Trypanosoma avium of raptors (Falconiformes): phylogeny and identification of vectors

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SUMMARY

Avian trypanosomes are widespread parasites of birds, the transmission of which remains mostly unclear, with various blood-sucking insects mentioned as possible vectors. A search for vectors of trypanosomes of sparrowhawk (*Accipiter nisus*), buzzard (*Buteo buteo*), lesser-spotted eagle (*Aquila pomarina*) and kestrel (*Falco tinnunculus*) was performed in Czech and Slovak Republics. Black flies (*Eusimulium* spp.), hippoboscid flies (*Ornithomyia avicularia*), mosquitoes (*Culex pipiens pipiens*) and biting midges (*Culicoides* spp.), trapped while attempting to feed on raptor nestlings, were found to contain trypanosomatids in their intestine. Trypanosomes from the raptors and blood-sucking insects were isolated, and their 18S rRNA sequences were used for species identification and for the inference of intra- and interspecific relationships. Together with the trypanosome isolated from a black fly, the bird trypanosomes formed a well-supported *Trypanosoma avium* clade†. The isolates derived from hippoboscid flies and mosquitoes are most likely also avian trypanosomes infecting birds other than the studied raptors. Analysis of the kinetoplast, that has features characteristic for the avian trypanosomes (minicircle size; dimensions of the kinetoplast disc), provided further evidence for the identification of vectors. It is suggested that all trypanosomes isolated from raptors included in this study belong to the *T. avium* complex and are transmitted by the ornithophilic simuliids such as *Eusimulium securiforme*.

Key words: phylogeny, *Trypanosoma avium*, *Herpetomonas*, transmission, blood parasites, insect vectors.

INTRODUCTION

Since 1885, when Danilewsky first described a flagellate from the blood of an owl (*Strix aluco*) and named it *Trypanosoma avium*, hundreds of bird species have been found to host trypanosomes. The generally high prevalence but low parasitaemia, and the lack of transparent clinical signs, indicate that the pathogenicity of most avian trypanosomes is low (Kučera, 1982; Atkinson & van Riper, 1991; Svobodová & Votýpka, 1998), although cases of a marked negative impact of parasites on wild birds, including morbidity and mortality, have been documented (Molyneux & Gordon, 1975; Molyneux, Cooper & Smith, 1983).

Worldwide, about 100 species of avian trypanosomes have been described, mostly on the basis of one host–one species paradigm, according to which a new species was assigned for every 'new' bird host (Bishop & Bennett, 1992). However, clear-cut evidence of strict host specificity is lacking, and since the validity of most species has been questioned, the descriptions are considered to be *nomina dubia*; frequently, they are referred to as trypanosomes of the *Trypanosoma avium* complex (Baker, 1976; Woo & Barlett, 1982; Apanius, 1991; Bennett *et al*. 1994; Sehgal, Jones & Smith, 2001).

A wide variety of blood-sucking arthropods (mites, hippoboscids, biting midges, culicine mosquitoes and simuliids) has been described as vectors of avian trypanosomes (reviewed by Baker, 1956*a*, *b*; Desser, 1977; Chatterjee, 1977; Molyneux, 1977; Miltgen & Landau, 1982; Chandenier, Landau & Baccam, 1988; Mungomba, Molyneux & Wallbanks, 1989). Most of these studies were based on laboratory experiments with various haematophagous insects being tested as trypanosome vectors. Only one direct comparison of trypanosomatids from naturally infected birds and those from their possible vectors has been reported (Dirie *et al*. 1990). Whether there is a single avian trypanosome or whether every bird species has its specific parasite has yet to be elucidated, as well as how specific the vectors of these trypanosomes are.

In this paper, using the 18S rRNA genes, we aim to address the transmission and host–parasite relationships of trypanosomes isolated from the blood of raptors and the naturally infected blood-sucking insects trapped in their nests. This novel approach explores the informative value of the 18S rRNA

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† The GenBank accession numbers of the determined sequences are: AF416559 (*Trypanosoma* '*avium*' APO1), AF416560 (*Herpetomonas* sp. CER1), AF416561 (*Trypanosoma* sp. CUL1), AF416562 (*Trypanosoma* sp. OA6), AF416563 (*Trypanosoma* '*avium*' SIM3).

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sequences that have been successfully employed for the inference of interspecific relationships of these parasites (reviewed by Stevens & Gibson, 1999; Stevens *et al*. 2001). Since the first 18S rRNA sequence of the raven trypanosome was published (Maslov *et al*. 1996), sequences of this gene from three more avian trypanosomes have become available. In the maximum likelihood (ML) and maximum parsimony (MP) trees, species parasitizing birds appear to be paraphyletic and their relations to other trypanosomes remain ambiguous (Stevens & Gibson, 1999; Stevens *et al*. 2001).

An alternative approach, which could contribute to the identification of probable vectors, is the analysis of kinetoplast (k)DNA. The order Kinetoplastida has been defined by the presence of a unique organelle, the kinetoplast, which is a DNA-containing compartment of the single mitochondrion. The kDNA of trypanosomes is composed of circular molecules (thousands of minicircles and dozens of maxicircles) interlocked into a single large network (reviewed by Lukeš *et al*. 2002). *In vivo*, the kDNA network is packed into a characteristic compact disc, located in the parabasal region of the mitochondrion. The size of minicircles is a characteristic feature for a trypanosome species (Ray, 1989). It was shown recently that the structure of the disc, as well as the size of the kDNA minicircles, varies significantly among avian trypanosomes (Yurchenko *et al*. 1999; Lukeš & Votýpka, 2000). We have, therefore, analysed the minicircle size and the kDNA structure in our set of isolates and assessed their potential as species-specific features.

