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Phylogeny of sheep and goat *Theileria* and *Babesia* parasites

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Abstract The phylogenetic relationship of *Theileria* and *Babesia* species infecting sheep and goats on the basis of their 18S RNA gene structure was addressed in the present study. For this purpose, the complete sequences of the small ribosomal RNA genes of a panel of sheep and goat piroplasm isolates, including *T. lestoquardi*, *T. ovis, T. separata, B. ovis*, *B. motasi*, *B. crassa* and several novel species, were sequenced and compared. The classification based on the established phylogenetic tree corresponded with traditional systematics and revealed that sheep/goat piroplasm species are of polyphyletic origin. The independent evolution of almost all sheep/goat piroplasms suggests that speciation may have occurred after transfer of the piroplasm-transmitting tick from a primal wild ruminant host to domestic sheep and goats. In accordance with recent reports, our study confirms the existence of at least two additional sheep/goat piroplasm species, designated *Theileria* sp. 1 (China) and *Theileria* sp. 2 (China). The recently reported pathogenic sheep/goat *Theileria* sp. 1 (China) seems to be identical with a *Theileria* sp. isolated from Japanese serow. Furthermore, our results suggest that *T. ovis* represents a single species.

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Introduction

Piroplasm species are tick-borne parasitic protozoa which are differentiated into the genera *Theileria* and *Babesia*. A number of these parasites are highly pathogenic for cattle, sheep and goats; and the diseases emerging from these infections are referred to as theileriosis and babesiosis, respectively. The economic losses due to theileriosis and babesiosis are enormous in tropical and subtropical areas (Mehlhorn and Schein 1984; Mehlhorn et al. 1994).

18S RNA genes have been successfully applied to identify and classify several previously unknown *Theileria* and *Babesia* parasites (Persing et al. 1993, 1995; Quick et al. 1993; Thomford et al. 1994; Herwaldt et al. 1997; Katzer et al. 1998; Gubbels et al. 2000; Schnittger et al. 2000a). Furthermore, the phylogenetic classification of cattle-infecting piroplasms by the analysis and comparison of 18S RNA genes has been shown to correspond with traditional taxonomy and provides additional refined information on their evolutionary relationship.

It was shown by molecular phylogeny of 18S RNA genes that the highly pathogenic cattle parasites *T. annulata* and *T. parva*, which share the ability to transform their host cells, are evolutionarily closely related and that parasites of the *T. buffeli* group (including *T. orientalis*, *T. buffeli*, *T. sergenti*) have a monophyletic origin and cannot presently be distinguished into different species (Allsopp et al. 1994; Chae et al. 1999; Gubbels et al. 2000, 2002). In contrast, cattle *Theileria* species (*T. mutans*, *T. velifera*) and *Babesia* species (*B. bovis*, *B. bigemina*, *B. divergens*) have been shown to be of individual origin and show no close relation to each or to other cattle piroplasms (Allsopp et al. 1994; Chae et al. 1999; Gubbels et al. 2000; Homer et al. 2000).

In comparison to cattle piroplasms, piroplasm species infecting sheep and goats have been somewhat neglected but increasingly attract attention due to their economic importance being higher than cattle-infecting piroplasms

in some countries (e.g. China, Iran; Luo and Yin 1997; Schnittger et al. 2000a; Ahmed et al. 2002; Bai et al. 2002; Yin et al. 2002, 2003).

The objective of the current study was to elucidate the evolutionary relationship of sheep/goat piroplasm species on the basis of their 18S RNA gene sequences. The sequences of the 18S RNA genes of a panel of isolates of the species *T. lestoquardi*, *T. separata*, *T. ovis*, *B. motasi*, *B. crassa*, *B. ovis* and two recently reported *Theileria* species originating from China were deduced. Establishment of a phylogenetic tree allowed integration and definition of sheep/goat piroplasm species in comparison with cattle piroplasms and piroplasms infecting other hosts.

Materials and methods

Parasite isolates

Information on parasite stocks used in this study and their vertebrate host, geographic origin and accession number under which the corresponding 18S RNA gene sequence was submitted to the GenBank data base is listed in Table 1. Parasite stocks isolated from sheep include *Theileria ovis* (Sudan), *T. ovis* (Turkey), *Babesia motasi* (Ameland), *B. crassa* (Iran), *Babesia* sp. (Turkey) and *B. motasi* (Turkey). Parasite stocks *Theileria* sp. G4 (Tanzania) and *Theileria* sp. G6 (Tanzania) were isolated from goats. Parasite stocks isolated after experimental infection of sheep with ticks collected in the field include *Theileria* sp. (Lintan 2), *Theileria* sp. (Lintan 3), *Theileria* sp. (Zhangjiachuan), *Theileria* sp. (Longde), *Theileria* sp. (Qinghai), *Theileria* sp. (Madang) and *Theileria* sp. (Ningxian), which all originate from *Haemaphysalis qinghaiensis* ticks, *T. separata* (South Africa) which originates from *Rhipicephalus evertsi* ticks, *B. motasi* (Texel) which originates from *H. punctata* ticks and *B. ovis* (Turkey) which originates from *R. bursa* ticks. All parasite isolates were stored as EDTA–blood stabilates in liquid nitrogen.

