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Trypanosoma melophagium from the sheep ked *Melophagus ovinus* on the island of St Kilda

W. GIBSON^{1*}, J. G. PILKINGTON² and J. M. PEMBERTON²

¹*School of Biological Sciences, University of Bristol, Bristol BS8 1UG, UK*

²*Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, West Mains Road, Edinburgh EH9 3JT, Scotland*

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SUMMARY

The sheep ked has been largely eradicated in the UK but persists in the feral Soay sheep of St Kilda in the Outer Hebrides. Sheep keds transmit *Trypanosoma melophagium*, but parasitaemias are typically cryptic and this trypanosome has not been recorded in the St Kilda sheep. Trypanosomes were detected by PCR in preserved keds and were also found in gut smears from live keds; one infected gut was used to establish the trypanosome *in vitro*. Examination of the morphology of bloodstream forms from culture confirmed its identity as *T. melophagium*. Most keds were found to harbour the trypanosome, particularly those collected from lambs. DNA was extracted from preserved keds and from trypanosomes grown *in vitro*. Sequence analysis of the small subunit ribosomal RNA (SSU rRNA) gene and the spliced leader transcript showed the *T. melophagium* sequences to be very similar to those from *T. theileri*. A partial sequence of the ked SSU rRNA gene was also obtained. The close genetic relationship of *T. melophagium* and *T. theileri* suggests that *T. melophagium* represents a lineage of *T. theileri* that adapted to transmission by sheep keds and hence became a specific parasite of sheep.

Key words: *Trypanosoma melophagium*, *Trypanosoma theileri*, sheep ked, *Melophagus ovinus*, St Kilda, Soay sheep.

INTRODUCTION

The sheep ked *Melophagus ovinus* Linnaeus, 1758 is an ectoparasite of sheep that has been largely eradicated in the UK as a consequence of the widespread use of pesticides (Small, 2005). However, it persists in the feral Soay sheep population of Hirta, St Kilda in the Outer Hebrides off the north-west coast of Scotland (Craig *et al.* 2008). The parasite fauna of these sheep has been monitored over the past 2 decades during an annual capture of study sheep each August and several nematode species, the tapeworm *Moniezia expansa* and the protists *Eimeria* spp., *Giardia duodenalis* and *Cryptosporidium parvum* have been recorded (Craig *et al.* 2006, 2007, 2008).

Sheep keds are wingless dipteran flies of the family Hippoboscidae that feed on host blood via their piercing mouthparts. The relationship with the host is intimate and obligatory, with the whole reproductive cycle taking place in the fleece of the host; the female ked produces a single full-grown larva periodically, which becomes firmly attached to the wool and forms a puparium *in situ* (Small, 2005). Transfer from sheep to sheep is by direct contact, typically from ewe to lamb (Small, 2005). The blood-feeding habit and intimate relationship with the host

make keds suitable vectors for blood-borne parasites and the sheep ked is known to transmit the protist, *Trypanosoma (Megatrypanum) melophagium* (Hoare, 1972).

At first there was controversy whether *T. melophagium* Flu, 1908 was a parasitic flagellate of the sheep ked, *M. ovinus*, rather than the sheep, because the trypanosomes were easy to find in the insect host whereas infection in the mammal was cryptic. The development of methods for culture of the trypanosomes direct from sheep blood, coupled with the meticulous description of the developmental stages in the ked, revealed the true life cycle of this digenetic parasite (Hoare, 1923). Transmission was demonstrated to occur when infective metacyclic trypanosomes from the hindgut were applied to the mucous membranes of the sheep's mouth; this would be achieved naturally by sheep removing infected keds from the fleece during grooming and chewing them in the mouth (Hoare, 1923). *T. melophagium* is regarded as non-pathogenic to the sheep host (Hoare, 1972), although heavy infestation with keds causes irritation and damage to the fleece and hide, potentially leading to economic losses (Small, 2005). Before the widespread use of pesticides eliminated keds from most of the UK flock, *T. melophagium* was commonly found in sheep; for example, diagnosis by blood culture demonstrated 80% prevalence in English sheep (Hoare, 1923). In other countries where sheep keds are common, *T. melophagium* is also found; for

* Corresponding author: School of Biological Sciences, University of Bristol, Bristol BS8 1UG, UK. Tel: 0117 928 8249. Fax: 0117 331 7985. E-mail: w.gibson@bris.ac.uk

example, a recent study in Turkey demonstrated 7·8% prevalence of *T. melophagium* in sheep by blood culture (Nalbantoglu and Karear, 2008).