In order to track the trypanosomes of the raptors investigated in this study in their possible vectors (blood-sucking dipterans *Eusimulium* spp., *Ornithomyia avicularia*, *Culex pipiens pipiens* and *Culicoides* spp.), we used both the 18S rRNA sequences and the structure of the kDNA as specific markers. Herein, we demonstrate that ornithophilic simuliids are the most probable vectors of trypanosomes isolated from a sparrowhawk, buzzard, kestrel and lesser-spotted eagle.

MATERIALS AND METHODS

Collection and cultivation of trypanosomes from raptors and insects

Blood samples were taken from the brachial vein of raptors during the breeding seasons of 1997–2000 (usually from the beginning of May until the end of July). Adult birds were trapped in a net when attacking a stuffed or living eagle-owl, while the blood collection from nestlings was performed directly in the nest. The blood samples from the raptor populations of sparrowhawk (*Accipiter nisus*), buzzard (*Buteo buteo*), kestrel (*Falco tinnunculus*), and lesser-spotted eagle (*Aquila pomarina*) from several localities in Central Bohemia, Southern Moravia and Eastern Bohemia (Czech Republic), and North-Eastern Slovakia, respectively, were analysed. For *in vitro* cultivation, the blood was inoculated into 2 ml vol. glass cultivation flasks containing the sloping rabbit blood agar medium overlayed with a liquid phase (SNB-9 of Diamond & Herman, 1954) and supplemented with gentamicin (80 μg/ml). Cultivation was performed at room temperature in a tilted position; the flasks were checked weekly for trypanosome presence and subsequently cultivated on blood agar or in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) foetal calf serum (Sigma). Non-contaminated isolates are stored in the cryobank of the Department of Parasitology, Faculty of Science, Charles University, Prague.

Blood-sucking insects attacking the nestlings of raptors were collected either directly (hippoboscid flies) or using air-sucking miniature CDC (Centre for Disease Control) traps without a light bulb placed at the level of the nest, 5–18 meters above the ground (black flies, mosquitoes and biting midges). Traps were installed overnight and captured specimens were kept in cages in humid and cold conditions. Dissection and light microscopy examination of the intestine and salivary glands for the presence of parasites was performed within 3 days after capture. Infected samples were streaked on the SNB-9 blood agar, stabilized and stored as described above.

Total DNA and kDNA isolation, PCR, and sequencing

Isolation of the kDNA network, total cellular DNA, PCR amplification of the 18S rRNA genes using specific primers S762 and S763, cloning, and sequencing with a set of conserved primers were performed as described previously (Jirků *et al*. 1995; Maslov *et al*. 1996). While the complete 18S rRNA gene was obtained for 5 selected isolates (APO1, SIM3, OA6, CUL1 and CER1), for 3 other raptor isolates (AN14, BUT15 and FT2) only the V4 hypervariable domain was sequenced. This domain, amplified using the primers S662 and S713 (Maslov *et al*. 1996), was shown to be specific for *T. avium* (positions 690–1262 in U39578). The size of minicircles was determined by digestion of the isolated kDNA with several restriction enzymes, out of which *Pvu*II cuts only once in most of the minicircles. Restriction digestion and agarose gel electrophoresis were performed according to standard protocols.

Phylogenetic analysis

Using the Clustal-X program (Thompson *et al*. 1997), the 5 complete 18S rRNA sequences were

aligned with the homologues from 5 *Trypanosoma* spp., 15 other trypanosomatids, 6 bodonids and the euglenids *Euglena gracilis* and *Petalomonas cantuscygni* (alignment I). The latter 2 species were used as outgroups. The tree was constructed using MP as the optimality criterion and the Tree-bisection-reconnection as the branch swapping method. All phylogenetic analyses were performed using PAUP 4.8b program (Swofford, 1998).

Another alignment contained 18S rRNA sequences of 52 trypanosomes, *Crithidia oncopelti*, *C. fasciculata*, *Leishmania major*, *L. donovani*, *L. amazonensis*, and *L. guyanensis*, and related outgroup bodonids *Bodo caudatus* and *Trypanoplasma borreli* (alignment II). This alignment was based on the secondary structure-derived alignment of Stevens *et al*. (2001).

Different portions of both alignments representing different levels of elimination of ambiguous or extremely variable regions were used for the MP analysis performed with random addition of sequences with 50 replicates. The nodal bootstrap support of all trees was computed out of 1000 replicates with 10 replicates of the sequences random addition.

The ML tree was inferred from a limited dataset containing 26 trypanosomes and *C. fasciculata* as an outgroup. The alignment used for the ML analysis was derived from the alignment II, by the exclusion of rapidly evolving sequences of the *T. brucei* clade. Moreover, the *T. cruzi* clade was partially reduced as well, and only several representatives of this cluster (*T. cruzi*, *T. vespertilionis* and *Trypanosoma* sp. 'kangaroo') were analysed. ML bootstraps were derived from 300 replicates. The Kishino-Hasegawa (K-H) and Shimodaira-Hasegawa (S-H) tests of 9 constrained trees were performed as implemented in PAUP 4.8b program. Both tests were computed using the RELL bootstrap (1000 replicates) with settings corresponding to the HKY85 model with starting branch lengths obtained via the Rogers-Swofford approximation method (Swofford, 1998).

Electron microscopy

For transmission electron microscopy, cells collected from culture in the exponential phase were washed in 0·1 M phosphate-buffered saline solution and fixed in 2·5% glutaraldehyde in the same buffer at 4 °C. Flagellates were further processed and the kDNA structure was analysed as described previously (Lukeš & Votýpka, 2000).

RESULTS

Trypanosomes obtained from raptors and insects

During 4 seasons from 1997 to 2000 we examined the blood of more than 1300 raptors from various localities in the Czech Republic and Slovakia. The following trypanosomes of raptors were successfully isolated into culture and cryopreserved: 31 isolates from buzzards, 21 isolates from sparrowhawks, 2 isolates from kestrels, and 7 isolates from lesser-spotted eagles. No conspicuous differences in growth and morphology between the isolates have been observed.

