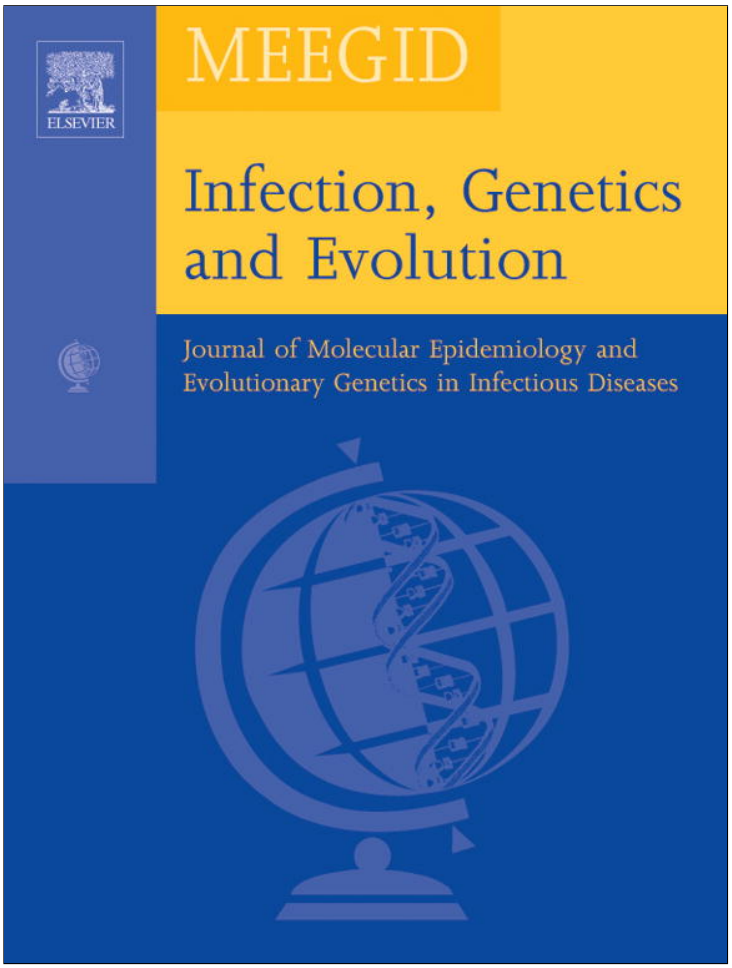

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Review

Babesia: A world emerging

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ABSTRACT

Babesia are tick-transmitted hemoprotozooans that infect mammals and birds, and which are acknowledged for their major impact on farm and pet animal health and associated economic costs worldwide. Additionally, *Babesia* infections of wildlife can be fatal if associated with stressful management practices; and human babesiosis, also transmitted by blood transfusion, is an increasing public-health concern. Due to the huge diversity of species reported to serve as *Babesia* hosts, all vertebrates might be potential carriers, as long as they are adequate hosts for *Babesia*-vector ticks. We here provide a comprehensive overview of the most relevant *Babesia* species, and a discussion of the classical taxonomic criteria. *Babesia*, *Cytauxzoon* and *Theileria* parasites are closely related and collectively referred to as piroplasmids. A possible scenario for the history of piroplasmids is presented in the context of recent findings, and its implications for future research avenues are outlined. Phylogenetic trees of all available 18S rRNA and hsp70 genes were generated, based on which we present a thoroughly revised molecular classification, comprising five monophyletic *Babesia* lineages, one *Cytauxzoon* clade, and one *Theileria* clade. Updated 18S rRNA and beta-tubulin gene trees of the *B. microti* isolates agree with those previously reported. To reconcile estimates of the origin of piroplasmids and ticks (~300 Ma, respectively), and mammalian radiation (60 Ma), we hypothesize that the dixenous piroplasmid life cycle evolved with the origin of ticks. Thus, the observed time gap between tick origin and mammalian radiation indicates the existence of hitherto unknown piroplasmid lineages and/or species in extant vertebrate taxa, including reptiles and possibly amphibians. The development and current status of the molecular taxonomy of *Babesia*, with emphasis on human-infecting species, is discussed. Finally, recent results from population genetic studies of *Babesia* parasites, and their implications for the development of pathogenicity, drug resistance and vaccines, are summarized.

Contents

Abbreviations: AFLP, amplified fragment-length polymorphism; BSC, biological species concept; CI, confidence interval; HPD, highest posterior density; ITS, intergenic spacer region; LD, linkage disequilibrium; Ma, million years ago; MLT, multilocus genotype; MOI, multiplicity of infection; MRCA, most recent common ancestor; PSC, phylogenetic species concept; RAPD, random amplification of polymorphic DNA.

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1. Introduction

Babesia are tick-transmitted protozoan hemoparasites, of great economic, veterinary and medical impact worldwide. They are considered to be the second most commonly found parasites in the blood of mammals after trypanosomes, and they have also been described infecting birds. In their vertebrate hosts they reproduce asexually inside erythrocytes, and together with *Theileria* spp. they are referred to as piroplasms or piroplasmids. The sexual phase of the *Babesia* life cycle typically takes place in Ixodid ticks, which acquire and transmit the parasites during their blood meals (Fig. 1) (Kakoma and Mehlhorn, 1994; Telford et al., 1993; Gray and Weiss, 2008).

Victor Babeş (1888) was the first to discover microorganisms inside bovine erythrocytes of Romanian cattle that presented hemoglobinuria; and he later observed a similar organism in sheep blood (Babeş, 1892). Five years later in the USA, Smith and Kilbour described that the presence of an intraerythrocytic parasite was the cause of tick-transmitted Texas Cattle Fever, a disease that had long stricken cattle ranchers in the Southern US states (Smith and Kilbourne, 1893). This turned out to be the first description of an arthropod-transmitted pathogen of vertebrates. The parasites described by Babeş, and Smith and Kilbour were later named *Babesia bovis*, *B. ovis* and *B. bigemina*, respectively (Starcovici, 1893; Mihalca, 2010). Soon afterwards, babesias parasitizing the blood of other domestic animals were observed, such as those that eventually became known as *B. canis* and *B. caballi*, described by Piana and Galli-Valerio (1895) and by Koch (1904), in dog and horse erythrocytes, respectively. Since these early findings, more than 100 different *Babesia* species have been discovered, and thanks to the advances in microscopy, cell biology and molecular biology techniques our knowledge of the *Babesia* world is rapidly expanding (Levine, 1988; Roncalli Amici, 2001; Criado-Fornelio et al., 2004; de Waal and Van Heerden, 2004; Uilenberg, 2006; Lack et al., 2012).

2. Distribution and pathological effects of some relevant *Babesia* spp.

The remarkable impact of babesia infections in three host groups: domestic animals, humans and, most recently acknowledged, some wildlife species, has inspired a great amount of research efforts in recent decades.

In general, babesia infections course with varying degrees of severity that can often be associated to the host's age, immunological status, concurrent infections with other pathogens, and/or genetic factors. Common manifestations of acute babesia infections in different hosts can include fever, anemia, hemoglobinuria, jaundice, malaise, lethargy and anorexia, while the chronic status is generally asymptomatic. Among domestic animals, babesia infec-

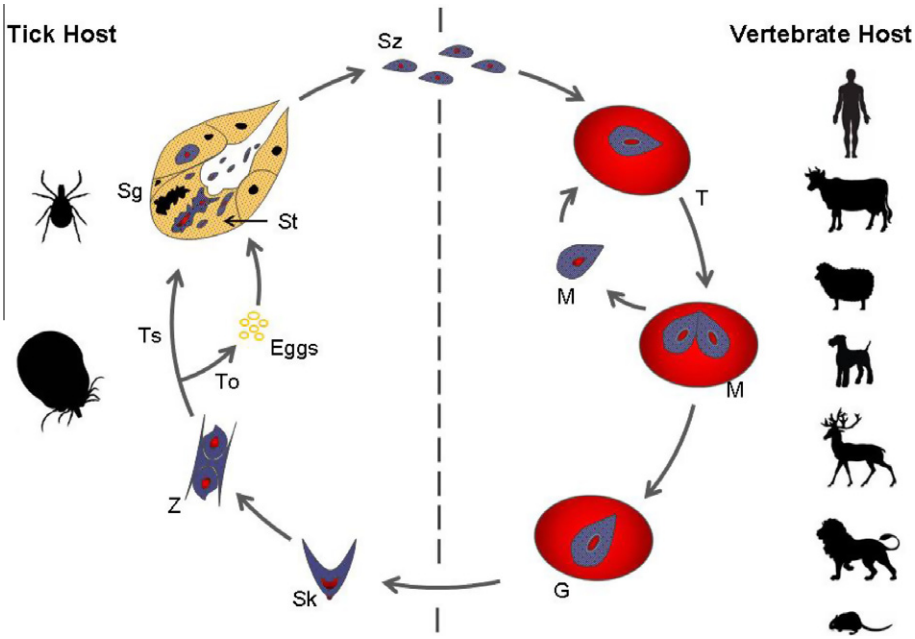

Fig. 1. Generic life-cycle of *Babesia* spp. *Babesia* sporozoites (Sz) are injected into the bloodstream of a vertebrate host with minute amounts of saliva, during the blood meal of an infected tick. After invading erythrocytes they differentiate into trophozoites (T), which divide asexually (merogony) into two or sometimes four merozoites (M). Merozoites exit the erythrocytes and invade new ones, continuing the replicative cycle in the host. A few merozoites stop division and transform into gamonts or pregametocytes (G). Gamogony and sporogony take place in the tick. When gamonts are taken up by a tick feeding on an infected host, they differentiate in the gut into gametes, also known as ray bodies or Strahlenkörper (Sk), that fuse forming a diploid zygote (Z, gamogony). Zygotes undergo meiosis giving rise to motile haploid kinetes, which multiply by sporogony and access the hemolymph, invading and continuing their replication in several tick organs, including the salivary glands (Sg). Here, a final cycle of differentiation and multiplication takes place, in which kinetes transform into sporozoites that will infect a vertebrate host after the tick has molted into the next stage, i.e. larvae to nymph or nymph to adult (transstadial transmission, Ts). In some *Babesia* spp. (*Babesia* sensu strictu), kinetes also invade the tick ovaries and eggs, and infective sporozoites are formed in the salivary glands of the next generation larvae (transovarial transmission, To) (Mehlhorn and Schein, 1984).

Table 1
Babesia spp. parasites of domestic animals.

Host(s)	Species	Distribution	References
Cattle	*B. bovis*	Africa, America, Asia, Australia, Europe	Babeş (1888), Starcovici (1893), Criado-Fornelio et al. (2003b)
	B. bigemina	Africa, America, Asia, Australia, Europe	Starcovici (1893), Uilenberg (2006)
	B. major	Asia, Europe	Uilenberg (2006), Criado-Fornelio et al. (2009a), Altay et al. (2008), Liu et al. (2008)
	B. occultans	Africa	Gray and De Vos (1981)
	B. ovata	Asia	Uilenberg (2006)
	B. divergens	Europe	Zintl et al. (2003)
	B. sp. Kashi	China	Luo et al. (2005)
Water buffalo	*B. orientalis*	Asia	Liu et al. (1997b)
	B. bovis	America, Asia	Liu et al. (1997a), Ferreri et al. (2008)
	B. bigemina	America, Asia	Liu et al. (1997a)
Horse	*T. equi*	Europe, America	Mehlhorn and Schein (1998), Pietrobelli et al. (2007), Criado-Fornelio et al. (2003b)
Horse, donkey, mule	*B. caballi*	Africa, America, Asia, Europe	Criado-Fornelio et al. (2004), Nagore et al. (2004), Uilenberg (2006)
Pig	*B. trautmanni*	Africa, Europe	De Waal et al. (1992), Yin et al. (1997)
Sheep, goat	*B. crassa*	Asia	Hashemi-Fesharki and Uilenberg (1981), Schnittger et al. (2003)
	B. ovis	Africa, Asia, Europe	Babeş (1888), Starcovici (1893), Bai et al. (2002), Schnittger et al. (2003)
	B. motasi	Africa, Asia, Europe	Uilenberg et al. (1980), Lewis et al. (1981), Schnittger et al. (2003)
Sheep	*B.* sp. Xinjiang	China	Liu et al. (2007)
Dog	*B. vogeli*	Africa, America, Asia, Australia, Europe	Carli et al. (2009), Reichenow (1937), Uilenberg et al. (1989), Carret et al. (1999), Criado-Fornelio et al. (2003a), Solano-Gallego et al. (2008), Matjila et al. (2004), Eiras et al. (2008)
	B. conradae	America (USA)	Kjemtrup et al. (2006)
	B. gibsoni	Asia, (Africa, America, Europe)	Birkenheuer et al. (1999), Criado-Fornelio et al. (2003c), Lee et al. (2009), Macintire et al. (2002), Meinkoth et al. (2002)
	B. vitalii	America (Brazil)	Da Silva et al. (2011)
	Babesia sp.	America (USA)	Birkenheuer et al. (2004)
	B. rossi	South Africa	Carret et al. (1999), Jacobson (2006), Keller et al. (2004), Leisewitz et al. (2001)
	T. annae	Spain, Portugal	Camacho et al. (2001)
	B. canis	Europe	Bourdoiseau (2006), Carret et al. (1999), Carli et al. (2009), Criado-Fornelio et al. (2003a), Solano-Gallego et al. (2008)
Cat	*B. felis*	South Africa	Conrad et al. (2006), Jacobson et al. (2000)
	B. (canis) presentii	Asia (Israel)	Baneth et al. (2004)

tions are of concern to both productive and companion species (Table 1).