DNA isolation and PCR amplification

Genomic DNA was prepared from blood, using a genomic DNA extraction kit (Qiagen) and following the protocol of the manufacturer; and the amount of isolated DNA was assessed by photometry. The PCR amplification protocol performed for the determination of 18S RNA gene sequences was in brief: 100 ng of genomic DNA was added to a reaction mixture (final volume of 30 µl) containing 1 µ*M* primer A and 1 µ*M* primer B which cover the eukaryotic 18S RNA gene sequence (Medlin et al. 1988), PCR buffer (10 m*M* Tris-HCl, pH 8.0, 50 m*M* KCl, 0.01% gelatine), 200 µ*M* each deoxynucleotide triphosphate, 1.5 m*M* $MgCl_2$ and 1.5 units of Taq polymerase; and the amplification was done in an automatic DNA thermocycler (Eppendorf). The reaction was incubated at 94 °C for 3 min to denature genomic DNA and the thermal cycle reaction programmed for 40 cycles of 1 min at 94 °C, 90 s at 40 °C and 90 s at 72 °C, with a final extension step of 5 min at 72 °C. Samples were held at 4 °C until analysed.

Sequence analysis of 18S RNA genes

After amplification of the complete 18S RNA gene with primers A and B as described above, the generated DNA fragments were either sequenced directly or ligated into pCR 2.1 and transformed into One Shot-competent *Escherichia coli*, using the TA cloning kit (Invitrogen) according to the manufacturer's instructions. Transformed bacteria were plated and qualified clones selected. A restriction digest was performed (with *Eco*RI), run on a 1% agarose gel and visualised using an ultra-violet transilluminator to recognise clones containing the desired 18S RNA gene fragment of approximately 1,750 bp. The sequences of coding and non-coding strands of four clones obtained in at least three independent PCR reactions were determined on an automatic sequencer according to standard procedures (Applied Biosystems). The sequencing output was analysed using the accompanying DNA Sequence Analyzer software (Applied Biosystems). Compilation, editing and assembly of multiple sequences generated from each template were performed with the EditSeq and SeqMan analysis program components of the Lasergene software package for Windows (DNASTAR, Madison, Wis.). The nucleotide sequence data for the analysed 18S RNA gene sequences of sheep and goat piroplasm isolates are available in the GenBank database under accession numbers AY262115, AY262119, AY262117, AY262118, AY260174, AY260173, AY260171, AY260172, AY260175, AY262116, AY262121, AY262120, AY260179, AY260180, AY260176, AY260177 and AY260178.

Sequence alignment and phylogenetic analysis

For sequence alignment and phylogenetic analysis, the MegAlign component of the Lasergene program ver. 4.01 (DNASTAR) was used. The MegAlign component performs multiple sequence alignments by the program algorithm of the Clustal method (Thompson et al. 1994) and phylogenetic analysis by the neighbour-joining method (Saitou and Nei 1987; Atteson et al. 1999). The constructed phylogenetic tree (cladogram; Fig. 1) is based on *Theileria* and *Babesia* 18S RNA gene sequences determined in our laboratory or obtained from the GenBank database (Table 1). To estimate the reproducibility of the tree, bootstrapping was done by tree reconstruction with random draws of sub-samples. While there was a minor change in the order of clades/clusters observed in the *Theileria* branch of the tree, the grouping of sequences into individual clusters/clades was in all cases identical, verifying that each cluster/clade represents an operational taxonomic unit (OTU). The phylogeny was established, assuming a biological clock; and the length of each pair of branches in Fig. 1 represents the distance between sequence pairs. The units on the horizontal axis indicate the number of substitution events. The matrix of pairwise sequence divergence and identity is not shown but is referred to in the Results and the Discussion.

Results

18S RNA gene sequences

Within the scope of this work, complete 18S RNA gene sequences were determined for isolates *Theileria separata* (South Africa), *T. ovis* (Sudan), *T. ovis* (Turkey), *Theileria* sp. G4 (Tanzania), *Theileria* sp. G6 (Tanzania), *Theileria* sp. (Lintan 2), *Theileria* sp. (Lintan 3), *Theileria* sp. (Zhangjiachuan), *Theileria* sp. (Longde), *Theileria* sp. (Qinghai), *Theileria* sp. (Madang), *Theileria* sp. (Ningxian), *Babesia motasi* (Ameland), *B. crassa* (Iran), *Babesia* sp. (Turkey), *B. motasi* (Turkey) and *B. ovis* (Turkey). Table 1 gives the parasite isolate, vertebrate host, geographic origin and the accession number under which the corresponding 18S RNA gene sequence was submitted to the GenBank database. Additionally, all other 18S RNA gene sequences from *Theileria* and *Babesia* parasites currently available in the GenBank database are registered in Table 1.