The high prevalence of keds recorded for the St Kilda sheep, particularly lambs (Craig *et al.* 2008), suggested that *T. melophagium* might also be present. Trypanosomes had not been detected in stained blood smears, but as parasitaemias are typically extremely low for *T. melophagium*, this is not surprising. Here, examination of keds removed from individual sheep during the annual catch-up was used to demonstrate infection of the sheep with trypanosomes and obtain an isolate of *T. melophagium* for molecular phylogenetic analysis.

MATERIALS AND METHODS

Collection of keds from sheep

Keds were removed from lambs, yearlings and adult sheep during February–March 2005 or during the annual catch-up in August 2008. When sampled in February–March, lambs, yearlings and adult sheep were approximately 10, 22 and ≥ 34 months old respectively. When sampled in August, lambs, yearlings and adult sheep were 4, 16 and ≥ 28 months old respectively. In 2005 insects were placed directly in alcohol to kill and preserve them before dispatch to Bristol, where they were stored at 4 °C until use. In 2008, keds were sent as live specimens on sheep's wool in individual tubes or were dissected in the field, the gut placed on a microscope slide and allowed to air dry before dispatch to Bristol.

Preparation of DNA

Preserved keds were removed from the alcohol and briefly air-dried in a Petri dish before extracting DNA using the method described by Hamilton *et al.* (2005). Briefly, the abdomen was removed and chopped into small pieces using a clean scalpel blade and placed in 200 μ l of insect digestion buffer (100 mM Tris-HCl, pH 8; 80 mM EDTA, pH 8; 160 mM sucrose; 1% w/v SDS) containing 50 μ g proteinase K. After macerating the abdomen with a pipette tip, the tube was incubated at 37 °C for 72 h, adding a further 20 μ g proteinase K every 24 h and mixing the contents. Following phenol-chloroform extraction and alcohol precipitation, the purified DNA was resuspended in 100 μ l of H₂O and frozen until use. DNA from trypanosomes grown in culture was purified using a DNA spin column kit (Qiagen).

PCR

PCR amplification of the small subunit ribosomal RNA (SSU rRNA) gene was carried out using primers complementary to conserved regions of the gene in genus *Trypanosoma* (Maslov *et al.* 1996). To

check the similarity of the primer sequences in trypanosomes and insects, SSU rRNA gene sequences of *T. theileri* (Accession no. AJ009164) and of the hippoboscids, *Lipoptena cervi* (Accession no. AF322426) and *Ornithoica vicina* (Accession no. AF073888), were aligned. Two of the primer sequences [C (S-713) 5' CCGCGGTAATTCC-AGCTCC; H (S-714) 5' CGTCAATTTCTTTA-AGTTTC] were well conserved (95% identity) in the insect SSU rRNA genes, and were used to amplify an approximately 600 bp fragment of the gene from the sheep ked. The other primer sequences showed much lower levels of conservation and could be used to specifically amplify trypanosome DNA from infected keds [B (S-823-F) 5' CGAACAACTGCCCTATC-AGC, 75% identity between trypanosome and insect sequences; D (S-825) 5' ACCGTTTCGGCTT-TTGTTGG and its complement, J (S-826) 5' CCAACAAAAGCCGAAACGGT, 35% identity between trypanosome and insect sequences].

DNA sequence analysis

The SSU rRNA gene was PCR amplified from purified *T. melophagium* DNA as previously described (Maslov *et al.* 1996). Four to six individual PCR reactions were pooled to yield sufficient DNA for automated DNA sequencing. After agarose gel electrophoresis, amplified bands were excised from the gel and the DNA purified before sequencing. The overlapping fragments were assembled into a single continuous sequence. An initial BLAST search was used to identify best matches with sequences held on database, and detailed alignments were performed using DNAMAN version 4.15 (Lynnon Biosoft). A 600 bp fragment of the SSU rRNA gene from uninfected ked DNA was obtained in a similar way. The gene for the spliced leader (SL; minixon) was amplified by PCR from purified trypanosome DNA as described previously (Gibson *et al.* 2000). The whole SL repeat unit was amplified using overlapping conserved primers within the SL itself (MED1: 5'-GGGAAGCTTCTGTACTATATT-GGTA; MED2: 5'-GGGAATTCAATATAGTACAGAAACTG) as described by Murthy *et al.* (1992). The amplified fragment was sequenced directly as described above, and also after cloning into a plasmid vector to obtain sequence close to the PCR primers.