Bovine babesiosis, or red water fever, as it is commonly called, is economically the most important arthropod-transmitted pathogen of cattle. The costs of bovine babesiosis are connected with mortalities, abortions, decreased meat and milk production, control measures, as well as losses of potential production, and cattle trade restrictions.

The economic burden imposed by this disease can be illustrated by the radical tick-control campaign that was mounted in the Southern USA in 1906, lasting for four decades, and demanding an investment of millions of dollars. This campaign ended with the eradication of the cattle tick and, hence, its transmitted parasites, thus making bovine babesiosis the first disease eradicated from continental USA. Since the country was declared free of bovine babesiosis, the estimated savings for the livestock industry are at least 3 billion dollars per year. Yet, most of the 1–2 billion cattle around the world are still exposed to babesiosis and, even in the USA, outbreaks occasionally occur and epidemiological surveillance is a permanent concern (Uilenberg, 1995; Bock et al., 2004; Guerrero et al., 2007; Perez de Leon et al., 2010; Ramos et al., 2010; Holman et al., 2011).

The most economically relevant bovine babesias are *B. bovis*, *B. bigemina* and *B. divergens*. The first two are found in tropical and subtropical regions of the globe, where their vector ticks – belonging to the genre *Rhipicephalus* and *Boophilus* – can survive. *B. divergens*, on the other hand, mainly affects cattle in Europe – from Scandinavia to the Mediterranean – and Northern Africa. Its distribution is connected to the ample range of temperatures tolerated by its tick vector, *Ixodes ricinus*. Clinical cases of bovine babesiosis occur when naïve adult cattle acquire the parasite through a tick bite, while calves can get infected, but do not get sick. The etiology of the disease varies according to the infecting species. *B. bovis* clinical cases are characterized by hypotension, respiratory stress syndrome and neurological symptoms, due to the accumulation of infected erythrocytes in lung and brain capillaries. Consequently, *B. bovis* infections normally show low parasitemia levels. Infections by *B. bigemina* and *B. divergens*, on the other hand, result in high parasitemias, and in these cases pathogenicity is mainly associated with massive erythrocyte destruction, leading to severe anemia. (Bock et al., 2004; Zintl et al., 2003).

Water buffalo (*Bubalus bubalis*) are mainly exploited as cattle in Asia, but have also been introduced into the Americas, Australia and Europe, amounting to over 160 million head worldwide. Their robustness, ability to thrive in poor pastures and floodable lands, and resilience to most infections make them particularly suitable as cattle in tick-endemic tropical and subtropical regions. Water buffalos have been reported to bear subclinical infections of *B. bovis* and *B. bigemina*; however *B. orientalis*, which is transmitted by *Rhipicephalus haemaphysaloides* in China, is highly pathogenic to these animals and produces great economic losses (Liu et al., 1997a,b; Uilenberg, 2006; Ferreri et al., 2008 Terkawi et al., 2011; He et al., 2011, 2012).

There is currently only one recognized *Babesia* species that causes equine piroplasmosis, *B. caballi*, which is transmitted by *Hyaloma*, *Dermacentor* and *Rhipicephalus* tick species. It has been estimated that only 10% of horses around the world are raised in piroplasmosis-free regions, while the disease is endemic in vast tropical and subtropical regions. After a weakening acute infection, the chronic state is characterized by decreased performance due to

a reduced capacity of erythrocytes to carry oxygen. Because of the threat posed to the pure-breed and racehorse industry, testing for equine piroplasmosis is a mandatory requirement for the international movement of horses (de Waal and Van Heerden, 2004).

Babesia infections of small ruminants (sheep and goats) have great economic importance in Southern Europe, the Middle East, and some African and Asian countries (Ranjbar-Bahadori et al., 2012; Yin and Luo, 2007). The infectious species include *B. ovis* and *B. motasi*, transmitted by *Rhipicephalus bursa* and *Haemaphysalis* spp. ticks, respectively. In addition, a *Babesia* parasite transmitted by *Haemaphysalis longicornis* ticks, has recently been reported to infect sheep in China, and has been temporarily assigned the name *Babesia* sp. Xinjiang. *B. ovis*, and *B.* sp. Xinjiang are highly pathogenic, while *B. motasi* shows moderate virulence (Hashemi-Fesharki, 1997; Yeruham et al., 1998; Bai et al., 2002; Uilenberg, 2006; Liu et al., 2007a; Guang et al., 2010, 2012a,b; Ekici et al., 2012).

Babesiosis in dogs is caused by a number of *Babesia* spp., including: (i) *B. vogeli*, transmitted by the widespread brown dog tick *Rhipicephalus sanguineus*, not only in tropical and subtropical regions but also in colder areas; (ii) *B. canis*, transmitted by *Dermacentor* spp. ticks, and described only in Europe; (iii) *B. rossi*, transmitted by *Haemaphysalis* spp. ticks, originally recognized in South Africa but later found in other African countries, such as Nigeria and Sudan; (iv) *B. conradae*, in the USA, with still undetermined vectors, and (v) *B. gibsoni*, transmitted by *Haemaphysalis longicornis*, and originally described in Asia, but also found in pit bull dogs of non-Asian countries, among which it is thought to be transmitted independent of ticks, through bites between infected and non-infected dogs during fighting. Note that separation of *B. canis*, *B. vogeli*, and *B. rossi* into subspecies (*B. canis canis*, *B. canis vogeli*, and *B. canis rossi*) is not substantiated, as they can be unequivocally differentiated by molecular phylogenetic analysis, their transmitting vector tick, and epidemiology (Zahler et al., 1998; Uilenberg, 2006).

Clinical manifestations of canine babesiosis vary with the species of the infectious agent, as well as with the age, immune status and concurrent infections of the affected dog. *B. rossi* is highly pathogenic, and its infections provoke hemolytic anemia and can lead to grave complications, including neurological signs, acute renal failure and pulmonary oedema, with poor prognosis. *B. vogeli* infections are normally subclinical in adult dogs, but can be fatal in 3- to 4-month-old puppies. *B. canis*, *B. gibsoni* and *B. conradae* infections elicit mild to severe disease according to the individual (Irwin, 2009, 2010; Penzhorn, 2011; Solano-Gallego and Baneth, 2011).

Clinical cases of cat babesiosis have mostly been related to infections by *B. felis* in Africa (Penzhorn et al., 2004; Solano-Gallego and Baneth, 2011). The *B. felis* vector tick has not been described yet. Although molecular evidence has shown that cats can be infected by *B. canis* and *B. vogeli*, clinical cases associated with these infections have not yet been reported (Criado-Fornelio et al., 2003a; Baneth et al., 2004; Simking et al., 2010).

Human babesiosis has lately received considerable attention as an emerging zoonosis. After the first finding of a babesia infection in a patient in the 1960s, an increasing number of human babesiosis infections, including fatal cases, have been reported. At first, it was believed that it was caused only by *B. microti* and *B. divergens*, restricted to the USA (over 300 reported cases) and Europe (over 30 reported cases), respectively. More recently, however, it has become evident that there is an array of *Babesia* spp. that can cause disease in humans (Table 2), and, in addition, the geographical range of *B. microti* and *B. divergens* is substantially larger than first thought. Rodents and cattle act as zoonotic reservoir species for these parasites and their *Ixodes* spp. vector ticks. Additionally, deer and other animals may contribute to the maintenance and spread of the infected vectors. Human babesiosis is mainly caused by tick bites; however, cases of transmission through blood transfusion are escalating (Leiby, 2011; Oz and Westlund, 2012). Infections vary in their manifestation from asymptomatic to life-threatening, according to the immunological status of the host. In asplenic or otherwise immunocompromised patients, as well as neonates or the elderly, acute infections can present complications involving severe hemolysis, hemodynamic instability, acute respiratory distress and multi-organ dysfunction, and can lead to death (Keirans et al., 1996; Gray et al., 2010; Leiby, 2011).

Finally, a great variety of wildlife species have been reported to be infected with babesia parasites, including among others, lion, lynx, panther, elephant, giraffe, antelope, buffalo, several deer species, wolf, raccoon, hyaena, mongoose, rhinoceros, and some birds, such as seagull and kiwi (Table 3). In most recent reports, the identification of these parasites has included molecular evidence. However, the species description of older findings cannot be relied upon. Babesias found in wildlife animals are of two types: parasites that seem largely specific to a particular host, e.g. *B. leo* in lions or *B. bicornis* in rhinoceros, and babesias typically found in closely related domestic animals, e.g. *B. bigemina* in gazelles or *B. canis* in wolves. Although the prevalence of babesia infections among wildlife populations seems to be rather high, subclinical infections appear to be the rule, and reported clinical cases are mostly associated with the recrudescence of existing infections due to stress, or exposure of naïve animals raised in tick-free areas to infected ticks. This type of situation can happen in the case of the capture of wild animals for relocation, or the release to the wild of animals kept in captivity. Thus, these findings need to be taken into account for wildlife management practices (Penzhorn, 2006).

Investigations of *Babesia* spp. have been mostly biased by the interest in controlling etiological agents of human and animal disease. However, taking into account the huge diversity of mammals and birds that have been described as carrying babesias, it can be expected that virtually all vertebrates are susceptible to infection by these parasites, as long as they are adequate hosts for the different babesia-vector ticks. In addition to the likely presence of yet to be discovered novel species, a large number of babesias are continuously found infecting non-expected or non-traditional hosts (Table 4). These might be either cryptic infections that have

Table 2
Babesia spp. parasites infecting humans.

Species	Distribution	References
B. microti	New England, USA	Gray and Weiss (2008), Zahler et al. (2000)
	Japan, Taiwan, Germany	Hunfeld et al. (2008), Hildebrandt et al. (2007)
B. venatorum	Austria, Italy, Germany, Belgium	Herwald et al. (2003), Häselbarth et al. (2007), Lempereur et al. (2011)
B. divergens	Europe, China	Zintl et al. (2003), Qi et al. (2011), Haapasalo et al. (2010)
B. duncani	USA	Conrad et al. (2006)
B. sp. CA1, CA3, CA4	USA	Kjemtrup and Conrad (2000)
B. divergens-like	USA (MO1)	Herwaldt et al. (1996, 2003), Beattie et al. (2002)
B. sp. (ovine)	South Korea	Kim et al. (2007)

Table 3
Recently described *Babesia* spp. parasites infecting wildlife mammals and birds.[a]

Species	Wild host	References
B. bennetti	Seagull (*Larus cachinnans*)	Criado et al. (2006)
B. kiwiensis	Kiwi (*Apteryx australis mantelli*)	Jeffries et al. (2008)
B. polea	Brown boobies (*Sula leucogaster*)	Yabsley et al. (2006)
B. vesperuginis	Bat (*Pipistrellus* sp.)	Concannon et al. (2005)
B. bicornis	Black rhinoceros (*Diceros bicornis*)	Nijhof et al. (2003)
B. capreoli	Roe deer (*Capreolus capreolus*)	Malandrin et al. (2010)
B. odocoilei	Deer (*Cervus elaphus*, *Odocoileus virginianus*, *Rangifer tarandus*)	Holman et al. (2000, 2003)
B. lengau	Cheetah (*Acinonyx jubatus*)	Bosman et al. (2010)
B. leo	Lion (*Panthera leo*, *P. pardus*)	Penzhorn et al. (2001)
B. sp.	Caracal (*Caracal caracal*)	Penzhorn et al. (2001)
B. sp.	Macaque (*Macaca fuscata*)	Hirata et al. (2011)
B. sp.	Olive baboons (*Papio cynocephalus*)	Reichard et al. (2011)
B. sp.	Black bear (*Ursus thibetanus*)	Ikawa et al. (2011)
B. sp	Brown bear (*Ursus arctos*)	Jinnai et al. (2010)
B. sp.	Raccoon (*Procyon lotor*)	Birkenheuer et al. (2006), Jinnai et al. (2009)

[a] All parasites included in this table have been identified using molecular tools. Those mentioned as *B.* sp. have not been biologically characterized.

