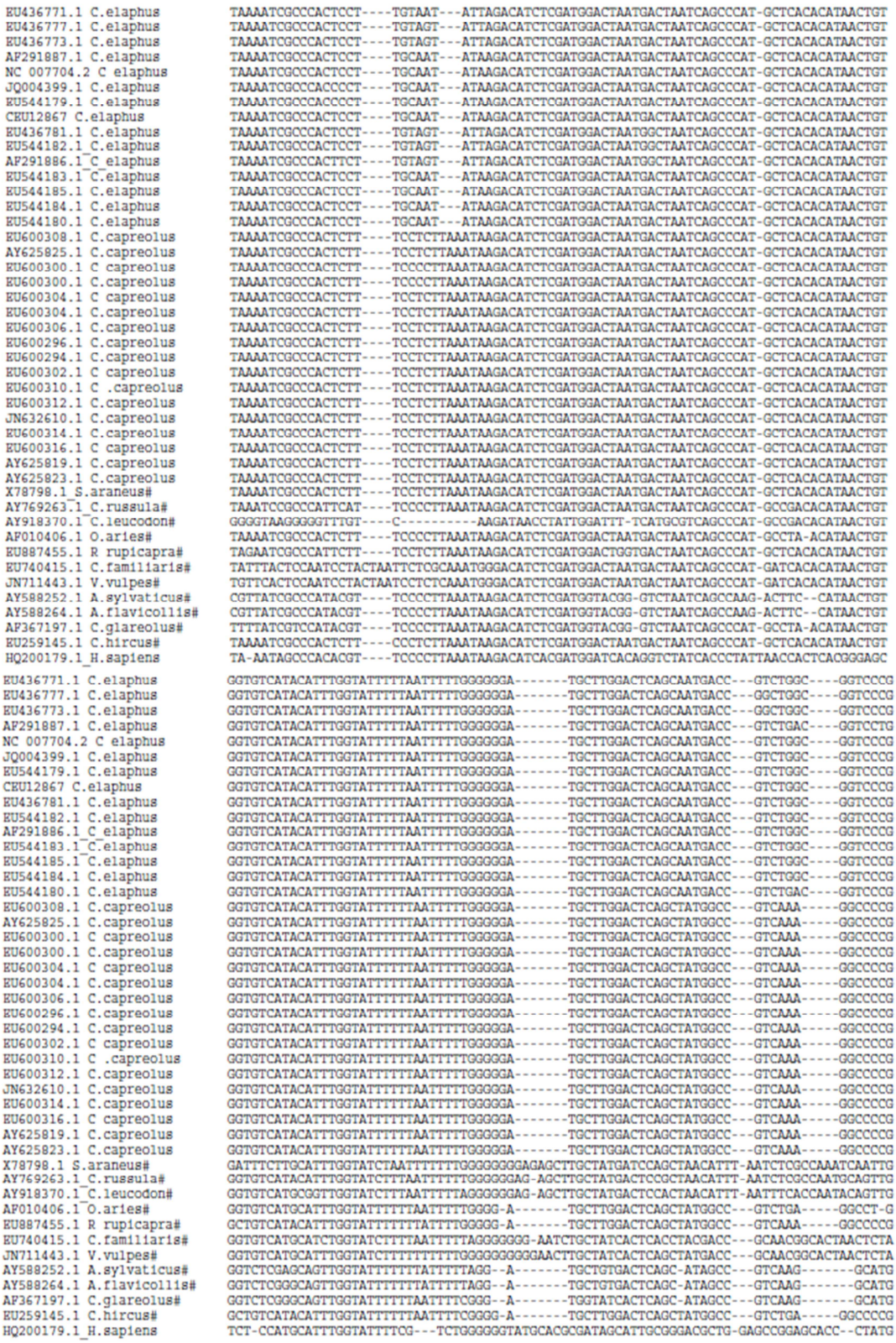
AY184439.1 C.capreolus AGCTGTAAAAAGCCATAATT--ATAATGAAAAT--AGATAACGAAAGTAACTTTAAAACAGC------TGA
AJ311164.1 A.flavicollis ATATGTGAAAATTCATTGTTC-GGCCCTAAAAT--CAATAACGAAAGTAGTTCTAATAATTT------TAC
AJ311131.1 A.sylvaticus ATATGTGAAAATTCATTGTTA-GGACCTAAGCT--CAATAACGAAAGTAGTTCTAATAATTC------TAC
AJ250356.1 C.glareolus ATATGTGAAAATTCATCGTT--GGACTTAAAAY--CAGTAACGAAAGTAATTCTAATTCACC------TGA
JN711443.1 V.vulpes AGCCGTAAAAAGCTACAGTT--ACCAT-AAAAT--ATACTACGAAAGTGACTTTAAAATTTT------CTG
EU740412.1 C.l.familiaris AGCCGTAAAAAGCTACAGTT--ATCAT-AAAAT--AAACCACGAAAGTGACTTTATAATAAT------CTG
AY012102 S.araneus AGCTGTAGAAAGCAACAGTT--AAAACTAAGAT--ACACTACGAAAGTGACTTTATTACAGC------TGA
AF484935.1 T.philomelos AGCTGTCATAAGCCCAAGATG-CCCATAAGGCCTCCGTCTTCAAAGAAGATCTTAGAACAACGATTAATTG
JN989561.1_Homo AGTTGTAAAAAACTCCAGTT--GACACAAAAT---AAACTACGAAAGTGGCTTTAACATATC------TGA

CERVIDAE_d-loop

HRM_Ung_DL_F >CGATGGACTAATGACTAATCA



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                                                                 CATCTTGA GCATCCCCAT  AA< HRM_Ung_DL_R

EU436781.1 C.elaphus          TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
EU544182.1 C.elaphus          TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
AF291886.1 C elaphus          TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
EU436771.1 C.elaphus          TCCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
EU436777.1 C.elaphus          TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
EU436773.1 C.elaphus          TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
AF291887.1 C.elaphus          TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGATAGGCATGG--GCATG--GCAGT-
NC 007704.2 C elaphus         TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
JQ004399.1 C.elaphus          TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
EU544179.1 C.elaphus          TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
CEU12867_C.elaphus            TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
EU544183.1_C.elaphus          TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
EU544185.1 C.elaphus          TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
EU544184.1 C.elaphus          TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
