Host specificity of *Trypanosoma (Herpetosoma)* species: evidence that bank voles (*Clethrionomys glareolus*) carry only one *T. (H.) evotomys* 18S rRNA genotype but wood mice (*Apodemus sylvaticus*) carry at least two polyphyletic parasites

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**SUMMARY**

The strongest evidence for host specificity of mammalian trypanosomes comes from parasites of the subgenus *Trypanosoma* (*Herpetosoma*). Laboratory studies have shown that *T. (Herpetosoma)* species will not infect an alternative host. However, this has not been demonstrated in wild populations. We screened 560 bank voles (*Clethrionomys glareolus*) and 148 wood mice (*Apodemus sylvaticus*) for trypanosomes by PCR amplification of the 18S rRNA gene. In total, 109 (19%) bank voles and 12 (8%) wood mice were infected. A *Hae*III restriction site was discovered that could be used to discriminate between *T. (H.) evotomys* of the bank vole and *T. (H.) grosi* of the wood mouse. All the parasites in the bank voles were identified as *T. (Herpetosoma) evotomys* by RFLP-PCR. Out of the 12 wood mouse infections 10 were due to *T. grosi*. Two of the wood mice were infected with parasites with a novel genotype that was most similar to those of *T. evotomys* and *T. microti* of voles. Fifty-six fleas collected from the rodents were also screened for trypanosomes; 9 were infected with *T. evotomys* and 1 with *T. grosi*. One of the fleas infected with *T. evotomys* was collected from a wood mouse.

Key words: host specificity, host restriction, *Trypanosoma microti*, *Microtus agrestis*, Siphonaptera.

**INTRODUCTION**

At least 472 specific names have been applied to members of the genus *Trypanosoma*, 153 to parasites of fish, 60 to those of amphibians, reptiles (79 names), birds (78 names) and mammals (102 names) (Hoare, 1972; Bardsley & Harmsen, 1973; Baker, 1976; Lom, 1979; Telford, 1995). The main characters that have been used for justifying the naming of species are morphology and host range. The morphology of *Trypanosoma* species is sufficiently variable and stable for it to be a useful character for identifying major groups of parasites, but within groups it is frequently difficult to discriminate between species on the basis of morphology alone. Therefore, at the species level, an assumption of host specificity has sometimes been invoked to justify the erection of new species. This is perhaps best exemplified within the subgenus *T. microti* (*Herpetosoma*). The great majority of the 45 named species within the subgenus *T. (Herpetosoma)* are parasites of rodents and are morphologically very similar to the type species *T. (H.) lewisi* of rats (*Rattus* spp.). The only comprehensive biometrical study of *T. lewisi*-like trypanosomes revealed highly significant variations in size between parasite populations from one and the same host species on the one hand and no significant differences between parasite populations from different host species on the other (Davis, 1952). Hoare (1972) concluded that: ‘most of the these trypanosomes have been given independent specific status, on the basis of alleged minor mensural or morphological differences, assumed host restriction, or merely because of their occurrence in a new mammalian host. Since in most cases the differential diagnosis on these lines was based on insufficient evidence, it has resulted in the creation of excessive numbers of species of doubtful validity.’

Despite these strictures there is strong evidence for host specificity of at least some species within the subgenus *T. (Herpetosoma)* (Molyneux, 1976). Intra-
peritoneal injections of *T. (H.) musculi*, *T. (H.) microti*, *T. (H.) grosi* and *T. (H.) evotomys* are only infective to their natural hosts (Molyneux, 1969; Maraghi & Molyneux, 1989; Santos-Gomes et al. 1993). German strains of *T. evotomys* isolated from *Clethrionomys glareolus* (Arvicolinae) are not infective to either Asian or North American species of *Clethrionomys* (Krampitz, 1959). Ionizing radiation and splenectomy did not modify the resistance of *Mus musculus* to *T. lewisi* of rats (Albright & Albright, 1985, 1991). This resistance can be overcome by coinjection of normal rat serum with *T. lewisi* or treatment of the rodents with silica dust and cobra venom factor prior to challenge. Immune suppression by co-injection with Ehrlich’s ascites sarcoma is also effective for establishing infections of *T. rabinowitschiae* and *T. Blanchardi* in heterologous hosts (Hommel & Miltgen, 1974). It should be noted that *T. rangeli* was believed to be an exceptional *T. (Herpetosoma)* species in that it is infective to a wide range of hosts. It has recently been shown that *T. rangeli* is a member of the subgenus *T. (Schizotrypanum)* which includes parasites such as *T. cruzi* with a wide vertebrate host range (Stevens et al. 1999a, b).