Table 4
Babesia spp. detected in unexpected hosts.[a]

Organisms	Host	Country	References
B. bigemina	Deer	Texas, Brazil	Holman et al. (2011), da Silveira et al. (2011)
B. bovis	Deer	Texas, Brazil	Ramos et al. (2010), Holman et al. (2011), da Silveira et al. (2011)
	Horse	Italy, Argentina	Criado-Fornelio et al. (2009b)
B. caballi	Zebra	South Africa	Zweygarth et al. (2002)
	Dog	Croatia	Beck et al. (2009b)
	Dromedary	Jordan	Qablan et al. (2012)
B. canis	Cat	Spain, Portugal	Criado-Fornelio et al. (2003a)
B. capreoli	Goat	Austria	Silaghi et al. (JN543183)
B. divergens	Cottontail Rabbit	USA	Goethert and Telford (2003)
	Cattle	Austria	Blaschitz et al. (2008)
		France, Germany	Delbecq et al. (AJ509158, AJ507802)
		Sweden, Ireland, Germany, France	Vogl et al. (EF458227, EF458187, EF458185, EF458183, EF458171, EF458169)
	Goat	Austria	Silaghi et al. (JN543174)
	Deer	France	Malandrin et al. (2010)
		Great Britain	Langton et al. (2003)
		Ireland	Zintl et al. (2011)
		Slovenia	Duh et al. (2005)
		Spain	Garcia-Sanmartin et al. (2007)
B. divergens-like EU1	Deer	Swiss Alps, Slovenia, Scotland	Hoby et al. (2007b), Duh et al. (2005), Langton et al. (2003), Schmid et al. (2008)
	Chamois	Swiss Alps	Hoby et al. (2007a)
	Cattle	Switzerland	Mathis et al. (2006), Hilpertshauser et al. (2006)
B. felis	Dog	Romania	Hamel et al. (2012)
B. microti	Dog	Spain	Zahler et al. (2000)
	Fox	Poland	Karbowiak et al. (2010)
	Horse	Italy	Pietrobelli et al. (2007)
B. microti-like	Monkey	Japan	Hirata et al. (2011), Voorberg-vd Wel et al. (2008)
	Squirrel	Japan	Nakajima et al. (2009), Tsuji et al. (2006)
B. odocolei	Sheep	USA	Schoelkopf et al. (2005)
B. ovis	Goat	Spain	Marco et al. (2000), Garcia-Sanmartin et al. (2007)
B. vogeli	Cat	Thailand	Simking et al. (2010)

[a] GenBank Accession numbers are provided for unpublished results.

not been recognized before, or accidental infections, where the life cycle cannot be completed. Some parasites initially classified as *Theileria* might in the future contribute to the *Babesia* spectrum, once further biological and molecular evidence has been gathered. Moreover, circumstantial evidence indicates that argasid ticks could also be babesia vectors. Thus, the argasid *Ornithodoros moubata* was able to transmit *B. gibsoni* to dogs, after being artificially infected with this parasite (Battsetseg et al., 2007). In addition, the English bat *Pipistrellus pipistrellus* was found to be infected with the potentially pathogenic babesia *B. vesperuginis*. Since no Ixodid ticks could be found to parasitize these bats, it has been postulated that the soft tick *Argas vespertilionis* could be the vector of *B. vesperuginis* (Gardner and Molyneux, 1987). A similar situation might be true for babesias infecting birds, since argasids are the main ticks that parasitize these vertebrates. These findings suggest that our picture of the species that harbor babesia parasites is far from complete, and a great number of interesting facts are still waiting to be unraveled.

In the following, an update is presented of *Babesia* natural history, taxonomy and population genetics, as well as a revised view of the phylogenetic relationships between major piroplasmid groups.

3. The natural history of *Babesia*

To gain some insight into the nature of co-evolution and interaction between apicomplexan piroplasmids and their invertebrate and vertebrate hosts, an inevitably tentative view of their evolutionary history is outlined here based on recent research findings.

Comparative morphology suggests that the most recent common ancestor (MRCA) of apicomplexans (which include gregarines, piroplasmids, *Plasmodium*, coccidians, cryptosporidia, etc.) possessed a prevailing presence in marine environments, with extracellular feeding stages (myzocytoic parasitism) and a life cycle involving a single invertebrate host (Leander, 2007).

In a variety of studies, the origin of Apicomplexa has been estimated in the range between 1100 and 500 Ma based on molecular clock estimations (Escalante and Ayala, 1995; Douzery et al., 2004; Berney and Pawlowski, 2006; Parfrey et al., 2011). Molecular clock approaches have dramatically improved in the last decade, and it is currently accepted that to reduce the dating error it is essential to use: (i) multi-gene trees, (ii) multiple calibration points ideally based on micro- and macrofossil records, (iii) a relaxed molecular clock, and (iv) a large number of taxa (Brinkmann and Philippe, 2007). A study by Parfrey et al. (2011) so far complies best with these criteria, and estimates the origin of apicomplexa around 1100 Ma (CI: 1250–1000 Ma).

Phylogenetic analysis indicates that members of the genus *Colpodella* form a sister group with the Apicomplexa (Kuvardina et al., 2002; Yuan et al., 2012). Colpodellids are predatory flagellates that feed on unicellular algae by attaching to the prey cell and extracting its cytoplasm via specialized structures, in a process called myzocytosis. These specialized structures are considered homologous to the apical complex which allowed the Apicomplexa to engage in a parasitic lifestyle by invasion of a host (prey) cell. Thus, the Apicomplexa likely developed from this myzocytoic predation to myzocytoic parasitism, as still exhibited by gregarines and cryptosporidia, and finally to intracellular parasitism as displayed by piroplamids, *Plasmodium*, and coccidians (Fig. 2). Accordingly, apicomplexans are parasitic; however, in a singlular case, a mutualistic apicomplexan, *Nephromyces*, has also been described, and it has been suggested that symbiotic associations might be an ancient feature of Apicomplexa (Perkins et al., 2000; Huang and Kissinger, 2006; Morrison, 2009; Okamoto and McFadden, 2008; Saffo et al., 2010). Many apicomplexans possess a remnant of an ancient algal plastid that they inherited from their common ancestor, the apicoplast (Lim and McFadden, 2010). This organelle is still present in piroplasmids, coccidians and *Plasmodium*, but has been lost in *Cryptosporidium* lineages, and possibly also in gregarines (Lang-Unnasch et al., 1998; Zhu et al., 2000; Fast et al., 2001; Huang et al., 2004; Moore et al., 2008; Sato, 2011). Although most reports seem to favor a red algae origin for the apicoplast, evidence for a green algae origin has also been presented (Moore et al., 2008; Lau et al., 2009; Janouskovec et al., 2010). Recently, *Chromera vella* has been described as the only known close relative of apicomplexans with a functional photosynthetic plastid. This plastid contains a genome with genetic features exclusively found in apicomplexan parasites (Moore et al., 2008). It may be assumed that the apicomplexan ancestor was a photosynthesizing unicellular organism that, possibly after adapting to myzocytosis and subsequently to a parasitic lifestyle, reduced its plastid to the now remnant apicoplast (Kuvardina et al., 2002; Moore et al., 2008).

The presumed origin of the piroplasmid tick host has been dated at about 300 Ma (CI, 325–275 Ma), and that of mammalian and bird hosts at 120 Ma and 220 Ma, respectively (Dunlop and Seldon, 2009; Jeyaprakash and Hoy, 2009; Parfrey et al., 2011). Molecular clock estimates of the origin of piroplasmids vary within a wide range from 820 to 17 Ma, and are obviously afflicted with considerable uncertainty. Available studies can be divided into (i) those designed to infer early eukaryotic diversification, resulting in higher estimates (820–430 Ma, Escalante and Ayala, 1995; Douzery et al., 2004; Berney and Pawlowski, 2006; Parfrey et al., 2011,), and (ii) those designed to infer piroplasmid evolution (or *Plasmodium*/piroplasmid divergence), resulting in lower estimates (300–17 Ma, Criado-Fornelio et al., 2003b; Silva et al., 2011; Lack et al., 2012). These latter estimates may be considered more reliable, as they focus on a smaller timescale, have been specifically calibrated for the purpose, and often take into account various _distinctive features of piroplasmid evolution (e.g. host switching and rate variation within genes or lineages). Further support for the validity of lower estimates (300–17 Ma) is the presence of the dixenous life cycle as a defining characteristic of piroplasmids, as its origin must be dated after the appearance of their tick and vertebrate host.

Based on the notion that the MRCA of Apicomplexa infected an invertebrate host, it may be most reasonable to assume that the ancient piroplasmid lineage parasitized an invertebrate tick-predecessor in a monoxenous lifestyle (Leander, 2007). An unexpected finding does strongly support this view: the apicomplexan *Cardiosporidium cionae* has lately been redescribed and shown to feature a monoxenous life style in the invertebrate ascidian *Ciona intestinalis*

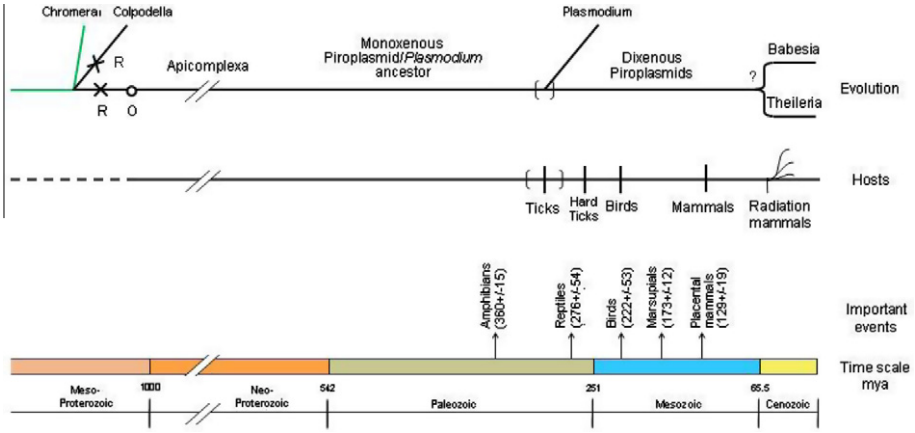

Fig. 2. The evolution of apicomplexan piroplasmids in relation to their vertebrate and invertebrate host. The lower timeline represents the geological timescale with the estimated origin of major vertebrate extant lineages (important events). The middle timeline marks the origin of ticks and their currently known vertebrate hosts (hosts). The upper timeline (evolution) depicts important evolutionary events during Apicomplexa and piroplasmid evolution. *Evolution:* independent loss of photosynthesis (R) according to Moore et al. (2008). Origin of apicomplexa (O) according to Parfrey et al. (2011). Divergence of piroplasmids and *Plasmodium* (300 Ma) as determined by Silva et al. (2011). *Hosts:* origin of ticks at about 300 Ma (ticks: Ixodida) and 240 Ma (hard ticks: Ixodidae) as estimated by Jeyaprakash and Hoy (2009). The depicted divergence time of *Theileria* and *Babesia* is hypothetical. Origin of birds and mammals as estimated by Kumar and Hedges (1998). Radiation of mammals (60 Ma) as determined by Young (1962), Kurten (1974) and Palmer (1999). *Important events:* the dating of important events are based on the molecular date estimates by Kumar and Hedges (1998), Heckman et al. (2001), Hedges and Kumar (2003) and Pisani et al. (2004). Fossil date estimates according to Dunlop and Selden (1998). Time scale: the stratigraphic time scale is given as recommended by the International Commission on Stratigraphy (ICS).

A phylogenetic and ultrastructural analysis identified this parasite as a piroplasmid, yet subsequent work suggests that *C. cionae* belongs with *Nephromyces* to a closely related sister clade of piroplasmids (Ciancio et al., 2008; Saffo et al., 2010). Thus, the monoxenous life cycle of *C. cionae* seems to be an ancient feature that likely was also exhibited by the MRCA ancestor of piroplasmids and the *C. cionae/Nephromyces*-sister clade.

Further biology and hosts of piroplasmids during the following rather long evolutionary timespan likely will remain speculative, as paleontological evidence cannot be expected. However, this lineage may have eventually led to the MRCA of ticks and their phylogenetic sister group, the Holothyridae mites. It has been proposed that their ancestor entertained a scavenger lifestyle like Holothyridae mites, by feeding on body fluids of dead organisms, before adopting a, presumably more profitable, blood-feeding tick lifestyle (Dobson and Barker, 1999). With the start of the parasitic life of the tick, piroplasmids may have evolved their invertebrate–vertebrate dixenous lifestyle, which greatly facilitated their rapid transmission within host populations.

The recent finding of the monoxenous apicomplexa *C. cionae* in the ascidian *C. intestinalis* does, however, allow an alternative view on the evolutionary lifespan before the start of the dixenous life history trait of piroplasmid parasites. *C. intestinalis* has been recently re-considered as a simplified chordate ancestor (Chourrout et al., 2006; Delsuc et al., 2006). It can thus not be excluded that the MRCA of *C. cionae* and piroplasmids may represent an early predecessor of a hypothetical vertebrate-parasitizing monoxenous piroplasmid (Ciancio et al., 2008; Saffo et al., 2010). Based on the probably ancestral state of their transstadial – compared to transovarial – transmission in the tick, a vertebrate origin of piroplasmids has also been recently suggested by Lack et al. (2012), because transstadial (in contrast to transovarial) transmission does not allow the passing on of piroplasmids in a vertical mode into the next tick generation.

Notwithstanding, the evolution of tick feeding as described above would have likewise resulted in the dixenous tick–vertebrate life cycle of piroplasmids. So far, systematic studies of the diversification and host switching of piroplasmids in relation to their tick vector have not been carried out, even though they might reveal new perspectives on their joint evolutionary history. Although host switching may obscure co-diversification, its dimension in the tick is unknown and may be in a range that allows obtaining valuable information as has been demonstrated for *Plasmodium* (Black and Piesman, 1994; Ricklefs and Fallon, 2002; Ricklefs et al., 2004; Mu et al., 2005; Martinsen et al., 2008; Garamszegi, 2009; Janouskovec et al., 2010; Mans et al., 2011).