Table 1 Isolate, vertebrate host, origin and accession number of 18S RNA genes used for phylogenetic tree constuction. Isolates isolated from sheep or goat are indicated by *Sheep* or *Goat* under *Host*. 18S RNA genes of sheep/goat piroplasms sequenced in this work are given in the Materials and methods. Other 18S RNA gene sequences were obtained from the GenBank database. All 18S RNA gene sequences of listed isolates were used to construct the phylogenetic tree shown in Fig. 1. Sequences which consistently behaved as a single operational unit were combined in clusters/subclusters, as listed here

Cluster	Isolate	Host	Origin	Accession number
1a	*Theileria* sp. (Lintan 1)[a]	Sheep	Lintan (China)	AF081136
	Theileria sp. (Lintan 2)[a]	Sheep	Lintan (China)	AY262115
	Theileria sp. (Qinghai)[a]	Sheep	Qinghai (China)	AY262119
	Theileria sp. (Madang)[a]	Sheep	Madang (China)	AY262117
	Theileria sp. (Ningxian)[a]	Sheep	Ningxian (China)	AY262118
	Theileria sp. CC3A Serow[a]	Serow	Japan	AB012201
	Theileria sp. CC1A Serow[a]	Serow	Japan	AB012200
	Theileria sp. CC3B Serow[a]	Serow	Japan	AB012202
1b	*Theileria* sp. CNY2A Sika deer[a]	Sika deer	Japan	AB012196
	Theileria sp. CNY3B Sika deer	Sika deer	Japan	AB012198
2a	*Theileria* sp. G4 (Tanzania)[a]	Goat	Lekoko Okarina (Tanzania)	AY260174
	Theileria sp. G6 (Tanzania)[a]	Goat	Lekoko Okarina (Tanzania)	AY260173
	T. ovis (Sudan)[a]	Sheep	Sudan	AY260171
	T. ovis (Turkey)[a]	Sheep	Turkey	AY260172
2b	*Theileria* sp. CNY1A[a]	Sika deer	Japan	AB012194
	Theileria sp. CNY1B Sika deer	Sika deer	Japan	AB012195
	Theileria sp. CNY3A Sika deer	Sika deer	Japan	AB012197
	Theileria sp. CNY3C Sika deer	Sika deer	Japan	AB012199
3	*T. velifera* (Tanzania)[a]	Cattle	Lugurni (Tanzania)	AF097993
4a	*T. buffeli* (Australia)[a]	Cattle	Warwick (Australia)	AB000272
	T. buffeli (China)	Cattle	China	AF236097
	T. sergenti (Japan)	Cattle	Japan	AB016074
	T. sergenti (China)	Cattle	China	AF081137
	Theileria sp. (China)	Cattle	China	AF036336
	T. sergenti (Ikeda, Japan)	Cattle	Ikeda (Japan)	AB000271
		Elk	Minnesota (USA)	
	Theileria sp. (Ipoh)	Cattle	Ipoh (Malaysia)	AB000273
	Theileria sp. (Medan)	Cattle	Medan (Indonesia)	AB000274
	Theileria sp. (Thung Song)	Cattle	Thung Song (Thailand)	AB000270
	Theileria sp. type A	Cattle	Japan, China, Korea, Kenya, Texas (USA)	U97047
	Theileria sp. type B	Cattle	Japan, Korea, Texas (USA)	U97048
	Theileria sp. type B1 (Korea, USA)	Cattle	Korea, Texas (USA)	U97049
	Theileria sp. type C	Cattle	Korea	U97051
	Theileria sp. type D	Cattle	Texas (USA), Korea, China	U97052
	Theileria sp. type E	Cattle	Korea	U97053
	Theileria sp. type H	Cattle	Korea	U97050
4b	*T. separata* (South Africa)[a]	Sheep	South Africa	AY260175
	Theileria sp. Sable[a]	Sable	South Africa	L19081
	Theileria sp. BK115 (USA)	Mhorr gazelle	California (USA)	AF158710
5a	*T. annulata* (India)[a]	Cattle	Hisar (India)	M64243
	T. lestoquardi (Lahr)[a]	Sheep	Lahr (Iran)	AJ006446
	T. lestoquardi (Fars)[a]	Sheep	Fars (Iran)	AF081135
	T. parva (Kenya)[a]	Cattle	Kenya	AF013418
	T. taurotragi (South Africa)[a]	Cattle	South Africa	L19082
5b	*Theileria* sp. type G (Canada, USA)[a]	Elk	Canada	
		White-tailed deer	Texas (USA)	U97055
	Theileria sp. type G1 (USA)	White-tailed deer	Texas (USA)	U97056
	Theileria sp. type G2 (USA)	White-tailed deer	Oklahoma (USA)	AF086804
	Theileria sp. type F (Canada, USA)	Elk	Canada	
		White-tailed deer	Texas (USA)	U97054
6	*Theileria* sp. (Lintan 3)[a]	Sheep	Lintan (China)	AY262116
	Theileria sp. (Zhangjiachuan)[a]	Sheep	Zhangjiachuan (China)	AY262121
	Theileria sp. (Longde)[a]	Sheep	Longde (China)	AY262120
7	*T. mutans* (South Africa)[a]	Cattle	South Africa	AF078815
	Theileria sp. MSD (South Africa)	Cattle	South Africa	AF078816
8	*T. equi* (South Africa)[a]	Horse	South Africa	Z15105
	Babesia microti (South Africa)[a]	Mice	South Africa	U09833
	B. rodhaini (South Africa)[a]	Mice	South Africa	M87565
9a	*B. motasi* (Ameland)[a]	Sheep	Ameland (Netherlands)	AY260179
	B. motasi (Texel)[a]	Sheep	Texel (Netherlands)	AY260180
9b	*B. ovata* (Korea)[a]	Cattle	Korea	AY081192
	B. bigemina (Mexico)[a]	Cattle	Mexico	X59604
9c	*B. crassa* (Iran)[a]	Sheep	Iran	AY260176
	Babesia sp. (Turkey)[a]	Sheep	Turkey	AY260177