The hypothesis of host specificity for *T. evotomys* and other *T. (Herpetosoma)* species is difficult to prove but it can be disproved by the discovery of *T. (Herpetosoma)* species in alternative hosts. A longitudinal survey of bank voles (Arvicolinae; *Clethrionomys glareolus*) and wood mice (Murinae; *Apodemus sylvaticus*) at Manor Wood on the Wirral peninsula in Cheshire, England, provided an opportunity to screen large numbers of these animals for heterologous trypanosomes that would disprove the host specificity hypothesis. At this site sympatric populations of bank voles and wood mice share burrow systems. The majority of *T. (Herpetosoma)* species are believed to be transmitted by fleas. Four species of flea have been recovered from wood mice and bank voles in Manor Wood, *Amalareas penicilliger*, *Ctenophthalmus nobilis*, *Rhadinopsylla pentacantha* and *Doratopsylla dasyneura*. Fleas of all these species have been recovered from both species of rodent. Consequently both species of rodent are likely to be challenged with heterologous parasites and therefore the samples from this site presented an ideal opportunity to test the host specificity hypothesis.

**Materials and Methods**

**Animal capture**

Bank voles (*C. glareolus*) and wood mice (*A. sylvaticus*) were collected at Manor Wood, Wirral, Cheshire UK, grid ref. SJ294816 as previously described (Hazel et al. 2000). Briefly, pairs of Longworth traps (Penlon, Oxfordshire, UK) were placed at 100 permanent trap stations in a 1 hectare 10 m × 10 m grid within the 8 hectare wood. Trapping sessions were at 4-weekly intervals, and in each session traps were set over 3 days and nights. Captured animals were identified using subcutaneous microchip transponders with a unique 9 digit number which could be detected using a handheld Power Tracker Reader II (Labtrac by AVID plc, East Sussex, UK). A 20–40 µl blood sample was taken from the tip of the tail into an untreated 1.5 ml Eppendorf tube and the animal was released at the capture site. Fleas were collected from animals that were found dead in the traps and, whenever possible, from living individuals.

**Sample processing**

Many animals were captured more than once. For this study only the sample taken the first time an animal was captured was retained for analysis. Consequently the population is biased towards younger animals. Blood samples were allowed to clot and the serum was separated for other studies. Red blood cell pellets were stored at −80 °C until DNA extraction. DNA was prepared from the blood clot by phenol–chloroform extraction and ethanol precipitation. Fleas were stored in 70 % ethanol and identified using a standard key (Smit, 1957). DNA from fleas was prepared using a previously published guanidine thiocyanate/phenol chloroform method (Livesley et al. 1994). DNA concentration was determined by spectrophotometry and concentration was standardized at 50 ng/µl. The most variable region of the *Trypanosoma* 18S ribosomal RNA gene was amplified as previously described (Noyes et al. 1999) by nested PCR using the following primers. External primers: TRY927F 5′GGAACAGAAA-CACGGGAG, TRY927R 5′CTACTGGGCAGC-TTGGA; internal primers SSU561F TGGGAT-AACAAAGGAGCA, SSU561R CTGAGACTG- TAACTCTCAAGGC.