EU544180.1 C.elaphus          TCCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
EU600308.1 C.capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--ACAGT-
AY625825.1 C.capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--ACATG--ACAGT-
EU600300.1 C capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--ACAGT-
EU600300.1 C.capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--ACAGT-
EU600304.1 C capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGA--GCATG--ACAGT-
EU600304.1 C.capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGA--GCATG--ACAGT-
EU600306.1 C.capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGA--GCATG--ACAGT-
EU600296.1 C.capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGA--GCATG--ACAGT-
EU600294.1 C.capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGA--GCATG--GCAGT-
EU600302.1 C capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGA--GCATG--ACAGT-
EU600310.1 C .capreolus       ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
EU600312.1 C.capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
JN632610.1 C.capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
EU600314.1 C.capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--ACATG--GCAGT-
EU600316.1 C capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--ACATG--GCAGT-
AY625819.1 C.capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
AY625823.1 C.capreolus        ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCATG--GCAGT-
X78798.1 S.araneus#           AAGCTGGGCTTATTCTTTATGGGGGCCGAAAAGATTATGTTATTATGGCTATTTCTCTTGCAGGGAGTAAATACGTTTAATGGTT
AY769263.1_C.russula#         TAGCTGGGCTTATTCTCTATGGGGGCCG-AAGTAGTTATTACAACCGTACTTAT----TCTCTTGATATGGTACATA---TGGTT
AY918370.1_C.leucodon#        TAGCTGGGCTTATTCTCTATGGGGGCCA-AAGTAATTATTCTTAGCGTACTTAT----TCTCTTGATAGAGTACATT---TAACT
AF010406.1_O.aries#           ACCCGGAGCATGAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCTCAT--AATGGTAAGCATGG--GCATAATATAAT-
EU887455.1 R rupicapra#       ACCCGGAGCATCAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAAGCATGG--GCAT---CGAGT-
EU740415.1 C.familiaris#      ACTTATCTTCTGCTCTCAGGGAATATGCCCGTCGCGGCCCTAATGCAGTCAAAT--AACTTGTAGCTGGAC-TTATTCATTATCA
JN711443.1 V.vulpes#          ACCTA-CATCTGCACTCAGGGAATATGCCCGTCGCGGCCCCGACGCAGTCAGAT--GATCTGTAGCTGGAC-TTATTCATTATCA