Dissection and culture

Live keds were decapitated and dissected by opening the abdomen; the gut was removed and placed in a drop of PBS for viewing as a wet mount by light microscopy. Live flagellates were transferred to Cunningham's medium (Cunningham, 1977) supplemented with 10% (v/v) heat-inactivated foetal calf

Table 1. PCR detection of trypanosome DNA in keds removed from St Kilda sheep

Host of ked	Trypanosome detection		Percentage of keds infected with trypanosomes
	PCR +	PCR -	
Lamb	12	0	100%
Yearling	10	1	90.9%
Adult	6	5	54.5%
Total	28	6	82.4%

serum, $5 \mu\text{g ml}^{-1}$ haemin and $100 \mu\text{g ml}^{-1}$ gentamycin (CM) and incubated at 30°C as recommended by Hoare (1923). Trypanosomes were transferred to a co-culture system with insect cells (*Anopheles gambiae*) in a 1:1 mixture of CM and Schneider's Insect medium (Sigma) with 10% (v/v) heat-inactivated foetal calf serum, gradually reducing the concentration of gentamycin to $10 \mu\text{g ml}^{-1}$ as medium was replenished. After 2 weeks in culture, flagellates were transferred to a feeder layer co-culture system designed for growth of bloodstream forms of *T. brucei* (Brun *et al.* 1981), comprising *Microtus montanus* embryo fibroblast-like cells in HEPES-buffered Minimal Essential Medium with Earle's salts supplemented with 20% (v/v) normal horse or lamb serum and $10 \mu\text{g ml}^{-1}$ gentamycin. Cultures were maintained at 37°C , 5% CO_2 for 2 months; every 2–3 weeks, approximately half the medium was exchanged for fresh medium. For morphological examination, flagellates were spread on microscope slides, air dried, briefly fixed in methanol and stained with Giemsa. Stained specimens were examined with a $100\times$ oil immersion objective by light microscopy.

RESULTS

Prevalence of trypanosomes in keds

DNA was extracted from a total of 34 alcohol-preserved keds collected in 2005 and trypanosome DNA was identified by PCR of the SSU rRNA gene using trypanosome-specific primers B and J in 28 (82.4%). All keds removed from lambs were infected, with a lower prevalence in yearlings of 90.9% and adults of 54.5% (Table 1). Thus, trypanosome infection of the keds decreased as the age of the sheep host increased.

In view of the observed high prevalence of trypanosomes in keds from young animals, to obtain specimens of the trypanosome, keds were collected from lambs in 2008. Few keds (<10%) remained alive after removal from the host and storage at ambient temperature for 1 week. Of 4 surviving keds that were dissected, 3 had swarming infections of flagellates (Fig. 1). These infections were used to establish *in vitro* cultures. Trypanosomes were identified in all

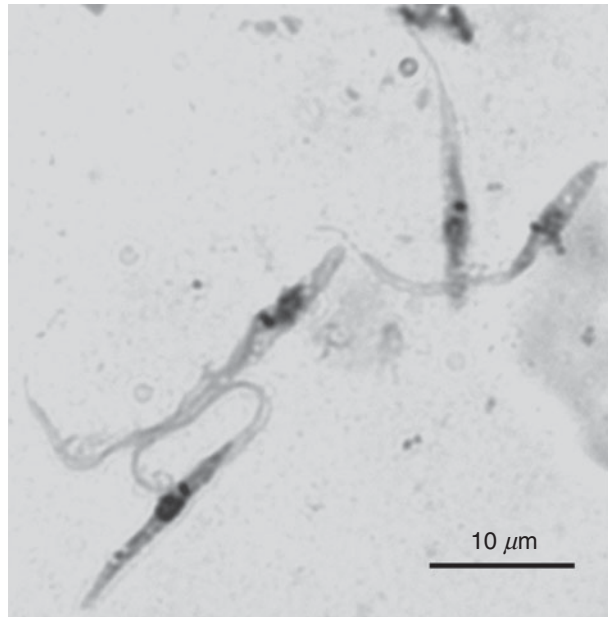


Fig. 1. Giemsa-stained trypanosomes (epimastigotes) from a dissected ked gut.