All samples were screened once by PCR. Positive samples were then re-tested with a negative control between each positive reaction for confirmation. Barrier tips were used throughout. PCR preparation, thermocycling and post-PCR processing were all conducted in separate rooms. Work was completed on the bank vole samples before work commenced on the wood mouse samples in order to reduce the risk of cross-contamination. Only samples that were positive in both the original and the confirmatory PCR test were included in the analysis. For analysis, 13 µl of each positive PCR reaction was digested with 1 unit of *HaeIII* (New England Biolabs) in a final volume of 15 µl according to the manufacturer’s instructions. The digest was analysed on a 1.5% 1:1 Nusieve:Normal agarose gel, stained with ethidium bromide, and observed under UV light. At least 1 sample of each genotype that was detected by
Host specificity of T. (Herpetosoma) species

Table 1. Polymorphic positions in the sequences of the 18S ribosomal gene

<table>
<thead>
<tr>
<th>Position in T. lewisi</th>
<th>Accession number</th>
<th>GenBank number</th>
<th>Host species</th>
<th>Host family</th>
<th>Position in T. lewisi</th>
<th>Accession number</th>
<th>GenBank number</th>
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<td>1016</td>
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<td></td>
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<tr>
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<tr>
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<td>Murinae</td>
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</table>

Only the taxa that are underlined were determined in this study; other sequences were obtained from GenBank as indicated in the fourth column. In the right-hand panel the position between bases 931 and 1426 is indicated; a dot indicates that the base at that position is identical to the one in the taxon above (not necessarily to the consensus). A dash indicates a deletion. The HaeIII restriction site that is absent in T. evotomys is at position 1063.

RESULTS

Bank vole data

In total 560 bank voles, 148 wood mice, 1 field vole and 56 fleas were screened for trypanosomes by PCR amplification of the 18S rRNA gene. Of these, 109 (19%) bank voles and 12 (9%) wood mice were positive by PCR. The PCR product from bank vole 025326018 was sequenced. This is the first report of any sequence data from a bank vole trypanosome. The sequence was assumed to be that of the 18S rRNA gene of T. evotomys, the trypanosome that is believed to be restricted to the bank vole. The T. evotomys sequence (AY043356) was aligned with other T. (Herpetosoma) species sequences that were obtained from GenBank (Table 1). The partial 18S sequence of T. evotomys differed from the published sequence of T. microti of the field vole (Arvicolinae; Microtus agrestis) (GenBank AJ009158) by 2/496 bases and from T. musculi and T. lewisi of murine rodents by 12 and 13 out of 496 bases respectively (Table 1). The aligned sequences were scanned for restriction sites that could be used to discriminate between T. evotomys and other T. (Herpetosoma) species and particularly between T. evotomys and T. grosi of wood mice. A transition (C → T) that disrupted a HaeIII restriction site (GGCC) was observed at position 1063 in the T. lewisi sequence (GenBank AJ009156). This indicated that T. evotomys could be distinguished from T. grosi, T. microti, T. musculi and T. lewisi by the absence of this HaeIII restriction site in T. evotomys. All the PCR products from vole DNA samples were digested with HaeIII. Only 1 out of 109 samples had the HaeIII restriction site (Fig. 1), this sample was from the only field vole (M. agrestis) in the study. The sequence of the PCR product of this sample was obtained and differed from the published sequence of T. microti by 1/496 bases, from T. evotomys by 3/496 bases and from T. grosi, T. musculi and T. lewisi by 12/496 bases (Table 1).

Wood mouse data

Twelve of the 148 wood mouse samples were positive for trypanosome 18S rDNA. HaeIII restriction digests of the PCR products indicated the presence of 2 genotypes; these were designated WM1 and
Fig. 1. *Hae*III digest of 93 trypanosome PCR products from vole DNA samples. The only sample that was cut by this enzyme is indicated by the arrow. This sample was from a field vole not a bank vole and was identified as *Trypanosoma microti*.