AY588252.1 A.sylvaticus#      AAGGGCAACTTATCATGTAGCTGGACTTTAAGTGAAGGGTCAT-TAATCCACAT--AACCAAATCATCGC--AGACTAATTAAT-
AY588264.1 A.flavicollis#     AAGGGCAACTTATCATGTAGCTGGACTTTAAGTGAAGGATCAT-TAATCCACAT--AACCAAATCATCGA--AGGCT-AATATCT
AF367197.1 C.glareolus#       AAGGTCAACTTATAGTCTAGCTGGACTTCCTATTAAGTATCAT-TTATCCCCAT--CAATACCCCTGCAAC-AGATTAATTAATG
EU259145.1 C.hircus#          ACCCGGAGCATAAATTGTAGCTGGACTT--AACTGCATCTTGA-GCATCCCCAT--AATGGTAGGCATGG--GCAT--TGCAGT-
HQ200179.1_H.sapiens          TCGCAGTATCTGTCTTTGATTCCTGCCTCATCCTATTATTTATCGCACCTACGTTCAATATTACAGGCGAACATACTTACTAAAG
```

Table A1 Identification results by HRMA and sequence BLASTn for questing ticks bloodmeal analysis during Real-time HRMA optimization; Tm, melting temperature; Tm1, melting temperature peak 1; Tm2, melting temperature peak 2.continued in the next page.

		Muroidea			Soricidae				Passeriformes			
	sample	Tm °C			Tm °C (n°rep)				Tm °C (n°rep)			
SITE	name	(n°rep)	HRMA genotype	BLAST genotype	Tm1	Tm2	HRMA genotype	BLAST genotype	Tm1	Tm2	HRMA genotype	BLAST genotype
CON	1_CO	74.2 (1)	out of range	no sequences obtained-too short					79.6		no match-out of range	148 bp-no similarity found
	2_CO								81.5		no match-out of range	103 bp-Ixodes sp
	3_CO								82.8	86.6	no match-out of range	575 bp-Ixodes sp
	4_CO											
	5_CO								82.1		no match-out of range	346 bp- no similarity found
	6_CO											
	7_CO											
	8_CO											
	9_CO											
	10_CO											
	11_CO				77.0	70.4 (1)	out of range					
	12_CO	81.9 (2)	*Apodemus sp.*	*A.flavicollis* 100%	81.8	74.4 (1)	*C. suaveolens*	*C. russula 99%*[a]				
	13_CO				83.1 (1)		out of range					
TRA	1_TR								83.6		strange profile	123 bp-Ixodes sp
	2_TR											
	3_TR								78.6	84.4	strange profile	154 bp double peaks-T.merula 99% NO POSITIVE
	4_TR								79.5	84.9	strange profile	double peak-not readable sequences
	5_TR											
	6_TR											
	7_TR											
	8_TR				81.2	83.9 (1)	late amplification-strange profile	no sequence obtained				
	9_TR											
	10_TR				81.6 (1)		*C. suaveolens*	*C. russula 97%*[a]				
	11_TR				82.4 (1)		*C. leucodon*	*C. leucodon* 99%				
	12_TR				81.9 (1)		*C. leucodon ??*	*C. leucodon* 99%				
CAD	1_CA								79.8	82.0	out of range	219 bp-Ixodes sp
	2_CA											
	3_CA											
	4_CA											
	5_CA											