More than 2400 haematophagous insects were trapped while attacking the nestlings of buzzards and sparrowhawks. Examination of their intestine and salivary glands resulted in the isolation and subsequent introduction into culture of a total of 41 isolates: 17 from mosquitoes (*Culex pipiens pipiens*), 12 from black flies (*Eusimulium securiforme* and *E. latipes*), 8 from hippoboscid flies (*Ornithomyia avicularia*), and 4 from biting midges (*Culicoides festivipennis*, *C. cubitalis* and *C. sylvarum*). In contrast to the trypanosomes isolated from raptors, the flagellates originating from insects exhibited significant differences in growth, motility and morphology.

From this large set of newly isolated flagellates, we selected 8 isolates (see Table 1 for a detailed molecular characterization). Each of them originates from a different raptor or insect host. The most important selection criterion was the age of the bird host, since the parasites of nestlings should be autochthonous for the breeding locality and may, therefore, rather correspond with trypanosomes isolated from the locally trapped blood-sucking insects. The trypanosome from kestrel represents an exception in this respect, since only isolates from adult birds were available. For further molecular analysis insect isolates with morphological features characteristic of the genus *Trypanosoma* (as described by Baker, 1976) were preferentially selected. Based on morphology, the appurtenance of parasites of *Culicoides* spp. to the genus *Trypanosoma* was questionable, and this notion was confirmed by molecular analyses (see below).

Phylogenetic analysis of the 18S rRNA genes

First, we studied the phylogenetic position of the newly obtained sequences within the kinetoplastids. The alignment I contained 2553 characters from which 857 were constant and 1208 variable characters were parsimony informative. Two most parsimonious trees constructed were 4393 steps long. Four newly obtained sequences branched within the *Trypanosoma* clade (*T.* '*avium*' SIM3 [black fly], *T.* '*avium*' APO1 [lesser-spotted eagle], *Trypanosoma* sp. CUL1 [mosquito] and *Trypanosoma* sp. OA6 [hippoboscid fly]), while the parasite isolated from the biting midge appeared within the *Herpetomonas* subclade. Further phylogenetic analyses were restricted to the *Trypanosoma* clade.

Table 1. Trypanosomatid isolates originating from raptor and insect hosts: the minicircle size (in kb) and kinetoplast thickness (in μm) are shown

(The kinetoplast thickness values are means ± standard deviation; *n*, number of kinetoplasts measured; N.I., not investigated.)

Isolate	Host species (Bird)	Host age	Minicircle size (kb)	Thickness (μm)	*n*
AACC/CZ/99/AN14	Sparrowhawk (*Accipiter nisus*)	Nestling	7	0·782 ± 0·055	40
ABUT/CZ/99/BUT15	Buzzard (*Buteo buteo*)	Nestling	6	0·691 ± 0·080	38
AAQU/SK/97/APO1	Lesser-spotted eagle (*Aquila pomarina*)	Nestling	7	0·848 ± 0·067	35
AFAL/CZ/99/FT2	Kestrel (*Falco tinnunculus*)	Adult	7	0·748 ± 0·058	34
	Vector species (Insect)	Collected on nest			
IEUS/CZ/99/SIM3	Black fly (*Eusimulium securiforme*)	Buzzard	7	0·888 ± 0·048	40
IORN/CZ/99/OA6	Hippoboscid fly (*Ornithomyia avicularia*)	Sparrowhawk	3	0·368 ± 0·015	30
ICUL/CZ/98/CUL1	Mosquito (*Culex pipiens pipiens*)	Buzzard	3	0·310 ± 0·031	35
ICUL/CZ/99/CER1	Biting midge (*Culicoides cubitalis*)	Buzzard	N.I.	0·174 ± 0·049	35

The alignment II contained 2562 characters, from which 700 ambiguous or extremely variable characters were excluded. Out of the remaining 1862 nucleotides, 359 characters were parsimony informative. In the MP tree of 1446 steps, trypanosomes split into several clusters, the species-composition of which was in good agreement with a recent extensive phylogenetic analysis (Stevens *et al*. 2001). The trypanosomes from aquatic hosts formed an early branching 'Aquatic' clade, followed by the well-defined *T. brucei* and *T. cruzi* clusters. All *T.* '*avium*' from birds formed, together with the isolate from the black fly (*Eusimulium securiforme*), a highly supported clade (bootstrap 99%). Sequence analysis of the V4 hypervariable domain of the 18S rRNA gene of 4 isolates from raptors and 1 isolate from black fly revealed identical sequences in this generally highly variable part of the gene (Fig. 2). Therefore, all obtained sequences confirmed the affiliation of respective isolates to the *T. avium* complex (see below). However, due to a polytomy, the relationship of these *T. avium* isolates to *T. bennetti* from an American kestrel, *T. scelopori*, *T. varani* and *T. grayi* from reptiles, and the newly obtained trypanosomes from insect (*Trypanosoma* sp. CUL1 and *Trypanosoma* sp. OA6) was not resolved (data not shown).