Table 1 (Contd.)

Cluster	Isolate	Host	Origin	Accession number
9d	*B. caballi* (South Africa)[a]	Horse	South Africa	Z15104
10a	*B. divergens* (Australia)[a]	Cattle	Australia	U07885
	B. divergens (USA)[a]	Cattle	Texas (USA)	U16370
	B. divergens (Ireland)[a]	Cattle	Drumaness (Ireland)	Z48751
10b	*B. canis* (South Africa)[a]	Dog	South Africa	L19079
	B. gibsoni (Oklahoma)	Dog	Oklahoma (USA)	AF205636
	B. odocoilei (South Africa)	White-tailed deer	Texas (USA)	U16369
11	*B. ovis* (Turkey)[a]	Sheep	Turkey	AY260178
12	*B. bovis* (South Africa)[a]	Cattle	South Africa	L19077
	B. bovis (Texas)[a]	Cattle	Texas (USA)	L31922
	B. bovis (Australia)[a]	Cattle	Samford (Australia)	M87566

[a] These isolates/sequences are representative of their cluster/subcluster; and only these are shown in the phylogenetic tree (Fig. 1)

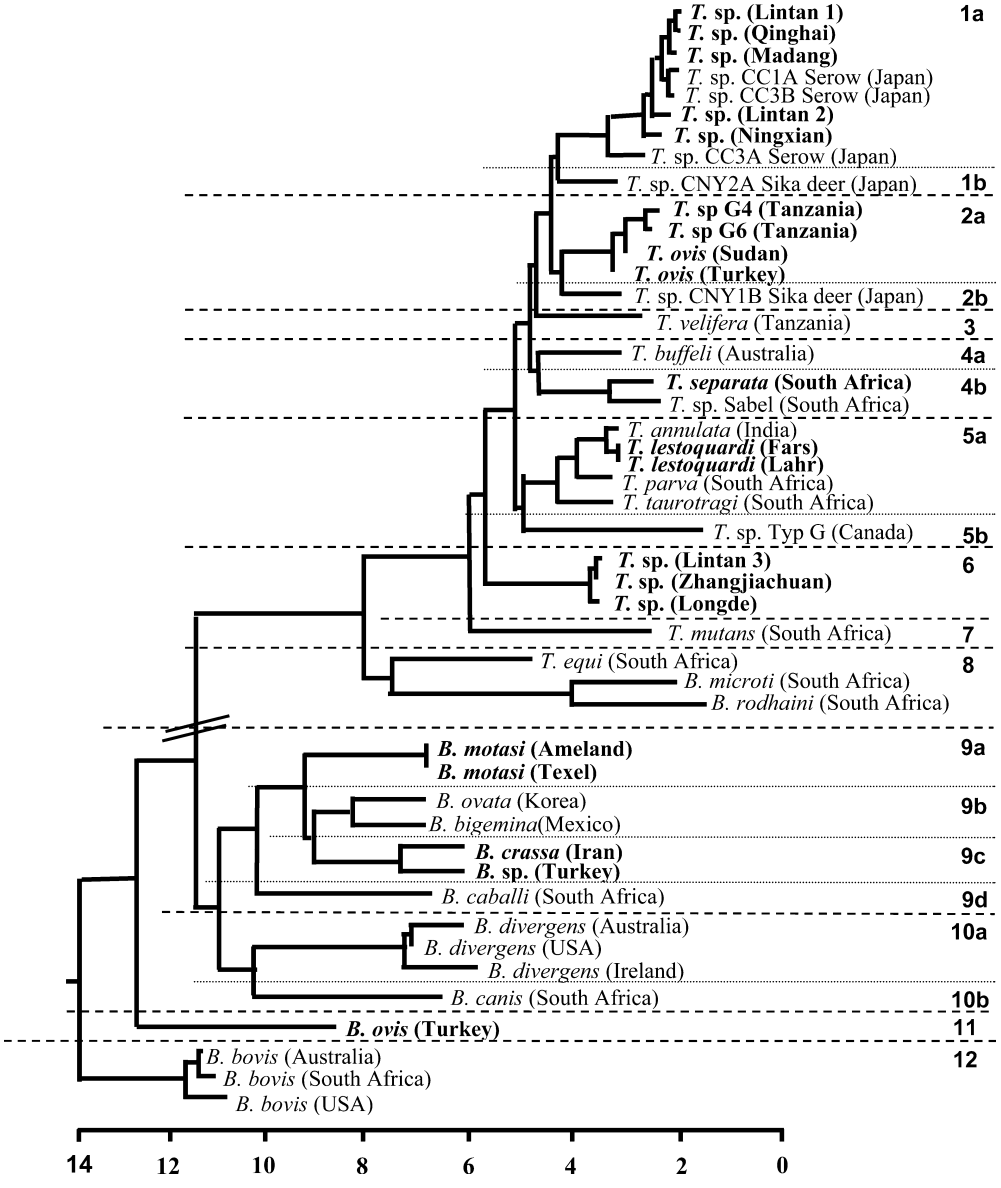

Fig. 1 Phylogenetic tree inferred from *Theileria* and *Babesia* 18S RNA gene sequences given in Table 1. Sequences combined in clusters/subclusters behave consistently as operational taxonomic units. For reasons of clarity only representative 18S RNA gene sequences of a given cluster/subcluster are shown (see footnote in Table 1). Sheep and goat piroplasm isolates are *printed in bold*. *The scale* gives the number of nucleotide exchanges. *The line-break* (//) marks the major division, as characterised by the highest phylogenetic diversity of 18S RNA genes between *Theileria* and *Babesia* parasites (with regard to this definition, *Babesia* sensu latu parasites *B. microti* and *B. rodhaini* belong to the genus *Theileria*). Clusters are *designated by numbers* and subclusters *by letters*

Phylogenetic tree construction

To integrate the small ruminant-infecting piroplasm parasites into the phylogenetic system of other *Theileria* and *Babesia* parasites, a phylogenetic tree was inferred from all 18S RNA sequences by neighbour-joining analysis (Table 1, Fig. 1). The robustness of the tree was tested by performing tree reconstructions with random draws of sub-samples. Some minor changes were observed in the ordering of clusters/clades within the *Theileria* branch of generated trees. Given the relatively short branch length in this part of the tree, this is not surprising and