	6_CA											
	7_CA											
	8_CA				82.3	75.3 (1)	*C. leucodon?*-strange profile	*C. leucodon* 99%, only F primer				
	9_CA	78.7	out of range	no sequences obtained-too short								
	10_CA				81.6	74.4 (1)	*C. suaveolens*	*C. russula 98%*[a]	58.1	84.3	late amplification-strange profile	double peak in the sequence-no readable
	11_CA											
	12_CA											
	13_CA											
	14_CA				80.5 (1)		*Sorex sp*	*S. antinorii* 100%				
PIE	1_PI	81.5 (1)	*Apodemus sp.*	*A. flavicollis* 100%								
	2_PI											
	3_PI				86.2 (1)		out of range	261 bp-Ixodes spp				
	4_PI											
	5_PI	81.3 (1)	*M. glareolus*	*A. flavicollis* 98% (2 del)	83.7 (1)		out of range	no sequences assembly				
	6_PI											
	7_PI				82.3 - 74.4 (1)		*C. leucodon*	*C . leucodon* 100%				
	8_PI				82.3 - 74.0 (1)		*C. leucodon*	*C . leucodon* 100%				
	9_PI				81.7 - 73.7 (1)		*C. suaveolens*	*C. russula 98%*[a]				
	10_PI				82.4 (1)		*C. leucodon*	*C . leucodon* 100%	84.1		late amplification-strange profile	
	11_PI				81.4 (1)		*C. suaveolens*	*C. russula 99%*[a]				
	12_PI				82.3 - 73.6 (1)		*C. leucodon*	*C . leucodon* 100%				
	13_PI				80.5 (1)		*Sorex sp*	*S. antinorii* 99%				

Table A1 continued

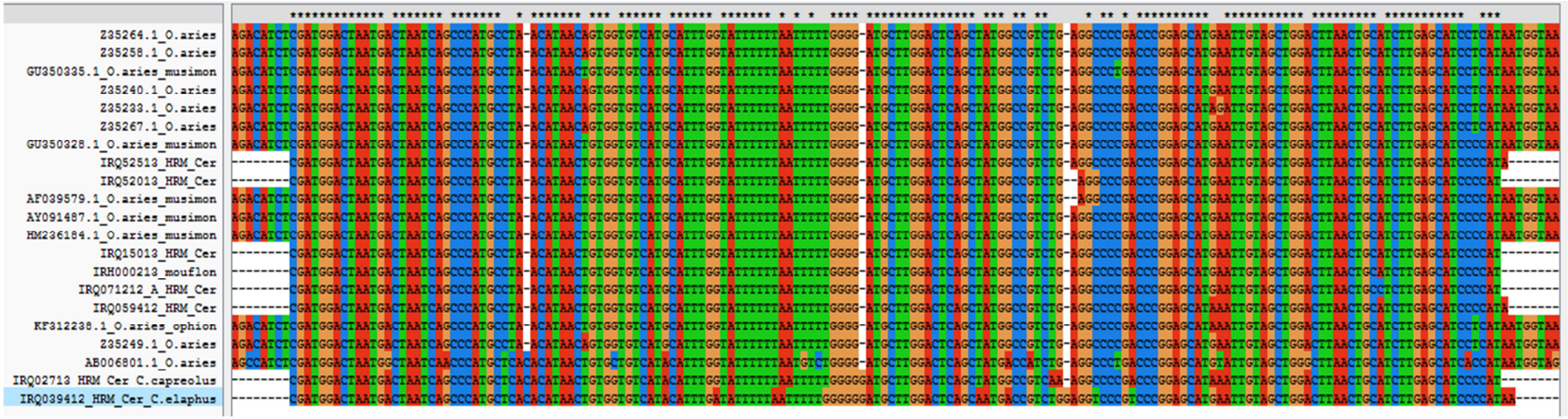
		Canidae				Caprinae					Cervidae				Mixed bloodmeal
	sample	Tm °C (replicate				Tm °C (n°rep)					Tm °C (n°rep)				
SITE	name	Tm1	Tm2	HRMA genotype	BLAST genotype	Tm1	Tm2	Tm3	HRMA genotype	BLAST genotype	Tm1	Tm2	HRMA genotype	BLAST genotype	
CON	1_CO	79.9 (1)		*C.l. familiaris*	*C.l. familiaris* 100%	80.7	83.9	88.2 (1)	no match-strange profile	double sequences not readable			-		
	2_CO	80.4 (2)		*C.l. familiaris*	*C.l. familiaris* 100%	80.8	83.9 (2)		no match-out of range	*Bos taurus* 99%			-		yes
	3_CO	80.8 (1)		*C.l. familiaris*	*C.l. familiaris* 100%	81.0	84.1 (2)		no match-out of range	*Bos taurus* 99%			-		yes
	4_CO												-		
	5_CO	80.4 (2)		*C.l. familiaris*	*C.l. familiaris* 100%	80.8	83.7 (2)		out of range	*Bos taurus* 99%			-		yes
	6_CO	80.5	82.7	no match-strange profile	no similarity found						83.5	80.7 (1)	*C. elaphus*	*C. elaphus* 100%	
	7_CO	80.2 (2)		*C.l. familiaris*	*C.l. familiaris* 100%								-		
	8_CO												-		
	9_CO	80.6 (1)		*C.l. familiaris*	*C.l. familiaris* 100%								-		
	10_CO										82.7	80.6 (2)	*C. capreolus*	*C. capreolus* 100%	