Recently it has been shown that *Nuttalliella namaqua*, the only species of the tick genus *Nuttalliella*, may represent a "living fossil" of ticks (Mans et al., 2011). Its semi-arid habitat (Namaqualand and the Karoo, Northern Cape, Africa) has been maintained since Permian times (300–250 Ma) and places the origin of ticks in South Africa. *N. namaqua* has been shown to feed on lizards but not on mammals. Mans et al. (2011) assume that *N. namaqua* fed originally on therapsids (synapsid mammal-like reptiles) that are reported to have lived at this time in this region. After therapsid lineages were replaced by diapsids, switching to lizards as preferential hosts may have occurred. Accordingly, lizards (and possibly other diapsids) should have been some of the major host species parasitized by hard and soft ticks, until much later a host switch to mammals occurred. The subsequent adaptive radiation of mammals might have resulted in the prevailing presence of piroplasmids as reported today in this vertebrate group. However, it cannot be excluded that the present picture is distorted due to an extreme sampling bias, since studies of piroplasmid infections are largely missing in reptiles and other vertebrate hosts such as marsupials and birds (Peirce, 2000; Criado-Fornelio et al., 2004; Lack et al., 2012; Paparini et al., 2012; Rong et al., 2012). In this context, it may be worth investigating whether *N. namaqua* harbors and transmits piroplasmid parasites. One might expect to find early branching ancestral piroplasmid species, which may shed light on the early evolutionary history of this group of organisms.

Essentially, two relatively well defined phenotypic characteristics may have been the product of more recent piroplasmid evolution: (i) the presence of a schizont propagation cycle as a derived character state *vs.* its absence, and (ii) the presence of transovarial transmission, which may be considered a derived character state *vs.* its absence (Kakoma and Mehlhorn, 1994; Mehlhorn et al., 1994; Freudenstein, 2005). Based on these character-state definitions, three lineages of piroplasms may be cautiously distinguished: a first lineage that lacks schizonts and undergoes a transstadial transmission (*Babesia* sensu latu (s.l.)) (note that the presence of schizonts has never been conclusively demonstrated in this group; Mehlhorn and Schein, 1984; Thomford et al., 1993; Kjemtrup et al., 2006; Conrad et al., 2006); a second group that displays a schizont propagation cycle and transstadial transmission (*Theileria*); and a third group that lacks schizonts and exhibits transovarial transmission (*Babesia* sensu strictu (s.s.)).

In an evolutionary context, the *Babesia* s.l. lineage may have an ancient origin, as it possesses two ancestral character states, while the *Theileria* and *Babesia* s.s. lineages each own a derived character state that probably facilitated the conquest of new ecological niches. The *Theileria* lineage possesses a pronounced schizont propagation cycle, thus creating an additional amplification cycle within the vertebrate host, promoting piroplasmid proliferation, and in this way securing parasite transmission into the feeding tick. The *Babesia* s.s. lineage lacks schizonts but evolved transovarial transmission, a feature that enables vertical transmission of the parasite when infected vertebrate hosts are not available. Each of these characteristics ensures a fast and efficient propagation and have likely contributed to the competitive edge of these two lineages to parasitize livestock populations. Based on the above evolutionary history of piroplasmids and their invertebrate and vertebrate hosts, how can current research findings accommodate this framework, and what can be learned?

First, the evolutionary timeframe of piroplasmid–invertebrate interaction is likely to be as long or even longer than the vertebrate–piroplasmid one. This allows the educated guess that the molecular interface between ticks and piroplasmids may be at least as intricate and complex as that between vertebrates and piroplasmids. Due to our anthropocentric view focused on the pathogenic effects caused by piroplasmid infection of livestock and humans, we may tend to underestimate the complex part of the piroplasmid lifecycle that happens in the tick, including sexual reproduction (Florin-Christensen and Schnittger, 2009).

Second, as outlined in the above evolutionary history, besides *Cryptosporidium*, which does not harbor a plastid organelle, piroplasmids have inherited the most reduced apicoplast known so far (Zhu et al., 2000; Sato, 2011). Compared to *Plasmodium* and *Toxoplasma*, the piroplasmid apicoplast is significantly further reduced, containing exclusively the [Fe–S] cluster assembly and isoprenoid biosynthesis pathways (Brayton et al., 2007; Fleige et al., 2010). Due to its origin, this organelle has bacterial characteristics, and the metabolic pathways it contains have been shown to be vital for parasite survival (Soldati, 1999; Roos, 1999; Yeh and DeRisi, 2011). Therefore it can be targeted by bacterial antibiotics which, as has been demonstrated in *Plasmodium* and *Toxoplasma*, result in a delayed-death response (Dahl and Rosenthal, 2007; Wiesner et al., 2008; Fleige and Soldati-Favre, 2008). Hence, particularly antibiotics that target the bacterial-like protein biosynthesis within the plastid like azithromycin, clindamycin, and tetracycline, are commonly used to treat human babesiosis and have also been shown to be effective in canine babesiosis (Krause et al., 2000;

Wijaya et al., 2000; Krause, 2003; Vannier et al., 2008; Barratt et al., 2010; Aboulaila et al., 2012; Di Cicco et al., 2012; Oz and Westlund, 2012).

Third, our picture of recent piroplasmid phylogeny is still very fragmentary. This is because piroplasmid sampling has been primarily targeted to species that cause pathogenic effects in livestock, dogs and humans and only marginally to wildlife of eutherian mammals (Tables 1–3). However, it is known that piroplasms are transmitted to birds and marsupials, and, as outlined above, possibly also to reptiles or even amphibians (Peirce, 2000; Paparini et al., 2012; Rong et al., 2012). It is therefore likely that hitherto unknown evolutionary lineages may still be identified. Only a more widespread taxon sampling across all potential vertebrate and tick hosts will finally reveal an authoritative and comprehensive phylogeny of piroplasmids (Reichard et al., 2005; Morrison, 2009; Lack et al., 2012).

4. Classical taxonomy

The group comprising *Babesia* and *Theileria* has been baptized "piroplasmids" due to the pear-shaped morphology of the multiplying parasite stage in the blood of the vertebrate host. As non-pigment forming hemoparasites, piroplasmids can be further distinguished from other erythrocyte-infecting genera, such as *Plasmodium* and *Haemoproteus*, which in contrast form pigment (hemozoin) in the parasitized cell (Uilenberg, 2006).

Before the application of molecular methodologies, no less than 111 valid *Babesia* and 39 valid *Theileria* species had been described; and up to now taxonomic classification of piroplasma has been based almost entirely on morphological and biological characteristics (Levine, 1988; Mehlhorn et al., 1994; Reichard et al., 2005). Particularly, the form of transmission and the existence or absence of schizonts have been shown to be valuable phenotypic features to distinguish *Babesia* sensu strictu (s.s.) from *Theileria* (Uilenberg, 2006). Hence, *Babesia* s.s. have been defined by the absence of schizonts and transovarial transmission, while the existence of schizonts and exclusive transstadial transmission placed a parasite in the genus *Theileria* (Mehlhorn et al., 1994; Kakoma and Mehlhorn, 1994).

A third group does exist, which is referred to as *Babesia* sensu latu (s.l.), originally comprising all piroplasmids that could not be unequivocally assigned to either the *Babesia* s.s. or *Theileria* groups. *Babesia* s.l. show only transstadial transmission and therefore do not belong to *Babesia* s.s. The best studied representatives of this group are *Theileria (Babesia) equi* and *Babesia microti*. After re-evaluation and final demonstration of schizonts in lymphocytes, the former *Babesia equi* was later renamed as *T. equi* (Mehlhorn and Schein, 1998). Lymphocyte-infecting schizonts have not been convincingly demonstrated in *B. microti*, and its classification within *Theileria* has remained doubtful due to ultrastructural findings (Mehlhorn and Schein, 1984). For other more recently described *Babesia* s.l. parasites like *B. conradae*, and *B. duncani*, that display a similar ultrastructure to *B. microti*, intra-lymphocytic schizont stages could likewise not be demonstrated (Kjemtrup et al., 2006; Conrad et al., 2006). Phylogenetic investigations have unequivocally confirmed that *B. microti* constitutes a third piroplasmid lineage; and, since then, additional lineages have been revealed and can be classified together with *B. microti* as *Babesia* s.l. (Mehlhorn et al., 1994; Zahler et al., 2000). *Cytauxzoon* is also a tick-transmitted piroplasmid infecting cats that has been separated from *Theileria*, based on the invasion of other types of host cells (endothelial cells) instead of lymphocytes (Barnett and Brocklesby, 1968; Kier et al., 1979). It is noteworthy; however, that Levine (1971) and Mehlhorn et al. (1994) have proposed to relocate *Cytauxzooon* into the *Theileria* taxon since it clearly meets the above outlined effective taxonomic criteria for this group. Possibly, molecular phylogenetic investigations may provide decisive evidence with respect to this matter (see below).

Phenotypic characteristics that often aid in the identification and differentiation of species during microscopical inspection, but have proven to be of more restricted taxonomic value, are piroplasmid size and the number of daughter cells that are formed by merozoite division. On one hand, piroplasms can be distinguished into two groups by their diameter: theileria and small babesia (<2.5–5 μm), and large babesia parasites (>1–2.5 μm). However, species identification can be hampered due to developmental changes in the form and size of parasitic stages in the erythrocyte, and due to the observation that some piroplasmid species may differ in form and size when infecting different vertebrate hosts (pleomorphism) (Homer et al., 2000). Interestingly, corresponding to their size classification, all small babesia (*Babesia* s.l.) as well as all *Theileria* parasites divide into four merozoite daughter cells (a maltese cross formation), while large babesia (*Babesia* s.s.) bud into two merozoite daughter cells. However, with regard to the above criteria, exceptions do occur (for example *B. gibsoni* and *B. divergens* are small in size yet belong to *Babesia* s.s.), and thus this feature is not entirely reliable for taxonomic classification.

Characteristics like invertebrate and vertebrate host specificity are of indicative value in epidemiological investigations but are of limited use for species definition. Although a single tick species is typically the principal vector of a certain *Babesia* or *Theileria* species, this transmission capacity is usually extended to the whole genus. Moreover, often two or even more tick genera can transmit any one piroplasmid species (Mehlhorn et al., 1994; Uilenberg, 2006). It has been also reported that a given tick species transmits two or even more different piroplasmid species (e.g. *Hyalomma a. anatolicum* can transmit *Babesia* sp. Xinjinang, *T. annulata*, and *T. lestoquardi*) (Hooshmand-Rad and Hawa, 1973; Mehlhorn et al., 1994; Yin et al., 2004; Guan et al., 2009).

One vertebrate host may be infected by several different piroplasmid species, and likewise "vertebrate host specificity" is not a reliable taxonomic criterion. Some *Babesia* spp. have a broad host range (e.g. *B. microti*), and numerous reservoir and accidental infections have been recently reported (Zamoto et al., 2004a,b; Criado et al., 2006), which may be facilitated and explained by oligophagy or polyphagy of their tick species (Balashov, 1989; Keirans et al., 1996). The restrictions on reliable parasite identification and classification based on traditional methods, as have been applied in the past, have been especially problematic in wild vertebrate hosts, as species description has not been sufficiently exact for their recognition (Penzhorn, 2006). This becomes evident through the observation that nowadays several species are routinely found by molecular methods in a single wildlife animal, while they could often not have been distinguished by microscopical observation (Nijhof et al., 2003; Reichard et al., 2005; Jinnai et al., 2009, 2010; Oosthuizen et al., 2009).

5. Molecular phylogeny

5.1. Tracing the ancestors: the 18S rRNA gene

In contrast to phenotypic characters, those derived from molecular sequences have the advantage of differentiating morphologically similar species and quantifying these differences. Recently, molecular phylogeny has made and will continue to make major contributions towards revealing evolutionary lineages and relations, and suggesting classification of piroplasmids. Importantly, these contributions have largely confirmed previous taxonomic classifications, based on a limited number of phenotypic characters. In turn, the unraveled monophyletic relationships should finally be reflected in a robust taxonomic classification (Reichard