was also observed by Gubbels et al. (2002). The observed change of cluster ordering is disregarded in the following because it is of no further relevance for the presented results. In contrast to *Theileria* sequences, the branching order of *Babesia* sequences stayed identical in all trials. In accordance with the high robustness of the *Babesia* part of the tree, the branch lengths were significantly enhanced in segregating *Babesia*, compared with *Theileria* 18S RNA sequences (Fig. 1). It was however essential that, in all generated trees, the grouping of sequences into individual clusters/clades stayed identical, verifying that each cluster/clade represents an OTU. Accordingly, the *Theileria* and *Babesia* 18S RNA sequence clusters, as presented, behaved as single OTUs and corresponded to clusters/clades described by other authors for *Theileria* and *Babesia* 18S RNA sequences (Allsopp et al. 1994; Chae et al. 1999; Gubbels et al. 2000, 2002; Homer et al. 2000). For reasons of clarity, only representative selected 18S RNA gene sequences (see footnote in Table 1) are shown in several clusters/clades/OTUs of the phylogenetic tree in Fig. 1.

Molecular phylogeny of sheep/goat *Theileria* and *Babesia* species

The affiliation of an isolate to a piroplasm species based on the segregation of its 18S RNA gene sequence within a phylogenetic tree is somewhat subjective (e.g. cluster 5a contains four different species, while cluster 6 contains one species only). To allow a more objective decision whether 18S RNA genes of two different isolates belong to the same or to separate species, a decision procedure was applied, based on the calculated matrix of pairwise sequence identity (see Materials and methods). The identity between 18S RNA genes is an estimate of the phylogenetic distance between corresponding isolates. Therefore, it is possible to determine an upper/lower identity limit of 18S RNA gene sequences above/below which corresponding isolates would be affiliated to the same or different species. These identity limits have to be established separately for the genera *Theileria* and *Babesia*, because each has a different evolutionary history, as apparent from the different branch lengths of the phylogenetic tree.