To study this relationship in more detail we have performed the ML analysis of a reduced dataset derived from the alignment II (see Materials and Methods section). The alignment contained 27 taxa with 257 variable characters from which 150 were parsimony informative. The obtained topologies displayed different phylogenetic positions for *T. scelopori*, *T. varani*, *T. bennetti*, and *T. grayi* and 2 newly sequenced trypanosomes from the insect vectors (CUL1 and OA6) (Table 2). The ML analysis placed *Trypanosoma* sp. CUL1 and *Trypanosoma* sp. OA6 (Fig. 3, cluster B) as a sister group of the highly supported *T. avium* clade (Fig. 3, cluster A). The joint cluster (A + B) was related to the clade of the reptile trypanosomes and *T. bennetti* (Fig. 3, clusters $C_1 + C_2$). However, the association between the clusters B, C_1, and C_2 was weakly supported and also the affinity between the clusters B and A was low (Fig. 3). Since the nodal support of this partial ML tree was generally low, we tested 9 constrained tree topologies using the K-H and S-H tests (Table 2). Both tests do not support monophyly of the cluster $C_1 + C_2$ as it appeared in the unconstrained tree. The K-H test supports a tree topology with the subclade C_2 (*T. bennetti* and *T. grayi*) related to the A + B cluster, while the subclade C_1 (*T. scelopori* and *T. varani*) is placed as a sister group to the cluster E (*T. lewisi* and *T. microti*). The affinity of the subclade C_2 with the avian trypanosomes is better supported by the S-H test (Table 2), mutual swapping of the subclades C_1 and C_2 being another feature of the S-H-based analysis.

Analysis of the kDNA disc structure and minicircle size

The DNA strands were packed in parallel to the axis of the disc in all analysed isolates. The cylindrical-shaped kDNA was observed by electron microscopy in the isolate from sparrowhawk (Fig. 4A), the other isolates derived from raptors (buzzard, lesser-spotted eagle and kestrel; data not shown), and also in the black fly isolate (Fig. 4B). In contrast, kinetoplasts of the other isolates obtained from insects (hippoboscid fly, mosquito and biting midge) have a low pitched and elongated shape (Fig. 4C–E). Based on the thickness of the kDNA disc, we were able to distinguish 2 different size categories. An exceptionally thick kinetoplast occurred in the trypanosomes obtained from raptor and the black fly isolates, while a significantly narrower kinetoplast was a feature shared by the remaining insect isolates

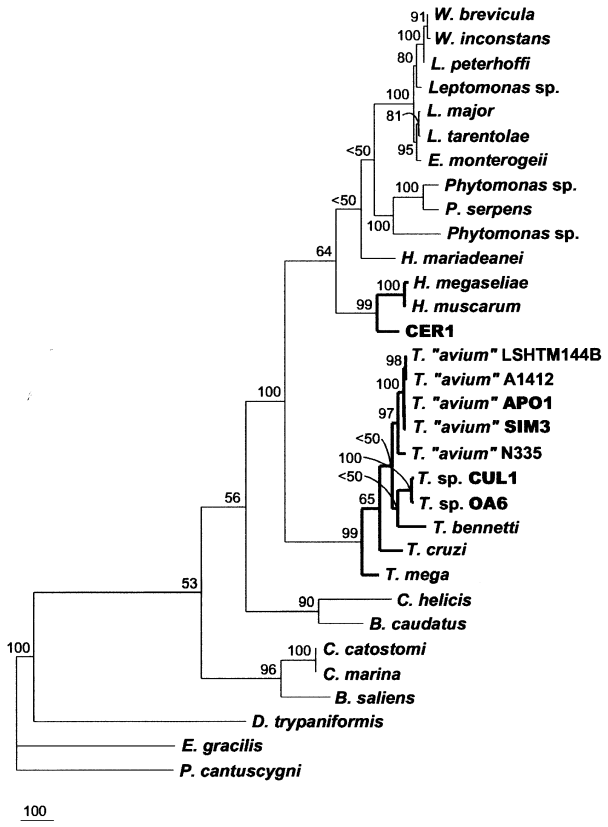

Fig. 1. Maximum parsimony tree based on the alignment I. Tree was constructed out of 1999 characters from which 763 were constant, 499 variable characters were parsimony uninformative and 737 sites were informative. Tree is 2940 steps long, CI = 0·6556, HI = 0·3444, CI excluding uninformative characters = 0·5596, HI excluding uninformative characters = 0·4404, RI = 0·7080, RC = 0·4641. Bootstrap support of selected nodes is indicated (1000 replicates).

(hippoboscid fly, mosquito and biting midge; see Table 1).

The existence of 2 different groups was reflected by the size of individual minicircles that constitute the kDNA network. Upon release from the network, the linearized minicircles were visible as a single band in the agarose gel (Fig. 5). This analysis revealed that the 'thick' discs, as they were observed in electron microscopy, are composed of large minicircles of 6 kb (the buzzard isolate) and 7 kb (the sparrowhawk, lesser-spotted eagle, kestrel and black fly isolates) (Fig. 5, lanes A–E). Similarly, the 'thin' discs of the hippoboscid and mosquito isolates were shown to contain 3 kb minicircles (Fig. 5, lines F–G). In all studied isolates a tight correlation between the minicircle size and the thickness of the kinetoplast was proven (Table 1).

DISCUSSION

In this work, a combined approach was used to identify vectors of trypanosomes that frequently infect various raptor species in Central Europe. We have obtained new isolates from the blood of raptors and, at the same time, we have trapped infected haematophagous insects directly at or in the vicinity of the bird nests.

Trypanosomatids originating from insects could belong either to the genus *Trypanosoma*, or to the monoxenous insect genera *Crithidia*, *Blastocrithidia*, *Herpetomonas*, *Leptomonas* and *Wallaceina*. Sequence analysis of the 18S rRNA genes of 4 selected isolates derived from the black fly, hippoboscid fly, mosquito and biting midge showed that, with the exception of the latter isolate, all flagellates are genuine trypanosomes. The sequences indicated that the parasite of *Culicoides cubitalis* is a *Herpetomonas* species, and therefore, as a member of a genus confined to insects, was not studied further. It seems to be the first report of a monoxenous trypanosomatid in the biting midge (see Podlipaev, 1990).

Sequencing of the highly polymorphic V4 domain of the 18S rRNA gene showed 100 % identity of all studied raptor isolates. This finding is supported by the electrophoretic patterns obtained by Kirkpatrick & Terway-Thompson (1986) who suggested that, to the exclusion of *T. bennetti*, all raptor trypanosomes under their study constituted a single *T. avium* group. These results clearly support the hypothesis that all raptor trypanosomes belong to the compact *T. avium* complex.