Fig. 2. *Hae*III digest of *Trypanosoma evotomys*, (TE); *T. grosi* (TG) and *Trypanosoma* sp. (Tsp). The column headings indicate the sample number, the genotype number in the case of wood mouse parasites. WM1 is assumed to correspond to *T. grosi* and WM2 is the wood mouse parasite genotype that is closer to *T. evotomys* of bank voles.

WM2 (Table 1, Fig. 2). At least 1 sample of each genotype was sequenced. Genotype WM1 was found in 10 different wood mice. The partial 18S rDNA PCR products of 2 of these samples were sequenced and were found to differ from the sequences of other murine rodent parasites by 2/496 bases but from arvicoline parasite sequences by 10 or 11 bases out of 496 (Table 1). In view of the close relationship of genotype WM1 to that of other murine trypanosomes it was assumed that WM1 represents the genotype of *T. grosi*, the trypanosome species that is believed to be restricted to wood mice (*Murinae; A. sylvaticus*). This is the first published 18S sequence from *T. grosi*. One representative of genotype WM2 was sequenced. This was found to differ from the *T. grosi* sequence at 11 out of 496 positions but only differed from *T. evotomys* of bank voles at 2 positions and from *T. microti* of field voles at 2 or 3 positions. This suggested that parasites with the WM2 genotype isolated from wood mice were much more closely related to the vole parasites. The same DNA sample that was used for the trypanosome PCR was genotyped using the microsatellite markers 55G7. This confirmed the identity of all host species from which positive PCR results were obtained (not shown).

**Flea data**

Ten out of the 56 fleas tested were positive for trypanosomes by PCR (Table 2). *Hae*III cut the PCR product of 1 of these samples producing fragments the same size as those of *T. grosi* WM1. The remainder were not cut by *Hae*III indicating that they lack the same *Hae*III restriction site that *T. evotomys* lacks. The PCR-RFLP data were interpreted to show that the fleas were infected by *T. evotomys* and *T. grosi*. The *T. grosi* sample and 3 of the *T. evotomys* samples from fleas were sequenced. The sequences obtained from the flea samples were identical to the *T. evotomys* and *T. grosi* WM1 samples collected from the rodents. The sequence data therefore confirmed the identities that had been assigned by PCR-RFLP. The *T. grosi*-infected flea had been collected from a wood mouse the natural host of this parasite. One of the 9 *T. evotomys*-infected fleas was also collected from a wood mouse, indicating that these animals may be challenged by heterologous parasites.

**Discussion**

All the PCR products from the bank vole samples lacked a *Hae*III restriction site that is found in all other rodent *T. (Herpetosoma)* 18S rDNA sequences including *T. microti* of field voles. *T. microti* is the
trypanosome of the most closely related host species that is found in the UK and only differs in sequence from *T. evotomys* by 2–3 out of 496 bases. The absence of this restriction site in *T. evotomys* alone is consistent with the bank vole alone being susceptible to infection with *T. evotomys* and is also consistent with the host specificity hypothesis. Any other species of trypanosome with a prevalence in the bank voles greater than 0.53% would have had a 95% chance of being detected in this sample of 560 animals (Post & Millest, 1991). The possibility that the primers used do not amplify other genotypes of *Trypanosoma* that infect bank voles cannot be excluded. However, this is considered unlikely since these primers amplify all species tested across the full range of *Trypanosoma* as well as *Leishmania* and monoxenous trypanosomatids (Noyes et al. 1999; Noyes, unpublished observations).

It is believed that the parasites are usually transmitted when fleas or flea faeces are eaten by the rodent during grooming (Hoare, 1972). Bank voles and wood mice share burrow systems that are infested with fleas and all 4 species of fleas were collected from both bank voles and wood mice. The finding that 1 of the infected fleas that was collected from a wood mouse was carrying the bank vole trypanosome *T. evotomys* is consistent with the assumption that both species of mammal are challenged by both species of parasites. The finding of both *T. evotomys* and *T. grosi* in *A. p. mustalae* indicates that the parasites are not restricted by vector species. All the infected fleas were collected in winter and 8 out of 10 were collected in January. Given that infections in bank voles may only be patent for 22–45+ days, and the low host abundance and parasite prevalence in winter and spring (Bajer et al. 2001; Molyneux, 1969; Turner, 1986), it is possible that fleas may act as an important reservoir host of the parasite during the winter.