9 stained smears of ked guts dissected in the field. These results, together with the results of PCR analysis of keds collected in 2005, demonstrate that most keds from sheep on St Kilda are infected with trypanosomes and that the prevalence of infection in keds removed from lambs (aged 4 or 10 months) was particularly high.

Culture of trypanosomes from keds

The live trypanosomes from dissected ked guts were transferred to *in vitro* culture at 30°C and continued to proliferate. After 2 weeks, trypanosomes were transferred to a *Microtus* embryo fibroblast (MEF) feeder layer at 37°C . Most of the trypanosomes disappeared within a day or two, but small numbers persisted for 2 months in medium supplemented with either 20% normal horse or lamb serum. The morphology of these cells was uniform and agrees with published descriptions and measurements of *T. melophagium* from the ovine bloodstream: mean length $39.3 \mu\text{m}$, distance from posterior end to kinetoplast $14.7 \mu\text{m}$ and from kinetoplast to centre of nucleus $5.1 \mu\text{m}$ (Buscher and Friedhoff, 1984); length range $52.0\text{--}60.5 \mu\text{m}$, distance from posterior end to kinetoplast $13.5\text{--}21.7 \mu\text{m}$ and from kinetoplast to centre of nucleus $4.0\text{--}5.0 \mu\text{m}$ (Hoare, 1923). The trypanosomes were large (approximately $40 \mu\text{m}$ in length) with a long, pointed posterior and large kinetoplast closer to the nucleus than the posterior end of the trypanosome (Fig. 2).

The finding of trypanosomes in large numbers in the sheep ked, coupled with the morphological characteristics of the bloodstream forms, are sufficient to identify this trypanosome unequivocally as *T. melophagium*.

Table 2. Similarity (%) of SSU rRNA gene sequences (1762 bp) from *Trypanosoma melophagium* and *T. theileri*

<i>T. theileri</i> D30 AJ009165	99.5%			
<i>T. theileri</i> K127 AJ009164	99.6%	99.8%		
<i>T. theileri</i> TREU 124 AJ009163	99.6%	99.8%	100%	
<i>T. theileri</i> KM AB007814	98.9%	99.3%	99.1%	99.1%
	<i>T. melophagium</i> FN666409	D30 AJ009165	K127 AJ009164	TREU 124 AJ009163

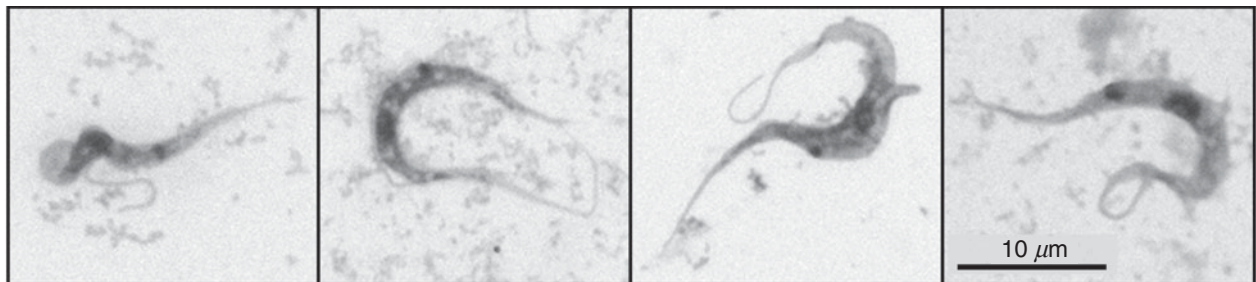


Fig. 2. Giemsa-stained trypanosomes (trypomastigotes) from a fibroblast co-culture incubated at 37 °C.

Sequence analysis

DNA from cultured trypanosomes was used as a template to amplify the SSU rRNA and spliced leader genes of *T. melophagium*. For the SSU rRNA gene, 1762 bp of sequence was obtained (Accession no. FN666409), which matched sequences from *T. theileri* almost perfectly (isolate TREU 124, Accession no. AJ009163; isolate K127, AJ009164; isolate D30, AJ009165; isolate KM, AB007814). The average within-group identity was 99.4% with closest similarity (99.6%) to sequences from *T. theileri* TREU 124 and K127 (Table 2).