The highest identity values of a pair of 18S RNA genes belonging to different species were observed between *T. annulata* vs *T. lestoquardi* (99.7%) and *B. divergens* vs *B. canis* (97.9%), respectively. Accordingly, isolates were considered to belong to a single species if their identity was found to be higher than 99.7%/97.9% for *Theileria*/*Babesia* 18S RNA genes. In contrast, the lowest identity values between 18S RNA gene sequences of isolates of a single species existed between *Theileria* sp. (Lintan 1) vs *Theileria* sp. (Ningxian) at 99.3% (both representing *Theileria* sp. 1 (China)] and between *B. bovis* (Australia) vs *B. bovis* (USA) at 96.6%. Therefore, isolates were considered to belong to different species if the identity was found to be lower than 99.3%/96.6% for *Theileria*/*Babesia* 18S RNA gene sequences, respectively. On the basis of these limits, it was possible to unequivocally assign all isolates to piroplasm species by applying the presented decision procedure (Table 2). In each case, the biological characteristics of assigned isolates were in accordance with that accepted and published by the known classic taxonomy of piroplasm species.

Five different *Theileria* species can be defined by the outlined phylogenetic classification of 18S RNA genes, each segregating in a single cluster/subcluster (Fig. 1, Table 2). *Theileria* sp. 1 (China) and *Theileria* sp. 2 (China) display identical biological characteristics in regard to tick and vertebrate host specificity, morphology and pathogenicity and can at present only be distinguished by molecular approaches. The similar aggregation of the 18S RNA sequences of four *T. ovis* isolates originating from different geographic regions into one cluster supports the view that *T. ovis* represents a single entity and does not represent a complex of different parasite species, as some authors suggested (Uilenberg 1981; Leemans et al. 1997).

Three different sheep *Babesia* species could be distinguished by phylogenetic classification: *B. motasi*, *B. ovis* and *B. crassa*. Furthermore, it could be shown that *B. crassa* is distributed not only within Iran but also in Turkey: *B. crassa* (Turkey). In Table 2, all investigated isolates are attached to the different defined species.

Discussion

The present study reports on the comparison and classification of sheep- and goat-infecting *Theileria* and *Babesia* piroplasms, based on the structure of their 18S RNA genes. The established phylogenetic tree can be divided in two major divisions, each having a single ancestor. One division represents the *Theileria* parasites, including the parasites *T. equi*, *B. rodhaini* and *B. microti* (*Babesia* sensu latu) and the other constitutes the true *Babesia* parasites (*Babesia* sensu strictu). Apparently, this division corresponds with biological characteristics: *Theileria* parasites can be distinguished from *Babesia* parasites by the presence of a schizont parasite stage and by their *trans*-stadial transmission by ticks. Both these features must have been a characteristic of a common

Table 2 Isolates assigned to piroplasm species by phylogenetic classification. Goat and sheep piroplasm isolates were assigned to different species by systematic classification of the 18S RNA gene, as described in the Materials and methods. Compare *Cluster/subcluster* data with Fig. 1. *n.k.* Not known

Isolate	Species	Tick	Cluster/ subcluster
Theileria sp. (Lintan 1)	*Theileria* sp. 1 (China)	*Haemapysalis qinghaiensis*	1a
Theileria sp. (Lintan 2)			
Theileria sp. (Qinghai)			
Theileria sp. (Madang)			
Theileria sp. (Ningxian)			
Theileria sp. (Lintan 3)	*Theileria* sp. 2 (China)	*H. qinghaiensis*	6
Theileria sp. (Zhangjiachuan)			
Theileria sp. (Longde)			
T. lestoquardi (Lahr)	*T. lestoquardi*	*H. anatolicum anatolicum*[a]	5a
T. lestoquardi (Fars)			
T. ovis (Turkey)	*T. ovis*	*Rhipicephalus evertsi*[a]	2a
T. ovis (Sudan)			
Theileria sp. G4 (Tanzania)			
Theileria sp. G6 (Tanzania)			
T. separata (South Africa)	*T. separata*	*R. evertsi*	4b
Babesia ovis (Turkey)	*B. ovis*	*R. bursa*	11
B. motasi (Ameland)	*B. motasi*	*H. punctata*	9a
B. motasi (Texel)			
B. crassa (Iran)	*B. crassa*	n.k.	9c
Babesia sp. (Turkey)			

[a] These tick species were not demonstrated in this work but were taken from the literature (Hooshmand-Rad and Hawa 1973; Mehlhorn et al. 1994)

Theileria ancestor. As *trans*-stadial transmission is a trait also displayed by some *Babesia* parasites, it is likely to be inherited by the genus *Theileria*.

As it is evident from the structure of the generated phylogenetic tree, the genera *Theileria* and *Babesia* have different evolutionary histories. In the *Theileria* parasites, the branch lengths are shortened and accordingly the identity between different species is much lower than in the *Babesia* parasites. Under the supposition that the evolutionary rate of 18S RNA genes is similar within both groups, this suggests that the genus *Theileria* developed later and has a shorter evolutionary history than *Babesia*.