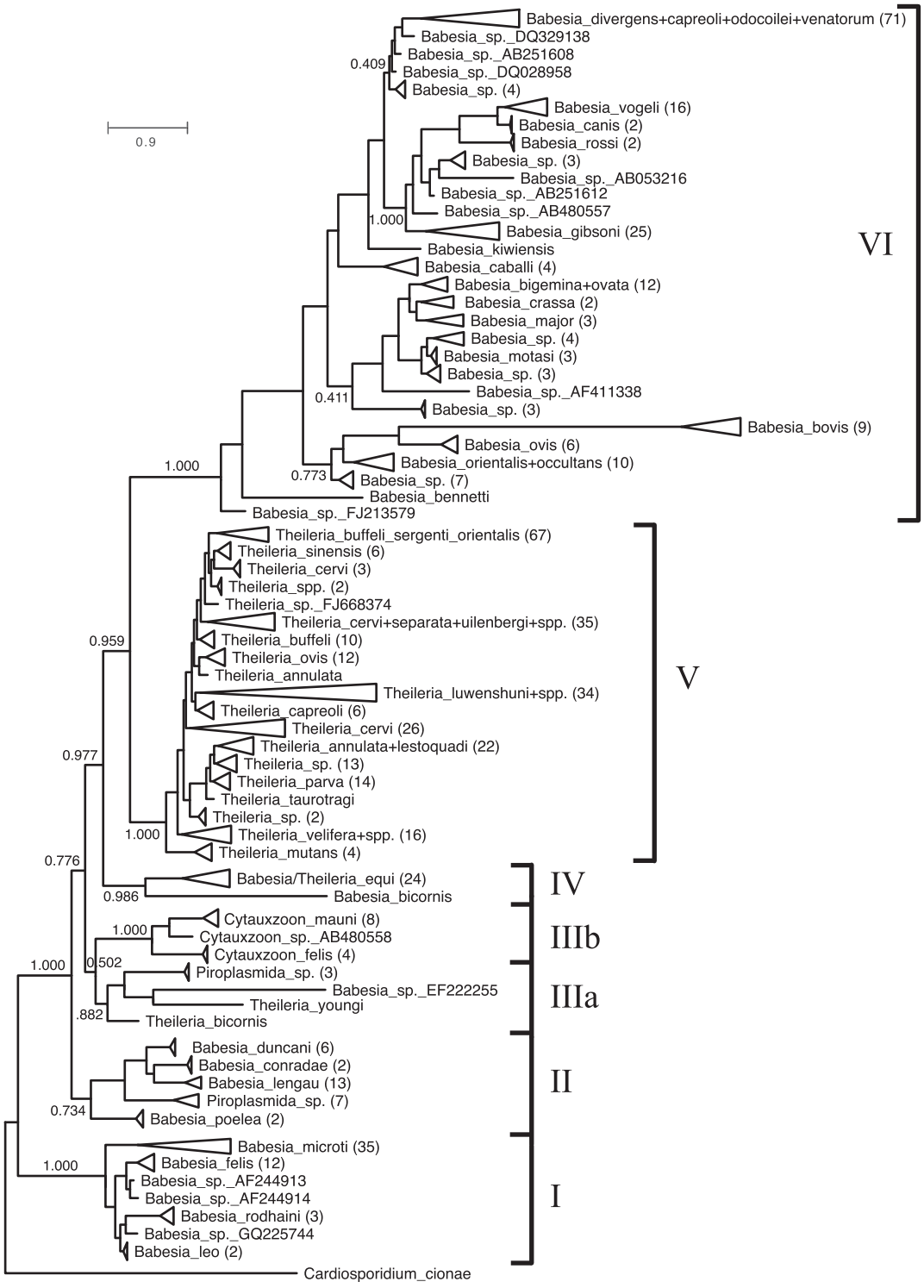

Fig. 3. Consensus tree from the 170,000 trees sampled by the bayesian analysis of 603 18S rRNA sequences of the piroplasmida, with *Cardiosporidium* as outgroup. The scale indicates the inferred number of substitutions. Each sequence label shows the sequence annotation, in some cases also with the accession number. Multiple sequences from the same (or closely related) species have been collapsed if they form a clade, with the numbers in brackets indicating the number of sequences involved. Posterior probabilities are indicated on the main branches. The sequence groups recognized in the text are also indicated.

et al., 2005; Morrison, 2009). This facilitates scientific communication and allows reasonable assumptions of a taxonomic group and its species to be based on their common natural history.

Based on the 18S rRNA gene, we have carried out a comprehensive molecular phylogenetic study using the most advanced methodologies (see Supplement). Almost 600 nearly complete 18S rRNA gene sequences deposited in the public databases were aligned based on all available homology criteria. A bayesian phylogenetic tree was established using a secondary structure model and, for the first time, with a close outgroup to determine the root (Fig. 3). Altogether, six major monophyletic piroplasmid lineages were inferred and are outlined below in the context of the results of other relevant studies (Criado-Fornelio et al., 2003b; Allsopp and Allsopp, 2006; Lack et al., 2012).

(i) *Clade I:* well supported (posterior probability of 1.000) and the sister to all other piroplasmid species, comprising notably rodent-infecting *B. microti* and *B. rodhaini*, and feline-infecting *B. leo* and *B. felis* parasites. Rodents and felines have been considered to be primordial evolutionary hosts (Penzhorn et al., 2001; Criado-Fornelio et al., 2003b). This clade is often referred to as the *B. microti*-group and contains *Babesia* parasites with supposedly ancient features (e.g. lower vertebrate host specificity), with many of its isolates originating from Africa. It is also recognized by Allsopp and Allsopp (2006) (their Group C), Criado-Fornelio et al. (2003b) (their Clade 1, although it is not a monophyletic group in all of their inferred trees), and Lack et al. (2012) (their Clade VIII).

(ii) *Clade II:* moderately supported (posterior probability of 0.734), including *Babesia* spp. isolated from humans, such as the *B. duncani* species isolates WA1, WA2, CA5, and CA6, and human isolates CA1, CA3, and CA4, the latter of which segregate with a *Babesis* sp. isolated from mule deer and bighorn sheep. Human isolates are considered to be accidental infections, and the genuine vertebrate hosts are as yet unknown (Persing and Conrad, 1995; Kjemtrup et al., 2006). Furthermore, this clade contains the canine *B. conradae* species, which corresponds to the former *B. gibsoni* California USA isolate, and *B. lengau* isolated from cheetah in South Africa (Zahler et al., 2000; Conrad et al., 2006; Kjemtrup and Conrad, 2006; Bosman et al., 2010). Apart from the latter, all piroplasmids from this clade have been isolated in the US Western states California and Washington, and the clade is therefore often referred to as the "Western clade". The best described species in this clade, *B. conradae* and *B. duncani*, are indistinguishable in their morphology and ultrastructure from the also human-infecting *B. microti*. Interestingly, *B. poelea*, isolated from a bird (brown booby), represents a sister species to the whole group. The invertebrate and genuine vertebrate hosts of many representatives of this group are poorly known. This clade is also recognized by Allsopp and Allsopp (2006) (their Group B) and Criado-Fornelio et al. (2003b) (their Clade 2), both of whom found greater support for it in their data. In contrast to our monophyletic Clade II, Lack et al. (2012) recognized two well supported clades (posterior probability of each $\geqslant$0.950) that are paraphyletic with respect to each other, namely the "Western clade" (their Clade VI) and a clade containing *B. poelea* (their Clade VII).

(iii) *Clade III:* poorly supported (posterior probability of 0.502), and composed of a Clade IIIa comprised of *Theileria youngi*, *Theileria bicornis*, and a *Babesia* sp. isolated from capybara and a Clade IIIb of *Cytauxzoon* spp. Evidence for the presence of schizonts only exists for *Cytauxzoon* spp. and could not be produced for *T. youngi* or *T. bicornis* (Kjemtrup et al., 2001; Nijhof et al., 2003; Reichard et al., 2005). Due to the presence of schizonts in some species of this group they have been assumed to be closely related to *Theileria* and are therefore sometimes referred to as *Theileria* s.l. Allsopp and Allsopp (2006) recognize Clade III as the monophyletic part of their Group A (which is paraphyletic on their tree), but also with poor support. Criado-Fornelio et al. (2003b) sampled only one species from it and placed it in their Clade 3. The corresponding clade of Lack et al. (2012) (their Clade V) is likewise poorly supported and *T. bicornis* is unplaced in their tree. Nevertheless, in the present analysis, the component Clades IIIa and IIIb are both well supported, notably recognizing the multiple samples of *Cytauxzoon* as a monphyletic group (IIIb).

(iv) *Clade IV:* well supported (posterior probability of 0.986), and containing the piroplasmids *B. bicornis* and *T. equi*. According to this result, *T. equi* can be considered neither a *Theileria* s.s. (Clade V) nor a *Babesia* s.s. species (Clade IV), but represents with *B. bicornis* a separate monophyletic group. Schizonts have been unequivocally identified in *T. equi*, and recognition of Clade IV underscores that schizonts are not a character trait confined to *Theileria* s.s. (Clade V), but represent a more ancient trait (Allsopp et al., 1994). The tree of Allsopp and Allsopp (2006) splits this clade across two of their groups (their *Theileria* group and Group A), while Criado-Fornelio et al. (2003b) sampled only *T. equi* and placed it in Clade 3. In the analysis of Lack et al. (2012), the relationship between *T. equi* (their Clade II), *Theileria* s.s. (their Clade III) and *Babesia* s.s. (their Clade I) is unresolved, and *B. bicornis* is unplaced.

(v) *Clade V:* a strongly supported monophyletic branch of true *Theileria* (*Theileria* s.s.), including the most prominent leukocyte-proliferative representatives *T. annulata*, *T. parva*, *T. lestoquardi*, a non-leukocyte-proliferative *T. buffeli* complex, and *T. ovis*. *Theileria* parasites of this group exclusively infect ungulates. Particularly, leukocyte-proliferative *Theileria* parasites affect the health of sheep and cattle in the Mediterranean region, Asia and Africa. Both Allsopp and Allsopp (2006) and Criado-Fornelio et al. (2003b) found good support for this clade, yet combined it with other species (i.e. *T. equi*, *Cytauxzoon felis*) into their *Theileria* clade and Clade 3, respectively, although with very poor statistical support in both cases. Neither the *Theileria* group of Allsopp and Allsopp (2006) nor Clade 3 of Criado-Fornelio et al. (2003b) were well supported in their analyses, and consist of species that are segregated into several better supported clades in our analysis (Clade IIIa, IIIb, and Clade IV). Thus, our tree topology provides strong evidence for the previously proposed reclassification of some *Babesia* species such as *T. youngi*, *T. bicornis* (Clade IIIa), *C. felis* (Clade IIIb), *B. bicornis* and *T. equi* (Clade IV) (Allsopp et al., 1994; Reichard et al., 2005; Allsopp and Allsopp, 2006). This clade is also well supported by Lack et al. (2012), however, as already mentioned above, the relationship of this clade (their Clade III) with respect to *T. equi* (their Clade II) and *Babesia* s.s. (their Clade I) is unresolved.

(vi) *Clade VI:* strongly supported group of *Babesia* s.s. (posterior probability 1.000). Clade VI contains a well supported branch (posterior probability of 1.000) that includes the canine-infecting *B. gibsoni*, *B. canis*, *B. rossi* and *B. vogeli* species, as well as recently described *Babesia* s.s. species infecting other carnivores (bear, cougar and raccoon) and field rodents, so that it can be referred to as the "carnivore/rodent clade". In the analysis of Criado-Fornelio et al. (2003b), the canine species grouped together with some species infecting ungulates, such as *B. divergens* and *B. odocoilei*, constituting their Clade 4. The latter was thought to represent a more ancient lineage with respect to other *Babesia* species included in other branches of our Clade VI, such as *B. bovis*, *B. bigemina*, *B. caballi* and *B. ovis* (their Clade 5) (Criado-Fornelio et al., 2003b). This is in contrast to our tree topology, which supports the view that this group represents a recently diverged lineage of *Babesia* s.s. The sister clade to the "carnivore/rodent clade" is relatively poorly supported (0.409). It contains the human isolate *B. venatorum*, as well as the cattle-infecting *B. divergens*, which also infects humans in Europe (Herwaldt et al., 2003). Furthermore, the clade contains *B. capreoli*, infecting roe deer, and *B. odocoilei*, which seems to infect a wide range of ruminant cervid and bovid hosts (white tailed-deer, reindeer, elk, yak, muntjac, markhor goat, desert bighorn sheep, musk oxen) (Schoelkopf et al., 2005; Bartlett et al., 2009). Many of these isolates do not group according to their species designation in the database (data not shown). It is not yet clear if 18S rRNA genes are suitable to delineate these species or whether many of the isolates are simply misnamed (Gray et al., 2010; Malandrin et al., 2010).

All other *Babesia* s.s form several clades with varying degrees of support. For example, the cattle-infecting *B. bovis*, the sheep-infecting *B. ovis* and the water buffalo-infecting *B. orientalis* form a group with moderate support (0.773) that is quite separate from the group containing the cattle-infecting *B. bigemina*, *B. major* and *B. ovata* and the sheep-infecting *B. crassa* and *B. motasi*. Interestingly, the horse-infecting *B. caballi* forms a separate group, as also do *B. bennetti* and *B. kiwiensis*, both isolated from birds (seagull and

kiwi, respectively). This paraphyletic assemblage includes piroplasmids of major economic importance (*B. bovis*, *B. bigemina*, *B. caballi* and *B. ovis*).

Clade VI is also recognized by Allsopp and Allsopp (2006) (their *Babesia* s.s. Clade) and by Criado-Fornelio et al. (2003b) (their Clade 4 + 5), both of which presented good support. The tree generated by Criado-Fornelio et al. (2003b) further subdivided the group into their Clades 4 and 5 but, as outlined above, our analysis finds no support for this split. Interestingly, while in our analysis *B. benneti* and a giraffe-infecting *Babesia* sp. form the base of Clade VI (*Babesia* s.s.), Lack et al. (2012) recognize them as a well supported sister clade (their Clade IV) to Clade V (*Theileria* s.s.).

Major features distinguish the presented molecular phylogeny from that of Criado-Fornelio et al. (2003b) and Allsopp and Allsopp (2006) as well as from studies of Persing and Conrad (1995), Homer et al. (2000), Kjemtrup et al. (2001), Penzhorn et al. (2001), Reichard et al. (2005), Birkenheuer et al. (2006), Oosthuizen et al. (2009) and Bosman et al. (2010). First, we have identified Clade IV, containing *T. equi* and *B. bicornis*, as a strongly supported monophyletic group on its own. Second, we have a moderately supported Clade III, which is comprised of two well supported monophyletic branches of some *Theileria/Babesia* spp. of uncertain classification (Clade IIIa), and *Cytauxzoon* spp. (Clade IIIb). Third, in our analysis Clade VI is composed of several small clades, which disagrees with the split of this clade into two equivalent sister groups (corresponding to Clade 4 and 5), as presented by Criado-Fornelio et al. (2003b). Likewise, this split has not been observed in the study of Reichard et al. (2005); and also a tree generated based on heat shock protein 70 (hsp70) does not support this split (see below).