The spliced leader (SL) transcript from *T. melophagium* was partially sequenced excluding primers used to amplify the fragment (109 bp; Accession no. FN666410) and the closest matches were obtained by BLAST search against sequences on database. The SL transcript has the conserved 39 nt SL at its 5' end, which is spliced onto the 5' end of every messenger RNA (mRNA) in trypanosomes. The remainder of the transcript is highly variable both in length and sequence, so that it is only possible to align SL transcripts from closely related trypanosome isolates or species (Gibson *et al.* 2000). The closest matches were SL sequences derived from *T. theileri* (isolate D30, Accession no. AJ250747; isolate K127, AJ250748), and *T. cyclops*, a primate trypanosome from Asia (Accession no. AJ250743) (Fig. 3). It can be seen from Fig. 3B that the SL transcripts of *T. melophagium* and *T. theileri* D30 show the highest level of similarity, differing at 10 positions excluding the pyrimidine tract, while the SL transcripts of

T. theileri isolates D30 and K127 differ at only 3 positions; these 2 are the only SL transcript sequences available for *T. theileri*. The SL transcript of *T. cyclops* aligns convincingly with this group of 3 sequences, but is the most distant (Fig. 3B). These results confirm the close relationship of *T. melophagium* and *T. theileri* evident from the SSU rRNA sequence comparison.

DNA from uninfected sheep keds was used as template to amplify an approximately 600 bp fragment of the SSU rRNA gene using primers C and H, which were well conserved in other hippoboscids flies. Two independently derived fragments were sequenced in both directions yielding a consensus sequence of 616 bp excluding primers used to amplify the fragment (FN666411). Comparison with SSU rRNA gene sequences from 2 other hippoboscids showed 98.9% similarity to that of the deer ked, *Lipoptena cervi* (Accession no. AF322426) and 97.2% similarity to that of *Ornithoica vicina* (Accession no. AF073888), a biting fly of birds.

DISCUSSION

We have isolated and characterized a trypanosome found in sheep keds collected from feral sheep on St Kilda and identified it as *T. melophagium*. Most keds collected were infected with this trypanosome and the live specimens dissected had abundant trypanosomes in the gut, confirming that they are the intermediate hosts of this trypanosome. Among sheep on St Kilda, keds were previously found to be most abundant on

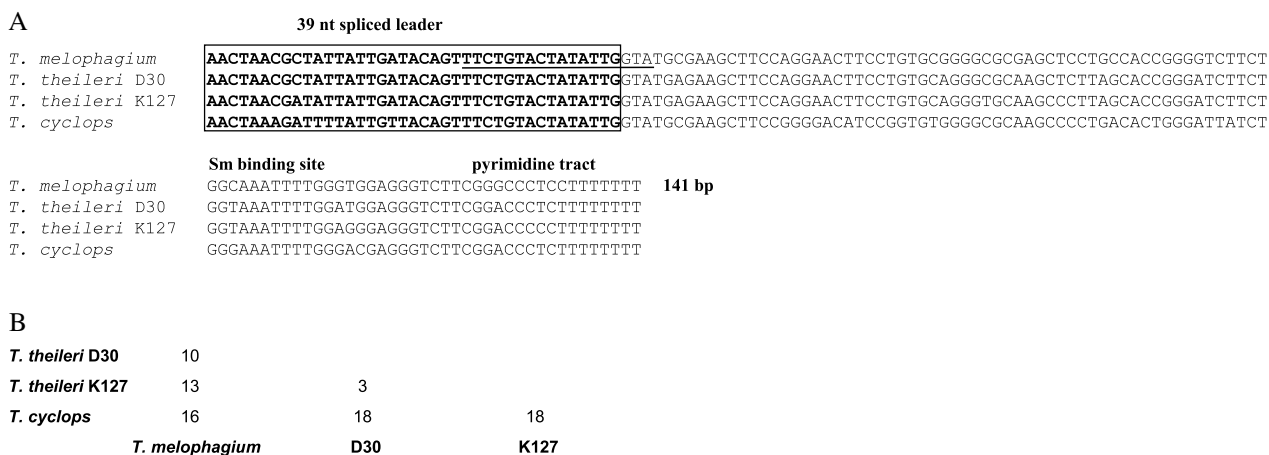


Fig. 3. Comparison of spliced leader (SL) transcripts. (A) Alignment of SL transcripts from *Trypanosoma melophagium*, Accession no. FN666410; *T. theileri* isolate D30, Accession no. AJ250747; *T. theileri* isolate K127, Accession no. AJ250748; *T. cyclops*, Accession no. AJ250743. The 39 nt SL is boxed; primer MED1 is underlined; neither of these sequences were directly sequenced in *T. melophagium*. The positions of the Sm binding site (consensus sequence RAT₄GG) and the pyrimidine tract that terminates the SL transcript are indicated. (B) Distance matrix showing numbers of nucleotide differences excluding the pyrimidine tract.