	11_CO												-		
	12_CO												-		yes
	13_CO												-		
TRA	1_TR	80.7 (1)		*C.l. familiaris*	*C.l. familiaris* 100%	74.3	83.7	87.9	no match-out of range	double sequences not readable	83.5	80.7 (1)	*C. elaphus*	*C. elaphus* 100%	
	2_TR	80.9 (2)		*C.l. familiaris*	*C.l. familiaris* 100%								-		
	3_TR	85.1		no match-out of range	no similarity found - Ixodes sp						83.6	80.7 (1)	*C. elaphus*	*C. elaphus* 100%	
	4_TR	80.3 (1)		*C.l. familiaris*	*C.l. familiaris* 100%										
	5_TR	83.4	80.7 (1)	*V. vulpes*	*V. vulpes* 100%										
	6_TR														
	7_TR														
	8_TR														
	9_TR														
	10_TR														
	11_TR	83.7	87.3 (1)	*V. vulpes*	*V. vulpes* 100%										yes
	12_TR														
CAD	1_CA	80.7		strange derivative profile	double sequences not readable	78.3	80.8	84.0	no match-out of range	double sequences not readable					
	2_CA					73.5	83.8	88.2	no match-out of range	double sequences not readable					
	3_CA	80.6		strange derivative profile	no similarity found						83.2		no match-strang	Ixodes sp.	
	4_CA	78.5	80.2	no match-strange profile	no similarity found						83.6	80.8 (1)	*C. elaphus*	*C. elaphus* 100%	
	5_CA														
	6_CA														
	7_CA										82.5	80.5 (2)	*C. capreolus*	*C. capreolus* 100%	
	8_CA														
	9_CA														
	10_CA														
	11_CA														
	12_CA	82.8		no match-strange profile	no similarity found										
	13_CA														
	14_CA														
PIE	1_PI			-							83.4	80.5 (2)	*C. elaphus*	*C. elaphus* 100%	yes
	2_PI	80.7	82.7	no match- strange profile	248 bp - no similarity found										
	3_PI	80.4 (2)		*C.l. familiaris*	*C.l. familiaris* 100%										
	4_PI			-							83.6	80.7 (2)	*C. elaphus*	*C. elaphus* 100%	
	5_PI			-											
	6_PI			-							83.5	80.6 (1)	*C. elaphus*	*C. elaphus* 100%	
	7_PI														
	8_PI										83.3	80.5 (1)	*C. elaphus*	*C. elaphus* 99%	yes
	9_PI														
	10_PI														
	11_PI														
	12_PI														
	13_PI														

FOOTNOTES Table A1:

[a] for C. suaveolens sequences BLAST give as maximum identity *C. russula*, because no sequence of *C. suaveolens d-loop* is available in the GenBank database; 100% C. suaveolens, after alignment with sequence obtained from DNA tissue samples of *C. suaveolens* by authors.