The generally observed higher sequence identity between *Theileria* 18S RNA sequences compared with those of *Babesia* may be best explained by an evolutionary process of adaptive radiation. That is the relatively recent and fast spreading of members of this group to different hosts, not allowing the accumulation of species-specific mutations within slowly evolving RNA genes (Tibayrenc 1998). This would furthermore explain why some *Theileria* parasites, as for example parasites belonging to the *T. buffeli* group, cannot be clearly separated into different species: they may presently be in a dynamic evolutionary process of diversification in which clear separation of species has not yet been completed (Chae et al. 1999; Gubbels et al. 2000, 2002). This assumption would also be consistent with the finding that the species *Theileria* sp. 2 (China) with sheep as the vertebrate host and *Theileria* sp. Serow (Japan) with serow as the vertebrate host show an overlapping sequence diversity of their 18S RNA genes and that *T. annulata* and *T. lestoquardi* have speciated evidently very recently, as outlined by some authors (Katzer et al. 1998; Schnittger et al. 2000a, 2000b).

Unfortunately, there is no general consensus on the extent of sequence variation in 18S RNA genes which must be present to consider organisms as different species. However, it would be somewhat subjective to assign isolates to species by mere visual inspection of a phylogenetic tree. In order to objectify the assignment of isolates to species, we decided to establish identity values as decision thresholds by an empirical method (see Results). Due to the dissimilar evolution of *Theileria* and *Babesia*, different threshold values were determined for each genus. When the identity of two 18S RNA genes exceeded the established maximum threshold value, the respective isolates were considered as separate species. In contrast, when the identity of two 18S RNA genes was lower than the established minimum threshold value, the respective isolates were considered as similar species. The assignment of isolates to species on the basis of sequence identities was revised with their biological characteristics and compared with the known classic taxonomy. In each case, the sequence data and biological characteristics of the isolates could be reconciled with classic taxonomy, verifying the validity of the applied decision procedure.

The 18S RNA sequence of *Theileria* sp. 1 (China) isolates [*Theileria* sp. (Lintan 1), *Theileria* sp. (Lintan 2), *Theileria* sp. (Qinghai) *Theileria* sp. (Madang), *Theileria* sp. (Ningxian)] all segregate in cluster 1a, together with sequences originating from *Theileria* isolates of Japanese serow: *Theileria* sp. Serow (Japan) (Schnittger et. al. 2000a; Gubbels et al. 2002). Thus, sequences of *Theileria* sp. 1 (China) isolates show an overlapping diversity to that of *Theileria* sp. Serow (Japan). Both probably descended from a single direct ancestor species; and it seems likely that the parasite was transferred very recently by a compatible tick from the wild Japanese serow to

domestic sheep, not yet allowing complete separation into different species. Unfortunately, biological data to support this hypothesis as the species of the transmitting tick have not been reported from *Theileria* sp. Serow. The very close relationship between both parasites has been already supposed by Gubbels et al. (2002).

The phylogenetic tree placed *T. ovis* 18S RNA sequences of isolates *Theileria* sp. G4 (Tanzania), *Theileria* sp. G6 (Tanzania), *T. ovis* (Sudan) and *T. ovis* (Turkey) together in cluster 2a. Until recently, *T. ovis* was identified mainly by its non-pathogenicity for sheep and goat and it was therefore supposed this species may constitute a complex of different species (Leemans et al. 1997; Uilenberg 1981). The observation that *T. ovis* isolates originating from three entirely different geographic regions cluster together suggests that *T. ovis* does represent a single species. Most closely related to *T. ovis* is the sequence of a *Theileria* species isolated from sika deer [*Theileria* sp. CNY1B Sika deer (Japan) in cluster 2b] and both may share a direct ancestor. Interestingly, there is another *Theileria* parasite which was also isolated from sika deer [*Theileria* sp. CNY2A Sika deer (Japan), but which clearly represents a different species (cluster 1b) more closely related to *Theileria* sp. CC3A Serow (Japan) and *Theileria* sp. 1 (China) in cluster 1a.

The sequence of the sheep piroplasm *T. separata* is most closely related to that of *Theileria* sp. Sable (cluster 4b). The finding that *T. separata* and *Theileria* sp. Sable are transmitted by the same tick species would provide support for the hypothesis that the parasite was passed from wild sable antelope to domestic sheep. However, the transmitting tick species of *Theileria* sp. Sable is not known (Thomas et al. 1982).

Apart from *T. taurotragi*, piroplasm species of cluster 5a are of great economic importance because of their pronounced pathogenicity for domestic animals. The cattle-infecting *T. annulata*, *T. parva* and the sheep-infecting *T. lestoquardi* parasites share the evolutionarily unique ability of host-cell transformation, which must have been developed by a *Theileria* parasite ancestral to these species, supporting the close relationship of these parasites as shown by the clustering of their sequences. *T. lestoquardi* seems to be closely related to *T. annulata*, as suggested by the high identity of their sequences, high antigenic cross-reaction, overlapping distribution patterns and the same transmitting tick species, *Hyalomma* sp. (Hooshmand-Rad and Hawa 1973; Leemans et al. 1997; Katzer et al. 1998; Schnittger et al. 2000a, 2000b). Interestingly, *T. lestoquardi* seems to be the only sheep/goat piroplasm which has likely evolved from a cattle-infecting piroplasm species. It is furthermore noteworthy that, except cluster 8, cluster 5a is the only cluster of the genus *Theileria* harbouring *Theileria* isolates which have been clearly defined as different species.