Two of the 12 infections of the wood mice were with trypanosomes that had partial 18S sequences that were most similar to that of the bank vole and field vole parasites. This may be the result of a relatively recent host-switching event of a parasite of Arvicolinae to Murinae. The partial 18S sequence of the novel parasite of the wood mouse (genotype WM2) differed from that of *T. evotomys* by 2 out of 496 bases and from the 2 *T. microti* sequences by 2 and 3 out of 496 bases respectively. This is the same amount of difference as is found between *T. microti* and *T. evotomys* of the field vole and bank vole. It is also the same amount of difference as is found between *T. grosi* and *T. musculi* of the wood mouse and house mouse. In contrast there are 44 differences between the corresponding sequences of 2 *T. cruzi* strains (GenBank accession numbers M31432, X53917). Hence genetic distance is not a good guide to species status in the genus *Trypanosoma* as a whole. However, within *T. (Herpetosoma)*, genotype WM2 is as different from *T. evotomys* and *T. microti* as these two are from each other. Therefore WM2 might be sufficiently unique to be a distinct species specific to wood mice. Samples from a much wider geographical range will be required to determine the true genotypic diversity of these species. Consequently, the finding of a parasite from a wood mouse that is most closely related to the bank vole parasite *T. evotomys* does not disprove the host specificity hypothesis, since it is not close enough to *T. evotomys* to be confident that it is included in this species. However, it does invalidate the assumption that host range is a sufficient taxonomic character to justify the erection of a new species. If the 2 genotypes of wood mouse parasites were both included in *T. grosi* then this species would be paraphyletic.

### Parasite developmental patterns

Previous studies using isoenzymes have indicated that *T. (Herpetosoma)* species may have co-evolved with their hosts (Mohamed, Molyneux & Scott, 1987). With the exception of WM2 the data

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**Table 2. Flea species, infecting trypanosomes and rodent hosts**

(*A. p. mustalae =* Alamaerae penicilliger mustalae; *R. pentacantha =* Rhadinopsylla pentacantha; *C. n. vulgaris =* Ctenophthalmus nobilis vulgaris; *D. dasycnema =* Doratopsylla dasycnema; *A. sylvaticus =* Alamaerae sylvaticus; *T. musculi =* T. musculi; *T. flavescens =* T. flavescens

<table>
<thead>
<tr>
<th>Flea ID</th>
<th>Flea species</th>
<th>Host</th>
<th>Parasite species</th>
<th>Date</th>
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<td>25085600</td>
<td><em>A. p. mustalae</em></td>
<td><em>A. sylvaticus</em></td>
<td><em>T. grosi</em></td>
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<tr>
<td>24809274</td>
<td><em>R. pentacantha</em></td>
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<td><em>T. evotomys</em></td>
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</tr>
<tr>
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<td><em>R. pentacantha</em></td>
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<tr>
<td>38</td>
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<td><em>Sox</em> sp.</td>
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</table>
Presented here are consistent with this observation with 2 distinctive parasite groups from Arvicolinae and Murinae respectively. The parasites of these 2 host groups have different developmental patterns. The parasites of Murinae divide by unequal multiple fission in the epimastigote stage while the parasites of Arvicolinae divide by multiple or binary fission in the amastigote stage (Molyneux, 1970). A description of the developmental patterns of the 2 wood mouse genotypes may indicate whether these developmental patterns are host or parasite driven.

In the case of T. (Herpetosoma) species the vast majority of samples are consistent with the host specificity hypothesis and possibly also with coevolution of host and parasite. The discovery of a novel genotype of wood mouse trypanosomes, however, indicates that this may not be a rigid rule and therefore that host distribution is not a sufficient character for defining T. (Herpetosoma) species.

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