Although clades generally agree between ours and the recent analysis of Lack et al. (2012), some differences in tree topologies are observed: (i) they report an unresolved relationship between Clades IV (*T. equi*), V (*Babesia* s.s.), and VI (*Theileria* s.s.) while we could resolve the placement of these three clades with strong support; (ii) *T. bicornis* and *B. bicornis* are both unplaced in their tree while in ours they are placed with good support into Clade IV and Clade IIIa, respectively; (iii) whereas we have the bird-infecting *B. benneti* and a giraffe-infecting *Babesia* sp. placed at the base of the well supported Clade VI (*Babesia* s.s.), these species are placed in their tree as a likewise well supported sister clade (their small Clade IV) to Clade V (*Theileria* s.s.); and (iv) they split our monophyletic Clade II (posterior probability of 0.734) into their well supported Clades VI ("Western clade") and VII (containing the bird-infecting *B. poelea*) that are paraphyletic with respect to each other.

It is noteworthy that these differences may have been caused by any of the following four methodological differences used in our analysis, as compared to theirs: (i) we used 594 sequences while they used 192; (ii) the closest outgroup for which data are available was used in our analysis while they used a distant outgroup; (iii) our alignment was guided by the use of rRNA secondary-structure information, while they used sequence similarity alone; and (iv) the RNA-structure based doublet model for paired nucleotides was used in our case, while they have used GTR for all nucleotides.

In summary, in our analysis Clade I ("*B. microti*-group"), Clade IIIa (*Theileria* s.l.: a group of *Theileria* and *Babesia* sp. of uncertain classification), Clade IIIb (*Cytauxzoon* spp.), Clade IV (*Theileria* s.l. composed of *T. equi* and *B. bicornis* only), Clade V (*Theileria* s.s.), and Clade VI (*Babesia* s.s. and *B. benneti*) each represent well established monophyletic groups, which may finally form a taxonomic classification. Additional studies may be needed to verify the validity of Clade II, which is only moderately supported in our analysis but is split in two separate clades (the "Western clade" and a *B. poelea*-containing clade) in the analysis of Lack et al. (2012).

Molecular phylogenetic studies greatly improve on studies of detailed biological information (e.g. the invertebrate and vertebrate hosts, transovarial or transstadial transmission, presence of schizonts, parasite ultrastructure) of many *Babesia* and *Theileria* species, and phenotypic characteristics that may support established molecular relationships are lacking for piroplasmids other than *Babesia* s.s., *Theileria* s.s. and *Cytauxzoon* spp. (i.e. piroplasmids pertaining to Clade I–IV), potentially delaying a stable taxonomic classification (Reichard et al., 2005; Lack et al., 2012).

However, *B. duncani* and *B. conradae*, both pertaining to Clade II, were found not to differ from *B. microti* (Clade I) in their known biological characteristics and ultrastructural features (Mehlhorn et al., 1986; Conrad et al., 2006; Kjemtrup et al., 2006). In case phenotypic differences that allow defining these groups are not identified, further evidence based on phylogenetic comparison of multiple concatenated genes may be needed to support their classification. The latter procedure may also allow clarification of whether Clades IIIa and IIIb have a single origin or whether they have different tree placements.

The information emerging from available phylogenetic trees of piroplasmids can significantly contribute to the discussion of the evolutionary history of this taxon that started in Section 3. Thus, by comparison of the inferred molecular phylogeny with known data on the evolution of mammalians, paleogeography and acarology, Criado-Fornelio et al. (2003b) sketched a scenario of piroplasmid evolution. To provide a time scale, calibration of a strict molecular clock was based on the assumption that the origin of *B. caballi* coincided with the appearance of modern equines (7 Ma). In this scenario, the origin of piroplasmids and the occurrence of novel piroplasmid lineages run parallel with the adaptive radiation of placental mammals, starting at about 57 Ma. At this timepoint, the major mammalian lineages consisted of rodents and carnivore ancestors. Correspondingly, Criado-Fornelio et al. (2003b) regard piroplasmids of Clade I as ancient survivors, as they infect felines and rodents, and represent a sister clade to all other piroplasmids. Felines have also been proposed by Penzhorn et al. (2001) as possible primordial piroplasmid hosts. Evidence that piroplasmid evolution might have started in Africa comes from the observation that isolates from African felids are sister to many other lineages. Piroplasmid clades are assumed to have originated with the appearance of their respective mammalian hosts. Clade II piroplasmids (hosts: dog, cervids) evolved with the emergence of carnivores and protoungulates 38 Ma, while piroplasmids of *Theileria* s.s (Clade V) co-evolved with bovines in Africa and Eurasia 20 Ma. The distribution of *Theileria* s.s. is confined to the Old World, corresponding to the geographic distribution of their original bovine host and the more limited host specificity of their tick vectors (Mehlhorn and Piekarsdki, 1993). *Babesia* s.s. (Clade VI) disseminated from Africa to Eurasia and America. Their transmission by tick vectors with broader host specificity possibly facilitated their spread, and their transovarial mode of transmission made them more independent from the availability of an infected vertebrate host, favoring their worldwide dissemination.

However, piroplasmid species infecting avians have not been integrated into the above scenario, yet their appearance in two clades with basic placement in our tree (cf. in three clades in the tree inferred by Lack et al., 2012) suggests that they may have played an important role in piroplasm phylogeny (Fig. 3, Clades II and VI). This is further supported by the recognition of at least thirteen additional avian infecting *Babesia* species (Peirce, 2000).

As mentioned above, Criado-Fornelio et al. (2003b) calibrated their molecular clock based on the assumption of codivergence of *B. caballi* and modern equines (7 Ma). However, piroplasmid phylogeny suggests frequent switching of the vertebrate host, thus not allowing the assumption of co-divergence to calibrate piroplasmid evolution (Schnittger et al., 2003; Lack et al., 2012).

Furthermore, Lack et al. (2012) demonstrated extreme rate variation between piroplasmid clades and lineages, making the assumption of a strict molecular clock erroneous.

To avoid these unwarranted assumptions and to improve the evolutionary time scale, Lack et al. (2012) used a relaxed molecular clock and calibrated piroplasmid evolution independently of the host phylogeny. For this purpose, a combined alignment of piroplasmid with mammalian *Plasmodium* 18S rRNA sequences was created and the inferred phylogeny calibrated with 12.8 Ma (CI: 19.5–9.9 Ma), as recently determined for the radiation of mammalian *Plasmodium* by Ricklefs and Outlaw (2010). Using this approach, the origin of piroplasmids was determined to be 20 Ma (HPD, highest posterior density: 32–11 Ma) and their diversification 17.1 Ma (HPD: 25–10 Ma). The latter time point coincides with the diversification of rodents and ungulate "grazers" during the middle Miocene, and they have been proposed as the MRCA of piroplasmid hosts (Fig. 3, Clade I).

Recently, a comprehensive study of divergence times of malaria parasites was carried out using 29 slowly evolving proteins (Silva et al., 2011). Co-speciation of *Plasmodium falciparum* and *Plasmodium reichenowi* with their hosts was tested and subsequently used to calibrate the phylogeny. Silva et al. (2011) provide evidence that, due to saturation of mitochondrial cytochrome *b*, sequences Ricklefs and Outlaw (2010) considerably underestimated *Plasmodium* divergence times, thus invalidating the timescale used by Lack et al. (2012). According to Silva et al. (2011), rather than at 12.8 Ma (CI: 19.5–9.9 Ma), mammalian *Plasmodium* radiation took place substantially earlier than 67–22 Ma and, by calibration with the corresponding factor, the origin of piroplasmids must be estimated well over 104–34 Ma. Correspondingly, Silva et al. (2011), also relaxing their molecular clock and calibrating their tree based on the estimated divergence of *P. falciparum* and *P. reichenowi*, determined the divergence time of piroplasms and *Plasmodium* at 310–290 Ma. Nevertheless, in either case the calibration of piroplasmid evolution based on divergence time estimates derived from *Plasmodium* harbors a considerable uncertainty, as the pattern of their evolution might differ significantly (e.g. with respect to the frequency of host switches, rate of molecular evolution, etc.). However, based on the above outlined considerations it seems to emerge that the time of piroplasmid origin may be considerably earlier than assumed by Lack et al. (2012) and Criado-Fornelio et al. (2003b).

Using 11 protein coding genes of the mitochondrial genome, Jeyaprakash and Hoy (2009) estimated the origin of ticks (Ixodida) at about 300 Ma (CI: 325–275 Ma), and suggested that amphibians might have been their first vertebrate hosts. According to this study, hard ticks (Ixodidae) appeared about 240 Ma, much earlier than the first placental mammals (120 Ma) and earlier than birds (220 Ma). The estimate of 300 Ma (310–290 Ma) as determined by Silva et al. (2011) places the origin of piroplasmids in a similar timeframe as that of ticks. Based on this estimate, piroplasmid lineages have co-evolved with early tick lineages and their vertebrate hosts rather than with modern placental mammalian hosts. Thus, major piroplasmid lineages may have already existed when modern mammals appeared. A multiple host switch of already evolved tick and piroplasmid lineages from other vertebrates (amphibians, reptiles, birds, marsupials) to placental mammalians may have occurred, facilitated by the oligophagy or polyphagy of ancient tick lineages. Evidence for such a multiple host switch across vertebrate lineages is the segregation of piroplasmid species infecting birds into different clades of the phylogenetic tree contributing to the high estimates of piroplasmid origin determined by some studies (820–430 Ma, see Section 3). Subsequent adaptive radiation of mammals may have further driven the evolution into the multiple piroplasmid lineages observed today, as outlined above. Most noteworthy, well studied mammalian host species are shown to be infected by a number of different piroplasmid species that are of polyphyletic origin, demonstrating that independent host switches of tick species at different time points resulted in piroplasmid speciation in the same vertebrate host. Previous lines of piroplasmids either may have been extinguished or may have not yet been sampled in vertebrate host lineages of more ancient origin. As mentioned in Section 3, ancient sister piroplasmid lineages might still be encountered in marsupials, birds, reptiles and amphibians as their potential progenitor vertebrate host lineages. This assumption is likewise corroborated by the recent isolation of *B. poelea* and *B. bennetti* from birds that both represent sister species of their respective Clade II and Clade VI (Fig. 3; Lack et al., 2012). As the time estimates are tentative, it is currently impossible to reconcile in detail all of these findings. However, a possibly improved estimation of the molecular divergence time of the origin of different piroplasmid species based on multiple protein coding genes, and detailed comparison of the origin and divergence of piroplasmid species lineages in relation to their host tick species, may reveal the occurrence of major past host switches, and shed further light on this issue.

5.2. Tracing the ancestors: other genes

Although the 18S rRNA gene has been utilized as the main data for elucidating phylogenetic relationships of piroplasmid parasites, other genes have also proved useful. Paraphyly of the genus *Babesia* has been demonstrated by phylogenetic analysis using the hsp70 gene (Ruef et al., 2000). In two recent studies, trees were established to analyse the phylogenetic position of canine *Babesia* spp. and *B. orientalis*, respectively (Yamasaki et al., 2007; He et al., 2009). The established trees largely correspond to those inferred by us from the 18S rRNA gene, and distinguish a group A (cf. Clade I), group B (cf. Clade IV and V), and a group C (cf. Clade VI) (Fig. 3). Clade II and III piroplasmids were not included as their hsp70 gene sequences are not yet available. Notably, hsp70 genes did not segregate into the sister clades (Clades 4 and 5) as defined by Criado-Fornelio et al. (2003b) but rather confirmed the topology of the Clade VI of the presented 18S rRNA tree. Furthermore, a tree established in the context of this review using all currently available hsp70 genes likewise agrees with the presented 18S rRNA gene tree topology of Clade VI except that (i) in the 18S tree *B. bovis* is the sister to *B. ovis*, and that (ii) in the 18S tree the "*B. ovis*-clade" is the sister to the rest of *Babesia* s.s (Fig. 4).

The intergenic spacer region (ITS) displays a significantly higher variability than does the 18S rRNA gene, and facilitates distinguishing isolates when differentiation by 18S rRNA gene is unreliable or impossible (Collins and Allsopp, 1999). Conversely, this DNA region is too variable to study the relationships between distant species. Zahler et al. (1998) used the ITS region to unequivocally distinguish between the closely related *B. canis*, *B. rossi* and *B. vogeli*. It is noteworthy, however, that the 18S rRNA genes can also clearly distinguish these canine *Babesia* species (Eiras et al., 2008; see also Fig. 3). Phylogenetic analyses using the ITS region was also performed to characterize the cheetah-infecting *Babesia lengau*, as well as to identify the previously unknown sheep-infecting *B.* sp. (Kashi) species (Niu et al., 2009; Bosman et al., 2010). Furthermore, bovine isolates could be unequivocally assigned to either the *B. major* or *B. ovata* species in a recent study employing ITS sequences (Liu et al., 2008).

The higher resolution power of the ITS sequence at smaller evolutionary distances compared to 18S rRNA genes was recently convincingly demonstrated in a study where cattle isolates were compared to isolates from chamois and roe deer (Schmid et al., 2008). 18S rRNA sequences were highly similar, not allowing confident differentiation between isolates. In contrast, comparison of their ITS region showed clear significant differences between cattle

Fig. 4. Consensus tree from the 54,000 trees sampled by the bayesian analysis of 36 hsp70 sequences of the piroplasmida. The scale indicates the inferred number of substitutions. Each sequence label shows the accession number and the sequence annotation. Posterior probabilities >0.3 are indicated on the branches. This tree agrees with the 18S tree except: (i) in the 18S tree *B. bovis* is the sister to *B. ovis*, which does not occur in any of the hsp70 trees sampled; and (ii) in the 18S tree the *B. ovis* clade is the sister to the rest of *Babesia*, which position has a posterior probability of 0.345 in the hps70 analysis.

isolates and isolates of free-ranging ruminants. This finding supports the use of the name *B. capreoli* for piroplasmids infecting free-ranging ruminants to distinguish them from *B. divergens*-like isolates of cattle (Schmid et al., 2008; Malandrin et al., 2010). Due to their high variability, ITS sequences cannot be used to construct a species tree (data not shown).