Appendix 2 *Ovis* spp. alignment and BLASTn results

ClustalX 2.0.12 alignment of *Ovis* spp sequences retrieved from GenBank and sequences otained with HRM_Cer primers from questing nymphs (IRQxxxxx) and from the engorged ticks collected while feeding on a mouflon (IRH000213). A *C. capreolus* and a *C. elaphus* sequence from questing nymphs were also included.



BLASTn results of HRM_Cer *Ovis* spp amplicons

	BLASTn: Sequences producing significant alignments	Max score	Total score	Query cover	E value	Ident %
HRM_Cer amplicon from engorged female from mouflon						
IRH000213	*Ovis orientalis* breed Asian mouflon	302	302	100	1E-78	100
	Ovis aries breed Jingzhong	302	302	100	1E-78	100
	Ovis aries	302	302	100	1E-78	100
	Ovis aries	302	302	100	1E-78	100
	Ovis aries musimon	302	302	100	1E-78	100
HRM_Cer amplicons from questing nymphs						
IRQ52013_Mez	*Ovis aries musimon*	300	300	100	5E-78	100
	Ovis aries	298	298	99	2E-77	100
	Ovis aries	298	298	99	2E-77	100
	Ovis aries	298	298	99	2E-77	100
	Ovis orientalis breed Asian mouflon	294	294	100	2E-76	99
IRQ52513_Mez	*Ovis orientalis* breed Asian mouflon	303	303	100	4E-79	100
	Ovis aries breed Jingzhong	303	303	100	4E-79	100
	Ovis aries	303	303	100	4E-79	100
	Ovis aries	303	303	100	4E-79	100
	Ovis aries musimon	303	303	100	4E-79	100
IRQ079212_A_Ala	*Ovis orientalis* breed Asian mouflon	296	296	100	6E-77	99
	Ovis aries breed Jingzhong	296	296	100	6E-77	99
	Ovis aries	296	296	100	6E-77	99
	Ovis aries	296	296	100	6E-77	99
	Ovis aries musimon	296	296	100	6E-77	99
IRQ15013_Vge	*Ovis orientalis* breed Asian	302	302	100	1E-78	100
	Ovis aries breed Jingzhong	302	302	100	1E-78	100
	Ovis aries	302	302	100	1E-78	100
	Ovis aries	302	302	100	1E-78	100
	Ovis aries musimon	302	302	100	1E-78	100
IRQ059412_Pin	*Ovis ammon hodgsoni*	303	303	100	4E-79	100
	Ovis aries ophion	298	298	100	2E-77	99
	Ovis orientalis breed Asian mouflon	298	298	100	2E-77	99
	Ovis aries breed Jingzhong	298	298	100	2E-77	99
	Ovis aries	298	298	100	2E-77	99
	Ovis orientalis anatolica	298	298	100	2E-77	99

For Taxonomy and Genbank NCBI database, the following synonyms refer to mouflon (*Ovis aries musimon* (Pallas, 1811): *Ovis orientalis musimon*; *Ovis aries mufflon*; *Ovis musimon*; *Ovis gmelini*; *Ovis ammon musimon*.

Appendix 3 Sequences alignment of HRM_Rod amplicons

Sequences alignment of HRM_Rod amplicons with special reference to deviating HRMA *Apodemus* spp. amplicons. IRHxxxx control samples sequences; IRQxxxx questing nymphs sequences.