lambs, with a prevalence of 82% recorded from the underbelly; the prevalence fell to 28% in yearlings and 12% in adults (Craig *et al.* 2008). Here, all but 1 of the keds taken from lambs were positive for trypanosome infection by PCR or dissection, and a lower prevalence was evident in keds removed from yearlings and adults. Thus it seems likely that the lambs on St Kilda are more susceptible to trypanosome infection than older animals and thus harbour the greatest proportion of infected keds.

According to Hoare (1923, 1972), there is no lasting immunity to *T. melophagium* and sheep can be readily re-infected by exposure to infected keds after an aparasitaemic period of several months. The duration of infection with *T. melophagium* in experimental sheep is usually about 3 months, sometimes much shorter, and after this trypanosomes can no longer be demonstrated in the blood (Hoare, 1923, 1972). However, it should be borne in mind that these data were derived from observations and experiments on English lowland sheep and resistance to trypanosome infection may vary in other sheep breeds such as the Soay of St Kilda. The level of transmission may also vary, as infection is dependent on the sheep grooming keds from its fleece and squashing them in its mouth, which must in turn depend on the number of keds, their accessibility by mouth grooming and the amount of irritation they cause. While numbers of keds on the St Kilda sheep are probably at their lowest during the summer when the sheep have just cast their winter fleeces, the lack of cover might also render the keds more susceptible to being eaten during grooming. This might paradoxically lead to a higher trypanosome infection rate in the summer despite lower ked numbers.

The phylogenetic relationships of *T. melophagium* with other trypanosomes were assessed by DNA

sequence comparisons of the SSU rRNA and SL genes. The closest relative was found to be *T. theileri*, a non-pathogenic trypanosome found ubiquitously in bovines throughout the world and transmitted by biting flies such as tabanids via the contaminative route (Boese *et al.* 1987b; Bose and Heister, 1993; Hoare, 1972). *T. theileri* is the type species of subgenus *Megatrypanum*, a group of non-pathogenic mammalian trypanosomes of large size, already acknowledged to be heterogeneous (Hoare, 1972) and recently confirmed to be markedly polyphyletic by molecular phylogenetic studies (Stevens *et al.* 2001). *T. theileri* and *T. melophagium* share a similar developmental route in the insect vector, with infective metacyclics produced in the hindgut (Bose and Heister, 1993; Hoare, 1923). In both cases, metacyclics have been demonstrated to infect via the mucosal surfaces of the mouth (Boese *et al.* 1987a,b; Hoare, 1923), and in nature, transmission is effected by ingestion of the whole insect or its faeces during grooming (Hoare, 1972).

Phylogenetic analysis of the D7-D8 region of the SSU rRNA gene of isolates of *T. theileri* from cattle, buffalo, wild antelope and tabanids from South America, Africa and Europe demonstrated only minor levels of sequence divergence and all isolates were considered to belong to the single species *T. theileri* (Hamilton *et al.* 2009). *T. melophagium* fits comfortably within this group, with 99.4% sequence identity over the 1762 bp region of the SSU rRNA gene sequenced here. The close genetic similarity of *T. melophagium* and *T. theileri* suggests that *T. melophagium* represents a lineage of *T. theileri* that adapted to transmission by sheep keds and hence became a specific parasite of sheep. Its mammalian host specificity was therefore driven by the intimate association of the vector with the mammalian host.

Likewise, it is probable that *T. theodori*, a non-pathogenic goat trypanosome, is another lineage of *T. theileri* that adapted to transmission by the goat ked *Lipoptena caprioli*, as its developmental cycle parallels that of *T. melophagium* in the sheep ked (Hoare, 1972). The divergence of these trypanosome lineages from *T. theileri* would have been driven by increasing dependency on the ked vector, so that they eventually became locked into a separate transmission cycle featuring particular host species. This predicts much greater levels of diversity among *T. theileri* and related trypanosomes, where numerous different tabanid vectors are responsible for transmission to a broad range of large mammals in many different countries throughout the world.

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