Although only a handful of sequences have been determined, the mitochondrial cytochrome *b* (*cob*) and cytochrome *c* subunit I (*cox1*) genes have been utilized to distinguish piroplasmid species in two studies. It was observed that sequence variation of the cytochrome *b* gene is higher than that of the 18S rRNA gene which may confer advantages for comparisons at or below the species level (Criado et al., 2006). In another study, *cob* and *cox1* aminoacid sequences were concatenated and a phylogenetic analysis carried out, yet the placement of *T. equi* with respect to *Theileria* and *Babesia* s.s. could not be resolved (Hikosaka et al., 2010).

5.3. A piroplasmid tree: the way ahead...

The establishment of a phylogeny of piroplasmid parasites is not an end in itself. In the last two decades, species classification of the overwhelming number of novel *Babesia* isolates has been carried out by phylogenetic analysis of their 18S rRNA sequences. However, it is evident that only a robust widely accepted phylogeny can serve as a sound backbone for species classification and identification. This calls for a further improved elucidation of the evolutionary relationships of piroplasmid parasites. In addition to phylogenetic analysis, cornerstones that need to be met to obtain a robust phylogenetic tree for apicomplexan parasites have been outlined in detail and comprise: (i) taxon sampling, (ii) character sampling, (iii) character interpretation, and (iv) directed data collection (Morrison, 2008, 2009). These cornerstones apply also for the construction of a robust and well-accepted phylogeny of piroplasmid parasites and will be briefly discussed.

Sampling of piroplasmid isolates is done in most cases with a focus on medical (human babesiosis) or veterinary (especially bovine, equine, ovine and canine babesiosis) research questions. However, this results in biased sampling of taxa, which may consequently lead to biased estimates of phylogenetic relationships (Zwickl and Hillis, 2002; Pollock et al., 2002). Only a phylogenetically adequate taxon sample array can finally result in well-defined taxonomic boundaries. This may be demonstrated by some

recently sampled novel piroplasmid species of wildlife mammals and birds (*Cytauxaoon* sp., brown bear, *Babesia bicornis*, black rhinoceros; *B. vesperuginis*, bat; *B. poelea*, brown booby; *Babesia kiwiensis*, kiwi; *B. benneti*, sea gull) (Nijhof et al., 2003; Concannon et al., 2005; Criado et al., 2006; Yabsley et al., 2006; Jeffries et al., 2008; Jinnai et al., 2010), some of which might be "seed crystals" of novel clades since they either form, as one single clade, a novel sister group to an already established clade, or they seem to be associated to less well-defined groups (*Theileria* s.l.) (cf. Fig. 3 and Lack et al., 2012). Thus, as already mentioned in other sections, sampling of piroplasmids in wildlife animals such as birds, marsupials, reptiles and amphibians, and also in diverse tick species, may result in a less biased and more complete phylogenetic tree displaying additional clades.

Primarily the 18S rRNA gene has been used to explore phylogenetic relationships of piroplasmids. However, one has to keep in mind that a single gene tree does not necessarily reflect a species tree. For this reason a tree should ideally be constructed using multiple genotypic characters of potentially different evolutionary histories. Recently, a tree of malaria parasites (*Plasmodium* and related genera) was generated based on four genes: a nucleus genome-encoded (adenylsuccinate lyase), an apicoplast genome-encoded (caseinolytic protease), and two mitochondrial genome-encoded genes (cytochrome *b*, cytochrome oxidase I) (Martinsen et al., 2008). Based on this work it was possible to reconcile phenotypic traits, like life history and host switches, of malaria parasites (*Plasmodium* and related genera) with the generated phylogenetic tree, allowing further insight into the evolutionary history of this hemosporozoan taxon (Perkins et al., 2011). Such an approach may have the potential to resolve the observed unclear phylogenetic relations with respect to the *Theileria* s.l. group, and possibly allow re-interpretation of contradictory phenotypic characters (Allsopp and Allsopp, 2006; Oosthuizen et al., 2009). A prerequisite for the corresponding investigations of piroplasmid parasites would be the availability of genomic DNA of known and characterized piroplasmid taxa. This could be facilitated by purposeful collaborative efforts among the piroplasmid research community.

Eventually, piroplasmid phylogeny should reconcile morphological and molecular data (Perkins et al., 2011). After robust trees have been established using genotypic characters, phenotypic characters need to be re-evaluated and, if necessary, convincingly re-interpreted in the context of the obtained molecular phylogeny (Allsopp and Allsopp, 2006; Martinsen et al., 2008). Only after a conclusive re-interpretation of traditional characters, the obtained phylogeny will be generally accepted by the research community (Morrison, 2009).

In summary, it is evident that with respect to taxon sampling and character sampling a collaborative effort would be needed to fast-track a comprehensive elucidation of the phylogenetic relationship of piroplasmids. Taxon sampling needs to be done independently of the pathogenicity of piroplasmid infection, in a wide variety of potential vertebrate and invertebrate hosts. Genomic DNA of the sampled taxa needs to be made available to determine multiple gene sequences that have been identified to be most apt for tree construction for these taxa. Aliquots of genomic DNA that have been isolated from piroplasmids in the past, or will be isolated in the future, must be stored and exchanged in a centralized way. Such a large undertaking will likely involve the creation of an informally or formally appointed dedicated consortium (Morrison, 2009).

6. Molecular taxonomy

As has been outlined above, the identification and definition of an increasing number of piroplasmid lineages and species cannot be achieved by the relatively few available phenotypic characters. As a steadily increasing number of piroplasmid species are described, molecular taxonomic approaches to define and outline species and higher taxon boundaries will become increasingly important (Blaxter, 2004; Blaxter et al., 2005; DeSalle et al., 2005; Hajibabaei et al., 2007). It is essential to realize that accepting this approach means also accepting the phylogenetic species concept (PSC) that "species are composed of a monophyletic group of the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent", rather than the biological species concept (BSC) stating that "species are members of populations that actually or potentially interbreed in nature" (Dobzhansky, 1937; Cracraft, 1983; de Meeûs et al., 2003; Tibayrenc, 2006). One inherent problem of this approach is to define the threshold of molecular difference under which two isolates belong to the same species. In fact, any threshold can be indefinitely shifted until molecular differences finally distinguish between individuals, as no universally valid criterion for threshold establishment exists.

A large number of methodologies are available for molecular taxonomy. They may be methodologically divided into those that use primers to amplify single or multiple organism-specific sequence regions (e.g. 18S rRNA gene comparisons, multilocus-typing using micro and minisatellites), and others that screen for polymorphisms along the genome using primers that do not target specific sequences of the organism under study (e.g. amplified fragment-length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD)). Of these two approaches, only the former has been successfully applied to piroplasmids, due to the simple fact that extracted parasite DNA is, in virtually all cases, heavily contaminated with host DNA. For this reason, molecular taxonomy in piroplasmid research is mostly based on a phylogenetic analysis using the 18S rRNA gene with the purpose to: (i) assign isolates to a known species, (ii) define and identify a species based on isolates, (iii) identify multiple piroplasmid species infections in a single host, and (iv) provide an evolutionary framework for taxonomic classification and comparative analysis (see Section 5.1). In addition, hypervariable regions can be used as DNA barcodes to (v) detect simultaneously the infection of different piroplasmid species in small and large ruminants by reverse line blotting, integrating molecular epidemiology and phylogeny (Gubbels et al., 1999; Schnittger et al., 2004; Hajibabaei et al., 2007). The following studies show the advantages of molecular taxonomy with respect to traditional methods of species identification:

(i) Clinical cases of babesiosis in water buffaloes have been exclusively reported in the Asian region, and were thought to be caused by *B. bovis* (Dwivedi et al., 1979; Shen et al., 1997). Only the recent use of a molecular phylogenetic analysis allowed the parasite to be recognized as a novel species, which was then named *B. orientalis* (Liu et al., 2005, 2007b). Applying traditional diagnostics, it had escaped notice for decades that another *Babesia* species rather than *B. bovis* causes clinical disease in water buffaloes. Accordingly, in South America in a region devoid of *B. orientalis*, *B. bovis* infects water buffaloes, but no babesiosis clinical signs have ever been reported in these animals (Ferreri et al., 2008).

(ii) Recently, a phylogenetic analysis revealed that altogether three *Theileria* parasites are transmitted by the tick *Haemaphysalis quinghaiensis*, one infecting yak (*T. sinensis*) and two infecting sheep (*T. luwenshuni*, *T. uilenbergi*) (Schnittger et al., 2003; Yin et al., 2004, 2007). The latter two are highly pathogenic for sheep, causing serious economic losses in China, and were thought to belong to a single species. Before a phylogenetic analysis was carried out, they could not be distinguished by any of the known traditional methods (Yin et al., 2004).

(iii) In an attempt to determine the cause of death of five South African giraffes, at least two novel *Babesia* spp. and three novel

Theileria spp. have been recently identified on the basis of their 18S rRNA sequences. One of the identified *Babesia* spp. may be conspecific with a sheep-infecting *Babesia* sp. recently described in China (Xinjiang) (Table 1). Due to the morphological similarity of piroplasmid parasites, these could not have been differentiated microscopically (Oosthuizen et al., 2009).

The following depiction may outline how molecular taxonomy of piroplasmids is in constant development and flux. Despite its unprecedented success in species designation, 18S rRNA gene sequence analysis has shown limitations to unequivocally define certain zoonotic piroplasmid strains that are likely concealed behind *B. divergens* and *B. microti* (Gray, 2010). Originally, *B. divergens* isolates from humans and deer were shown to differ in their 18S rRNA sequences by three or more nt from those of bovine, and were found not to infect splenectomized calves or bovine erythrocytes *in vitro* (Olmeda et al., 1997; Centeno-Lima et al., 2003; Duh et al., 2005; Langton et al., 2003; Hilpertshauser et al., 2006). Recently though, variations of 1–2 nt have been observed within *B. divergens* isolates from bovines, obscuring the minor sequence differences with respect to human *B. divergens* isolates (Vogl, S.J., Zahler-Rinder, M.M., unpublished, Accession Nos. EF458219–EF458229; Beattie et al., 2002; Holman, 2006). It has been furthermore observed that a *B. divergens* isolate from humans differing only in 1 nt from a bovine isolate was infective for Mongolian gerbils, which are considered highly susceptible to bovine isolates of *B. divergens* (Zintl et al., 2003). Taken together, it seems that 18S rRNA differences may not be suitable to clearly differentiate between *B. divergens*-type parasites from these different hosts, and other more sensitive molecular markers need to be developed and employed. However, it has been recently shown that *B. capreoli* infecting roe deer can clearly be distinguished from *B. divergens* based on a 3 nt difference in their 18S rRNA genes, and do not pose a threat to humans (Malandrin et al., 2010).

In the case of *B. microti*, similar limitations have already led to the use of other genes able to further subdivide parasites with different zoonotic potential, and thus possibly unmask novel species. *B. microti* as a causative agent of emerging human babesiosis has motivated the study of potential host reservoirs in several countries (USA, Switzerland, Spain, Russia, Japan and China) (Rar et al., 2010a,b). During the last decade, it has become clear that *B. microti* infects a large variety of different hosts worldwide (many different small rodent species; carnivores, such as raccoon, skunk, canine, fox, river otter; macaque; and humans), and does not constitute a single species as previously assumed, but a variety of different species currently referred to as the *B. microti*-species complex. This was first shown by comparison of 18S rRNA and tubulin-β genes of different *B. microti*-isolates and their segregation into two clades and a third paraphyletic group (Goethert and Telford, 2003). One clade (A) contained rodent piroplasmids and most of the zoonotic strains, while a second clade (B) comprised presumed non-zoonotic rodent parasites. The third paraphyletic group (C) consisted of parasites isolated from carnivores, including dogs. The higher variability of the tubulin-β gene with respect to the 18S rRNA gene allowed the generation of a more robust and better-supported tree, suggesting the existence of two new *B. microti*-lineages, Kobe- and Hobetsu-type, while confirming a US-type (cf. clade A and B) (Tsuji et al., 2001; Zamoto et al., 2004a,b). Later, an additional fourth *B. microti* "Munich-type" lineage of European isolates was suggested, since their sequence identity was low in comparison to the isolates that were included in the other three groups (Gray, 2006; Tsuji et al., 2006).