Our MP analysis included all 18S rRNA genes known for *Trypanosoma* spp., several representatives of the monoxenous genera, and two outgroup bodonids. *Trypanosoma* species that split from the rest of the genus form, in accordance with earlier studies (Stevens *et al.* 2001), the 'Aquatic' clade that comprises species associated with the aquatic environment. The branching order of the other clades remained unresolved, creating a 10-way polytomy (data not shown). In this tree, a trypanosome isolated from the black fly is closely related to several bird trypanosomes, thus creating a new clade for which we propose the term *T. avium* clade. The homogeneous enlarged group of the *T. avium* complex contains species derived from the raven (*Corvus frugilegus*), chaffinch (*Fringilla coelebs*), and lesser-spotted eagle (*Aquila pomarina*), all originating from Central Europe, the Asian Java sparrow (*Padda oryzivora*), and one isolate from the vector (*Eusimulium securiforme*). Moreover, the ML tree revealed an affinity of the vector subclade, composed of flagellates from the mosquito (*Culex pipiens pipiens*, isolate *Trypanosoma* sp. CUL1) and the hippoboscid fly (*Ornithomyia avicularia*, isolate *Trypanosoma* sp. OA6), with the *T. avium* clade. Although the bootstrap support for this relationship is not high, the clustering of trypanosomes from birds and insects was significantly better supported by the K-H and S-H tests than were alternative topologies.

In the unconstrained ML tree (obtained using heuristic search), *T. bennetti*, *T. grayi*, *T. scelopori*,

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                         1051                                                1100
Eagle (APO1)             GATTATGG-G GCTGTGCGAC AAGC-GGCTG GGTGTA--TT CC-------- ----------
Sparrowhawk (AN14)       .......... .......... .......... .......... .......... ..........
Kestrel (FT2)            .......... .......... .......... .......... .......... ..........
Buzzard (BUT15)          .......... .......... .......... .......... .......... ..........
Black fly (SIM3)         .......... .......... .......... .......... .......... ..........
Raven                    .......... .......... .......... .......... .......... ..........
Chaffinch                .......... .......... .......... .......... .......... ..........
Java Sparrow             .......... .......... .......... .......... .......... ..........
Mosquito (CUL1)          .........A A.....T... ...T...... .....TAT.C .......... ..........
Hippoboscid fly (OA6)    ........GA A.....T... ....C..... .....TAT.C .......... ..........
T.bennetti               .......... ....C..... .......... .....C.... .T........ ..........
T.grayi                  .......... .......... .......... .....T...C .......... ..........
T.scelopori              ...C...... ......T... ........C. ..C.AC..CC .TTGAATCCC CTCTCCACAA
T.cruzi                  .......... ..A...T... .......... ....GTTA.. ..ACACACAC ACGCACACT.

                         1101                                                1150
Eagle (APO1)             CCTTTACTGG GGG------- ---------- CACCCGTCGC CTT-TGTGAG AAATCAGTGG
Sparrowhawk (AN14)       .......... .......... .......... .......... .......... ..........
Kestrel (FT2)            .......... .......... .......... .......... .......... ..........
Buzzard (BUT15)          .......... .......... .......... .......... .......... ..........
Black fly (SIM3)         .......... .......... .......... .......... .......... ..........
Raven                    .......... .......... .......... .......... .......... ..........
Chaffinch                .......... .......... .......... .......... .......... .....C....
Java Sparrow             .......... .......... .......... .......... ....C..CG. .....C...T
Mosquito (CUL1)          .......... ...G...... .........G .......... ...TC...G. .....C...T
Hippoboscid fly (OA6)    ----.CAA.. A......... .......... ..A....... ........G. .....C...T
T.bennetti               ---A.T.G.. A......... .......... .......... ......GTG. .....C...T
T.grayi                  .G.C.G.GAA ...GAGGAG. ......GAGC .GT...C... ......G.G. GGT..--.C.
T.scelopori              .....TT... AT.TTGTGTC TTGTGTGTGG ...T...... ........G. .....C...T

                         1151                                                1200
Eagle (APO1)             CG-------- -----TTGTC GT--AGCTTC GGCTAT---- -----CG-AT TTC------G
Sparrowhawk (AN14)       .......... .......... .......... .......... .......... ..........
Kestrel (FT2)            .......... .......... .......... .......... .......... ..........
Buzzard (BUT15)          .......... .......... .......... .......... .......... ..........
Black fly (SIM3)         .......... .......... .......... .......... .......... ..........
Raven                    .......... .......... .......C.. .......... .......... ..........
Chaffinch                .......... .......... .......C.. .......... .......... ..........
Java Sparrow             .......... .......... T.TA...... ....T..... .......... ..........
Mosquito (CUL1)          ..G....... ....G..AAA .G..G..... ....T..... ......TTGA ..........
Hippoboscid fly (OA6)    ..A....... ....G..AAA .G..G..... ....T..... ......TTGA ..........
T.bennetti               .CA....... .....-.TC. .GG.G..... ...ATCT... ......GTG. ..-.......
T.grayi                  ..G....... .....-.A.. .C.GG..... ....CG.... .....-TCGA ..........
T.scelopori              ..G....... ......C.CG .A..CA.AC. .T.C.C.... .....---.. CC.......-
T.cruzi                  G.CACTTGTT TGGTG....T .GCAGA.T.. ..TCTTGCCT TCACA.ACG. ...ACATGT.