Besides *Theileria* sp. 1 (China), a second parasite pathogenic for sheep and goat has been identified (Yin et al. 2003). The species is designated *Theileria* sp. 2 (China); and 18S RNA gene sequences of isolates *Theileria* sp. (Lintan 2), *Theileria* sp. (Zhangjiachuan) and *Theileria* sp. (Longde) segregated in cluster 6. *Theileria* sp. 2 (China) is relatively isolated in the phylogenetic tree and shares with the most closely related *Theileria* sp. CNY1B Sika deer (Japan) sequence (cluster 2b) an identity of 96.1% only. It is remarkable that *Theileria* sp. 2 (China) has a similar morphology and is transmitted by the same tick species (*Haemaphysalis qinghaiensis*) as *Theileria* sp. 1 (China). Moreover, differentiation of both species is complicated by overlapping endemic regions and the occurrence of mixed infections. Possibly for these reasons, the two were not distinguished until molecular classification methods were applied (Yin et al. 2003).

The parasites *B. motasi* (Ameland) and *B. motasi* (Texel) were isolated in the Netherlands and, accordingly, both segregate into cluster 9a. The species *B. crassa* is represented by two geographic variants having highly related 18S RNA gene sequences: *Babesia* sp. (Turkey) and *B. crassa* (Iran) in cluster 9c. So far *B. crassa* has been identified in Iran only. However, the present report of *B. crassa* in Turkey may not be surprising, since Turkey and Iran share borders.

The species *B. ovis* (Turkey), like *Theileria* sp. 2 (China), is also somewhat isolated in the phylogenetic tree (cluster 11). It represents a small *Babesia* species and is based on 18S RNA gene identity most closely related to *B. crassa* (93%).

All sheep/goat piroplasm species investigated in this work developed independently by polyphyletic evolution. Each of the following pairs of parasites descended most likely from a single direct ancestor: *Theileria* sp. 1 (China)/*Theileria* sp. Serow (Japan), *T. ovis*/*Theileria* sp. CNY2A Sika deer (Japan), *T. separata*/*Theileria* sp. Sable (South Africa) and *T. lestoquardi*/*T. annulata*. Possibly, the parasite has been transferred from the wild serow, sika deer and/or sable antelope to imported domestic sheep by transfer via compatible tick species. While the known transmission of *T. annulata* and *T. lestoquardi* by the same tick species is consistent with this assumption, the tick species transmitting *Theileria* sp. Serow (Japan), *Theileria* sp. CNY2A Sika deer (Japan) and *Theileria* sp. Sable (South Africa) are unfortunately not known at present (Thomas et al. 1982; Stoltsz and Dunsterville 1992; Takahashi et al. 1992).

In contrast, there are no close relatives to the species *Theileria* sp. 2 (China), *B. crassa* and *B. ovis*; and all three are placed relatively isolated in the tree as exemplified by the low identity of 18S RNA genes with their closest relatives: *Theileria* sp. CNY1B Sika deer (Japan) (97.5%), *B. ovata* (96.2%) and *B. bigemina* (Mexico) (91.8%), respectively.

In summary, five sheep/goat *Theileria* and three sheep/goat *Babesia* piroplasm species have been recognised in this study: *Theileria* sp. 1 (China), *T. ovis*, *T. separata*, *T. lestoquardi*, *Theileria* sp. 2 (China), *B. motasi*, *B. crassa* and *B. ovis*. Of these, the species *Theileria* sp. 1 (China) and *Theileria* sp. 2 (China) have

been reported very recently (Schnittger et al. 2000a, Yin et al. 2003).

Two additional sheep/goat piroplasm species, *T. recondita* and a very recently reported unknown *Babesia* parasite from China [*Babesia* sp. (China)] have not been included in this study (Alani and Herbert 1988; Bai et al. 2002). *T. recondita*, in contrast to *Theileria* sp. 1 (China), *T. lestoquardi* and *Theileria* sp. 2 (China), is non-pathogenic and certainly not identical with the species *T. ovis* and *T. separata* (Uilenberg and Andreasen 1974; Uilenberg 1981). Hence, *T. recondita* is most likely an additional species and it must therefore be concluded that at least six sheep/goat *Theileria* species do exist. *Babesia* sp. (China) seems to be most closely related to *B. motasi*. However, in contrast to *B. motasi*, it is transmitted by the tick species *H. longicornis*, is highly pathogenic, infects both sheep and goats and exhibits a smaller morphology (Bai et al. 2002). Due to these attributes, it certainly represents an additional fourth sheep/goat *Babesia* parasite species.

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