Noteworthy, in our hands the 18S rRNA genes did faithfully segregate into the above mentioned clades/groups with the exception of the Kobe-type, yet sometimes with relatively low support (Fig. 5). Although the tubulin-β genes have been reported to be better suited to differentiate these isolates than 18S rRNA genes, bootstrap values are still not satisfactory for all branches (Fig. 6, Supplement). To allow a better discrimination, the chaperonin-containing t-complex polypeptide 1 (CCT7) gene was sequenced and used for a phylogenetic analysis of *B. microti* isolates (Nakajima et al., 2009). Indeed, four zoonotic *B. microti*-lineages referred to as US-type clade, Munich-type clade, Kobe-type clade, and Hobetsu-type clade, could thus be convincingly distinguished. The US-type isolates were found to be highly variable, and possibly represent an overlapping array of variant isolates distributed in North America, Europe and Asia, including isolates of clades A and B described above (Zamoto et al., 2004b; Goethert and Telford, 2003). The Kobe-type isolates are confined to a few spotted regions

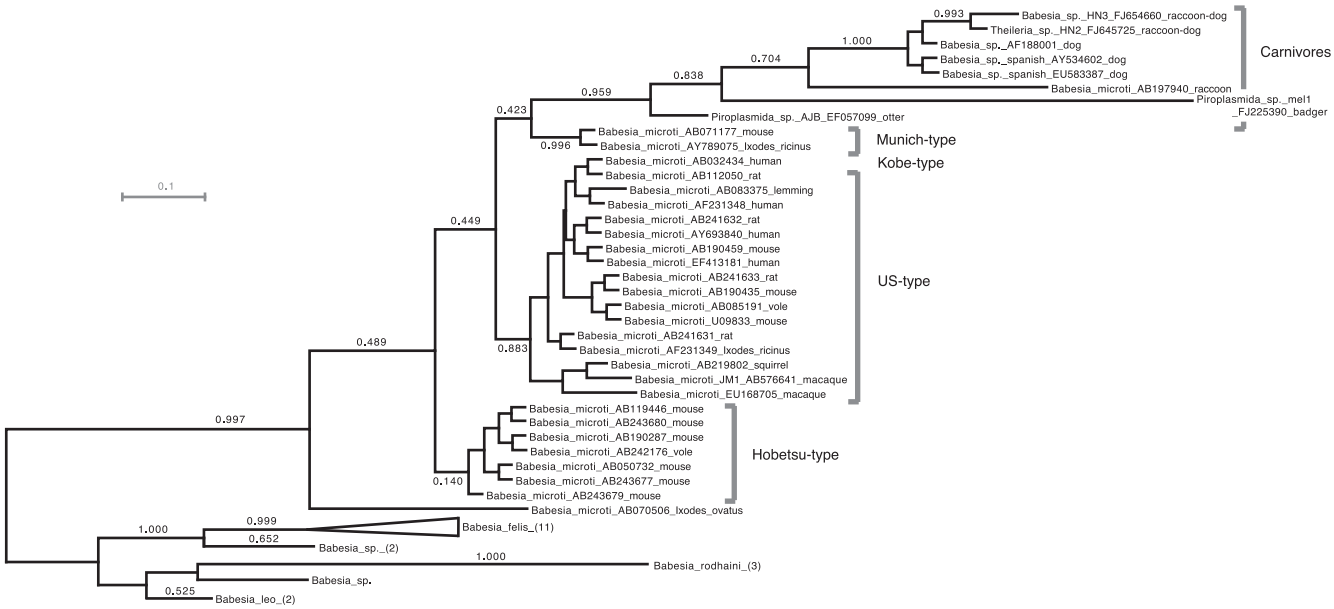

Fig. 5. Details of the *Babesia microti* part of the 18S rRNA gene tree. The scale indicates the inferred number of substitutions. Each sequence label shows the identification, the accession number and the reported host. The outgroup sequences have been pooled by species, with the number in brackets indicating the number of sequences pooled. Posterior probabilities >0.4 are indicated on the branches. The sequence groups based on the tubulin gene are also indicated.

of Japan, while the Hobetsu-type seems to be common throughout this country. In contrast, isolates of the Munich-type are endemic in Europe (UK, Germany, Poland). In the same study, the CCT7 gene was shown to contain six introns, of which the fifth differed in length between isolates of the four lineages (US-type, Munich-type, Kobe-type, and Hobetsu-type) while it was found to exactly correspond in size within each lineage, providing strong evidence that these four lineages represent species (Tsuji et al., 2001; Zamoto et al., 2004a; Nakajima et al., 2009). In a subsequent work, a phylogenetic analysis compared concatenated highly variable intron sequences of the CCT7 gene between isolates of each of the four identified *B. microti* species (Fujisawa et al., 2011). It could be demonstrated that the US-type isolates divided into three geographical subtypes (North America, Western to Central Eurasia, and Northeastern Eurasia), and the Kobe-type isolates into two subtypes (corresponding to two geographical regions in Japan), while the Hobetsu- and Munich-type isolates showed no variation.

Apart from the above four clades defined by Nakajima et al. (2009), an additional fifth group (corresponding to group C above) exists and is composed of different carnivore isolates (dog, fox, cat, raccoon, skunk and river otter) (Fig. 5). One of the well-supported clades within this group comprises *B. microti*-like organisms that have been isolated from dog, fox and cat, including a dog isolate that was named *Theileria annae* at the time of its finding (Zahler et al., 2000; Camacho et al., 2001). However, in accordance with the current nomenclature, it is clearly a *Babesia* species (Gray et al., 2006). Uncertainty with respect to this name is reflected by the fact that, in a later publication, this isolate was referred to as *Babesia annae* (Camacho et al., 2005). No evidence exists so far that members of this group are zoonotic.

7. Population genetics

Population genetics provides important insights into the genetic diversity, population dynamics, and structure of parasite populations. As these parameters describe the adaptation of the parasite population for survival in response to environmental challenges, they have a major impact on vaccination strategies, as well as on the understanding of drug resistance, epidemiology, and pathogenicity (Beck et al., 2009a). The advent of molecular genetics and genomics, specifically PCR-based methodologies, has afforded the means to determine genetic variation along the genome of several pathogens. As they allow a quantitative assessment of genetic exchange, micro- and minisatellite marker systems have been developed and successfully applied to a number of studies of the piroplasmids *B. bovis*, *T. parva* and *T. annulata* (Oura et al., 2003; Katzer et al., 2006, 2011; Weir et al., 2007, 2011; Perez-Llaneza et al., 2010; Simuunza et al., 2011a).

Similar to other apicomplexan hemoparasites like *Plasmodium*, piroplasmids are haplonts featuring the zygote as the exclusive diploid stage during their sexual reproduction in the tick host. This has been ascertained by DNA content measurements of different parasite stages for some *Babesia* (*B. bigemina*, *B. divergens*, *B. canis*) and *Theileria* (*T. annulata*, *T. parva*) species (Mackenstedt et al., 1990, 1995; Gauer et al., 1995). Thus, in the vertebrate host exclusively a single allele can be identified, and the detection of two or more alleles is indicative of dual or multiple infections, respectively. It follows that heterozygosity cannot be directly determined in individual parasites, but is estimated based on observed allele frequencies in the study population.

Among *Babesia* spp., population genetic studies have been restricted so far to *B. bovis*. A set of 14 micro- and minisatellite markers was developed for this parasite, and showed that the multilocus genotype (MLG) distances between five reference isolates (R1A, S2P, Argentina; RAD, Mo7, Mexico; and T2Bo, USA) correlated with the geographic distance of their origin (Perez-Llaneza et al., 2010). Further studies were performed in which MLGs of a larger number of reference isolates from Argentina, Brazil, Mexico, USA, Australia and Israel were determined. Preliminary results suggested that isolates originating from each country segregated into their own clades, corroborating the finding of Perez-Llaneza et al. (2010) (Flores et al., 2011; Minichiello et al., 2011).

An additional typing system of eight micro- and minisatellite markers has also been established and applied to carry out a comparative population genetic analysis of *B. bovis* in Zambia and Turkey (Simuunza et al., 2011a). A high multiplicity of infection (MOI) ranging from 1.5 to 4.8 in Zambia and from 1.1 to 3.3 in Turkey has been observed and corresponds with findings in various *Theileria* spp. populations, revealing an extremely high diversity of MLGs in any given population sample (Oura et al., 2003, 2005; Odongo et al., 2006; Weir et al., 2007, 2011; Simuunza et al., 2011a). The significantly higher MOI of *B. bovis* in Zambian cattle may be explained by the higher mean age of Zambian with respect to Turkish cattle that participated in the study. Possibly, older cattle may have suffered more tick bites, causing the accumulation of an increasing number of *B. bovis* genotypes. Also, for *T. annulata* the MOI in the bovine host correlated positively with an increased age but also with previous vaccination (Weir et al., 2011).

In *B. bovis*, *T. annulata* and especially *T. parva* populations linkage disequilibrium (LD) has been observed, and populations are thus non-panmictic. Observance of LD may be due to a low rate of genetic recombination, selection, recent immigration, inbreeding (including self-fertilization) and/or population sub-structuring. Studies to assess genetic exchange in experimental settings have been exclusively carried out for *T. parva*, where a high rate of recombination was determined (Katzer et al., 2006, 2011). For *Theileria* spp. it has been therefore proposed that, except for a low recombination rate, all of the above mentioned factors may have variable degrees of importance depending on the epidemiological situation, and this seems to apply also to *B. bovis* (Oura et al., 2005; Odongo et al., 2006; Weir et al., 2007, 2011; Simuunza et al., 2011a). Accordingly, for *B. bovis* and *T. annulata* populations studies suggest that genetic exchange by recombination is unhampered under the precondition of a relatively low geographic distance between populations, a high transmission rate, and that no exchange of infected animals (e.g. by trade) takes place (Weir et al., 2007). With regard to *T. annulata* populations, it was furthermore suggested that the observed LD may be due to inbreeding (including self-fertilization) (Weir et al., 2011).

Substructuring has been demonstrated between *B. bovis* populations of Zambia and Turkey, as well as between the Aydin and Izmir regions in Turkey (Simuunza et al., 2011a). Most likely, this sub-structuring is due to a complete absence of cattle movement between these two countries or regions. In contrast, isolates from the province of Lusaka in Zambia represent a subset of those of the Eastern province of Zambia rather than a sub-population. This is in agreement with the observation that cattle movement seems to be primarily responsible for the westward spread of both the vector tick and *B. bovis* in Zambia (Simuunza et al., 2011b).

In some countries (e.g. Argentina, Australia and Israel) attenuated live vaccines are used to protect against bovine babesiosis. Attenuation is achieved by rapid passage of virulent strains in splenectomized calves, and vaccination with attenuated parasites results in solid protection against disease. Lau et al. (2011) compared changes at the genome level between three virulent parent strains (T2Bo_vir, USA; L17_vir Argentina; and T_vir, Australia) and their attenuated daughter strains (T2Bo_att, USA; L17_att, Argentina; and T_att, Australia). Most importantly, attenuation resulted in a strong reduction of genome diversity with 81% of base pairs shared between attenuated strains compared to only 60% be-

tween virulent strains. A simplified population structure of the attenuated strains resulted in a reduced diversity of the variant erythrocyte surface 1 (ves) multigene family. No consistent association of virulent factor-encoding genes or other genes was observed with either the attenuated or virulent stage. The latter observation corresponds with other reports that both the (i) genome-wide protease expression and content, as well as the (ii) allelic length and sequence variants of the highly polymorphic single copy Bv80 gene, were likewise not associated with attenuation or virulence (Mesplet et al., 2011; Mazuz et al., 2012). The findings suggest that loss of pathogenicity is associated with an overall decrease of virulence factor-encoding genes in the parasite population during passages in splenectomized calves (Lau et al., 2011). In other words, pathogenicity is strongly defined by the parasite population diversity underscoring the importance of genetic recombination and high MOI as observed in the above outlined population genetic studies.

Recently, the genotypic diversity of the merozoite surface antigen 1 (MSA-1), a member of the variable merozoite surface antigens (VMSA) of *B. bovis*, has been determined in an endemic population at the beginning and the end of a 6-month period (Lau et al., 2010). Of the 31 animals, 20 showed a genotypic change over the study period. Altogether, 28 different MSA-1 genotypes could be identified, and changes of their prevalence during the study time were observed in the herd. It was suggested that co-infection of ticks with multiple genotypes resident in the bovine host, their recombination and subsequent re-infestation of bovines during tick bite, resulted in the observed changes of antigenic variation and diversity. The observed high MOI in individual animals, and the frequent genetic exchange inferred in the study of Simuunza et al. (2011a), strongly supports this notion.

Taken together, genetic exchange is frequent in *Babesia* as well as in *Theileria* parasites, and it may have the following significance with respect to disease control. First, drug resistance, if it occurs, will most likely spread rapidly in the entire population. Second, inter-genetic recombination between and intra-genetic recombination within polymorphic surface antigen-coding genes should highly promote the diversity of the parasite surface and its surface antigens. This may result in continuous creation of immune escape variants in a given parasite population, advocate vaccine breakthroughs, and complicate vaccine development.

8. Concluding remarks

We have presented a comprehensive summary of recent research findings that outline and integrate the important historic events of piroplasmid evolution in relation to their tick and vertebrate hosts. A novel improved classification of major piroplasmid lineages has been proposed based on an extensive molecular phylogenetic analysis of the 18S rRNA gene. The classic taxonomy of *Babesia* parasites, as well as *Theileria* and *Cytauxzoon*, was discussed, and recent developments of molecular taxonomy, in particular of *Babesia* parasites infecting humans, illustrated. Furthermore, the significance of recent population genetic findings were summarized. As obligate hemoparasites, *Babesia* species have adapted over hundreds of millions of years to exist as stealthy cryptic inhabitants within their vertebrate and invertebrate hosts. As such, in their overwhelming majority they are invisible to the naked eye. Usually, man-made natural upheavals or unbalances make them conspicuous by causing pathogenicity and mortality of domestic animals, wildlife, and humans as accidental hosts. The increasing identification and description of novel species in a great array of hosts allows us to foresee that *Babesia* research will become a highly dynamic field in the years to come.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2012.07.004.

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