                         1201                                                1251
Eagle (APO1)             TCAT--CTTC TCGCAACTCG CGGCATCCAG GAATGAAGGA GGGTAGTTCG GGGGAGAACG
Sparrowhawk (AN14)       .......... .......... .......... .......... .......... ..........
Kestrel (FT2)            .......... .......... .......... .......... .......... ..........
Buzzard (BUT15)          .......... .......... .......... .......... .......... ..........
Black fly (SIM3)         .......... .......... .......... .......... .......... ..........
Raven                    .......... .......... ........G. .......... .......... ..........
Chaffinch                .......... .......... ........G. .......... .......... ..........
Java Sparrow             .......... .......... .......... .......... .......... ..........
Mosquito (CUL1)          G..C...... ..ATG..... ...TT..... .......... .......... ..........
Hippoboscid fly (OA6)    G..C...... C.ATG..... ...TT..... .......... .......... ..........
T.bennetti               G.GC...... C.T.A..... T...T..... .......... .......... ..........
T.grayi                  G.GC...... CAT.G..... ....A..... .......... .......... ..........
T.scelopori              G..G..GCC. ..T.A....T .......... .......... .......... ..........
T.cruzi                  ....GC.... C.T.A....A .......... .......... .......... ..........
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Fig. 2. Alignment of the variable rDNA region (positions 1051–1250) specific for putative *Trypanosoma avium* isolates. This region is conservative for trypanosomes isolated from eagle (reference sequence, APO1), sparrowhawk (AN14), kestrel (FT2), buzzard (BUT15) and black fly (SIM3). Raven (A1412), and chaffinch (LSHTM144B) differ in 2 positions (1177 and 1229); the isolate from the Java sparrow (N335) represents the most variable putative *T. avium* and differs in 5 positions (1146, 1171, 1173, 1174, 1185) from the reference sequence. The GenBank accession

Table 2. The Kishino-Hasegawa (K-H) and Shimodaira-Hasegawa (S-H) tests of constrained trees

(Both tests were computed using RELL bootstrap (1000 replicates). These settings correspond to the HKY85 model with starting branch lengths obtained using the Rogers-Swofford approximation method. The tests were performed as implemented in the PAUP program version 4.8b. Clusters A–H are indicated in an unconstrained maximum-likelihood tree (see Fig. 5). K-H and S-H tests were computed out of the dataset used for the maximum-likelihood analysis. 'Best' = the tree with the best likelihood score.)

				K-H-test	S-H-test
Tree no.	Tree topology	−ln L	Diff −ln L	*P*	*P*
1	((((((A,B),C),(D,E)),F),G),H)	4492·31333	42·20057	0·004	0·004
2	(((((A,B,C),(D,E)),F),G),H)	4499·40976	42·29700	0·001	0·001
3	(((((A,B),(D,E)),C),F),G),H)	4492·31333	42·20057	0·005	0·006
4	((((((A,(D,E)),(C,B)),F),G),H)	4498·43633	48·32357	0·002	0·002
5	((((((A,(D,E)),C),B),F),G),H)	4499·40976	49·29700	0·002	0·004
6	((((((A,(D,E)),C),F),B),G),H)	4496·55358	46·44082	0·004	0·007
7	((((((A,C),(D,E)),F),B),G),H)	4496·52254	46·40978	0·000	0·004
8	(((((((A,B),C_1),(D,E)),C_2),F),G),H)	4450·52717	0·41441	0·000	0·773
9	(((((((A,B),C_2),(D,E)),C_1),F),G),H)	4450·11276	Best	Best	Best

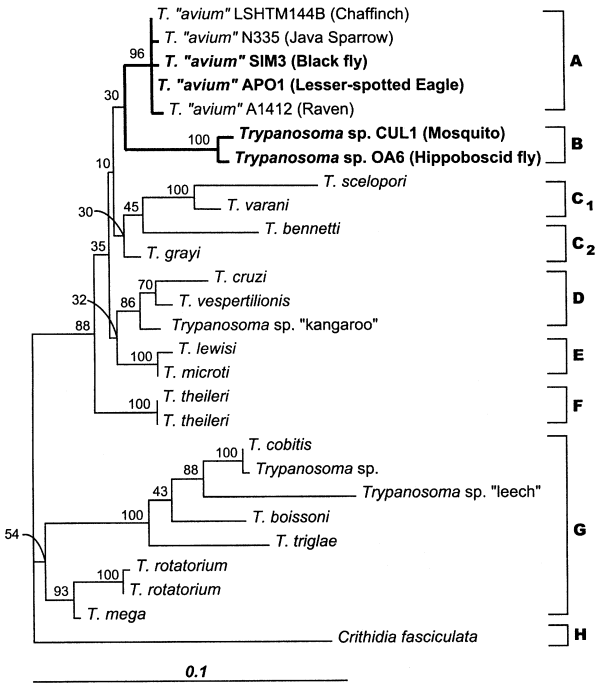

Fig. 3. Maximum likelihood tree based on the narrowed alignment II. Tree was constructed out of 854 characters (1708 ambiguous, highly variable or gapped characters were excluded from the primary alignment), 232 characters were variable. Likelihood score of the best tree found = 3877·41273. Bootstrap support was computed out of 300 replicates. Likelihood settings correspond to the HKY85 model.

and *T. varani* form a monophyletic group closely related to the cluster of *T. avium* and the insect-derived species. Both likelihood (K-H and S-H) tests of constrained tree topologies do not support the monophyly of the 4 above-mentioned trypanosomes and prefer the affinity between *T. grayi* and *T. bennetti* on one side, and trypanosomes from the *T. avium* complex and the insect isolates on the other side. In previous studies, *T. bennetti* formed a separate branch thus rendering the trypanosomes from bird hosts polyphyletic (Hagg, O'Huigin & Overath, 1998; Stevens & Gibson, 1999). Although our MP analysis of an extended set of species showed the same results, the likelihood tests prefer a common origin of the bird trypanosomes. The picture is, however, complicated by the fact that *T. grayi* from crocodiles is also part of this clade, which is in accordance with its position in the tree of Hagg *et al.* (1998), where it is inserted between *T. bennetti* and *T. avium*. Since Molyneux (1973) described that tse-tse fly could serve as a transmitter of the avian trypanosomes, it is possible that the close relationship of *T. grayi* and the avian trypanosomes reflects their (ancient) co-transmission by tse-tse flies in Africa. Alternatively, since crocodiles are more closely related to birds than to lizards, this relationship may reflect possible co-evolution. Importantly, the addition of new 18S rRNA sequences of trypanosomes from raptors and insects brought the avian species together, thus favouring the scenario of a certain degree of co-evolution of these flagellates with their hosts. (But note the illustrated polyphyly of the reptile trypanosomes.) The addition of more trypanosomes from birds, reptiles and insect vectors is needed in order to establish whether all bird trypanosomes including *T. bennetti* form a monophyletic clade.

Sehgal *et al.* (2001) recently reported partial SSU rDNA sequences of trypanosomes isolated from the

numbers of reference sequences are: AY099318 (*T.* '*avium*' AN14), AY099319 (*T.* '*avium*' FT2) and AY099320 (*T.* '*avium*' BUT15).

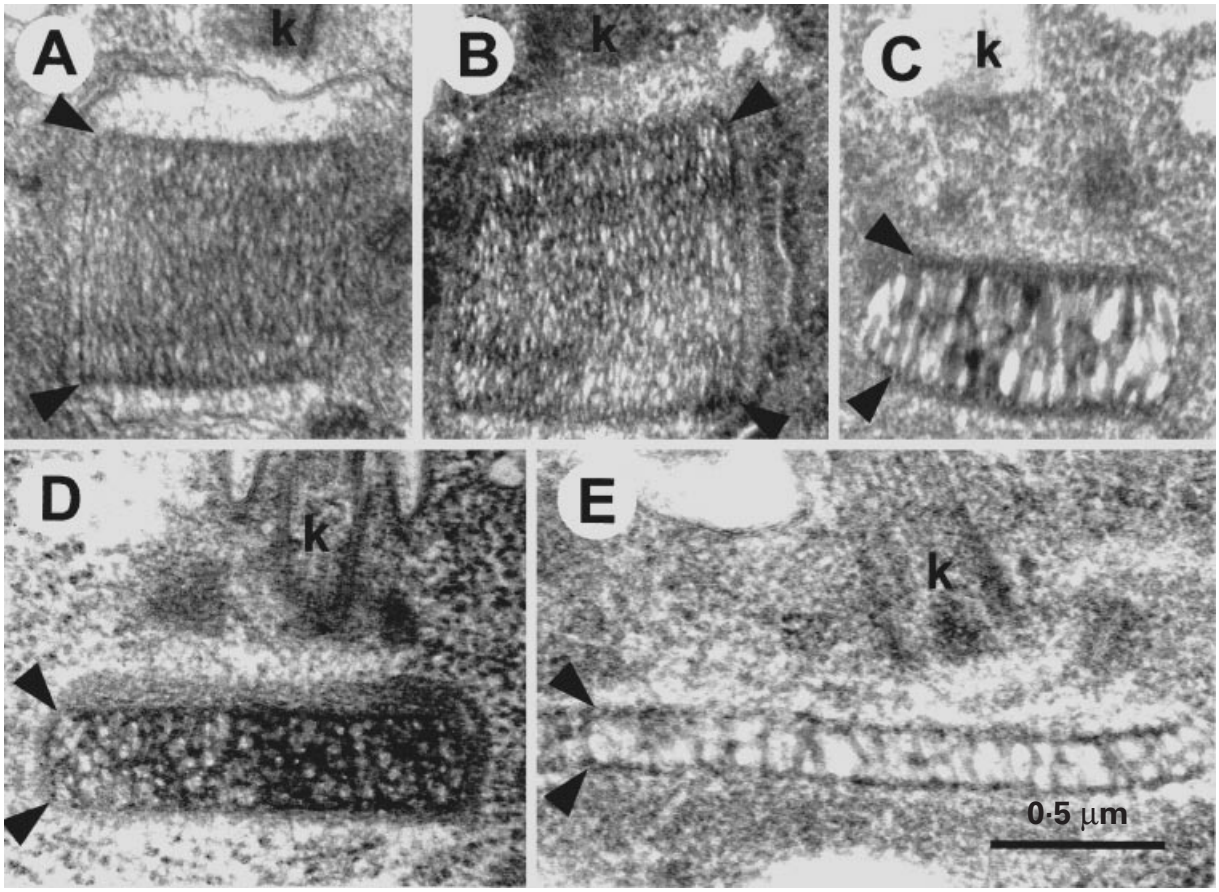

Fig. 4. Transmission electron microscopy of kinetoplasts from trypanosomatids isolated from raptor and insect hosts. Trypanosomes with the characteristic cylindrical-shaped kDNA are represented by isolates from sparrowhawk AN14 (A) and black fly SIM3 (B). kDNAs of trypanosomes from hippoboscid fly OA6 (C), mosquito CUL1 (D) and *Herpetomonas* sp. from biting midge CER1 (E) have a low-pitched and elongated shape. Arrowheads indicate thickness of kinetoplasts; k, kinetosome of the flagellum. All micrographs are to the same scale.

African singing birds. The position of these sequences is generally unstable in our phylogenetic trees, which is caused by their short length and low informative value. Some of these trypanosomes appear to be related to *T. avium* (clade A) and others to *T. bennetti*, while 1 is strongly affiliated with our isolates from insects (CUL1 and OA6; clade B) (data not shown). This unexpected relationship indicates that mosquitoes and hippoboscid flies are transmitters of trypanosomes parasitizing birds of the order Passeriformes.

Based on the phylogenetic analysis and the strictly ornithophilic feeding preferences of *Ornithomyia* flies and *Culex pipiens pipiens* mosquitoes, we conclude that isolates obtained from these vectors are almost certainly avian trypanosomes. Since several nucleotide differences exist between the 18S rRNA genes of these isolates and those obtained by us directly from raptors, we assume that *Culex* and *Ornithomyia* serve as vectors of as yet unidentified trypanosome(s) of birds. Such a scenario would be supported by the fact that these haematophagous insects are opportunistic feeders attacking various bird species. Buzzards and especially sparrowhawks are hunters of singing birds, and are therefore in frequent physical contact with other birds. Such a behaviour considerably increases the chance that hippoboscid flies found on these raptors may have previously fed on another bird species. This would explain an unexpected finding of a typical *T. avium* isolate (7 kb minicircles; AN14) in the blood of a sparrowhawk nestling infested at the same time by a hippoboscid fly parasitized by another trypanosome species (3 kb minicircles; OA6). Likewise, the bank vole trypanosome *T. evotomys* was found in a flea caught on a wood mouse (Noyes *et al*. 2002). Therefore, blood-sucking vectors seem to be less specific towards their vertebrate hosts than the trypanosome parasites they transmit.

We have previously shown that some bird trypanosomes have extremely large kDNA minicircles (Yurchenko *et al*. 1999), this feature being reflected in a unique cylindrical shape of their kinetoplast (Lukeš & Votýpka, 2000). In search for this specific marker, we have isolated kDNA networks from all 8 studied isolates and performed their restriction analysis, since minicircle size can be deduced from the *Pvu*II digestion that linearizes most of the minicircles. Similar size (6 and 7 kb) of the kDNA minicircles of the avian trypanosomes and those of

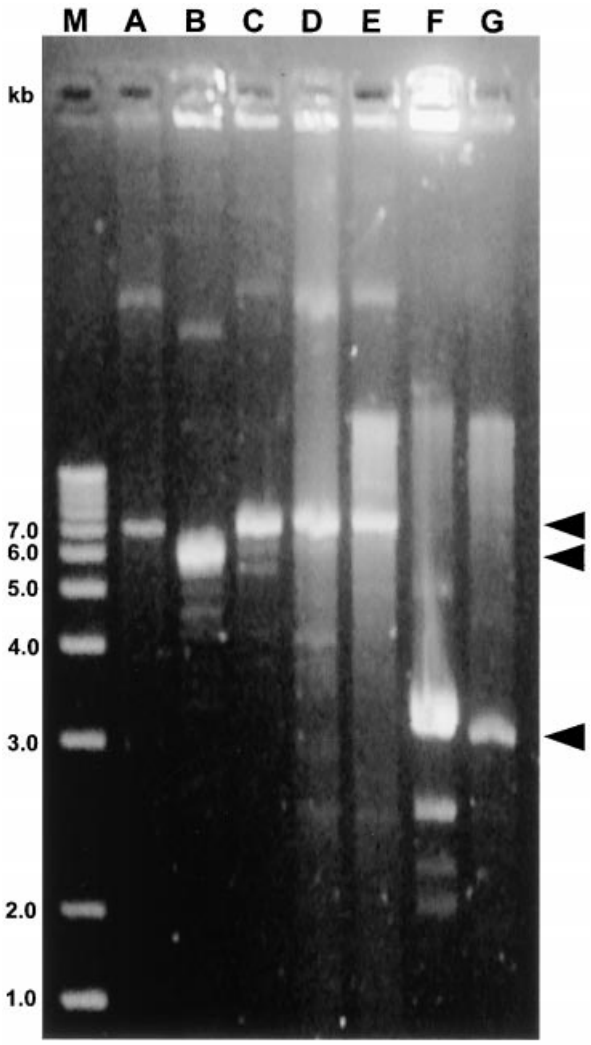

Fig. 5. Agarose gel (0·75%) electrophoresis run in the presence of ethidium bromide of the kDNAs of trypanosomes isolated from raptor and insect hosts, digested with *Pvu*II. Kb ladder (BRL) (lane M), the sizes of linearized minicircles of trypanosomes from sparrowhawk AN14 (lane A), buzzard BUT15 (lane B), lesser-spotted eagle APO1 (lane C), kestrel FT2 (lane D), black fly SIM3 (lane E), hippoboscid fly OA6 (lane F) and mosquito CUL1 (lane G) are indicated by arrowheads.

the isolate from *Eusimulium* further supports the conclusion that they all belong to one species or a complex of very closely related species. As expected, the ultrastructure of their kinetoplasts matches well with the size of minicircles, and the kDNA discs of the *Eusimulium* and raptor trypanosomes have almost identical size and morphology.

In search for vectors of the trypanosomes of raptors, we have selected parasites derived from nestlings, because they should be indigenous to the breeding locality. Insect isolates with morphological features characteristic of the genus *Trypanosoma* were selected. Along with above-mentioned 8 bird- and insect-derived isolates studied in detail, we have analysed the kDNA of 11 other isolates from raptors (4 from buzzards, 2 from sparrowhawks and 1 from a kestrel) and insect vectors (1 from black fly, mosquito, hippoboscid fly and biting midge each). Electron microscopy study and restriction enzyme digestion of these isolates further supported our conclusion that the black flies are genuine vectors of the raptor trypanosomes (data not shown). Nevertheless, there is still a possibility that trypanosomatids that parasitize raptors are transmitted not only by black flies, but also by other blood-sucking arthropods.

There are 4 main possibilities regarding the specificity of the host–parasite relationship. Avian trypanosomes are (i) bird and vector specific, (ii) bird specific but not vector specific, (iii) vector specific but not bird specific, and (iv) not specific to either host. Questions concerning host specificity of avian trypanosomes remain open, and one has to await analysis similar to that performed with trypanosomes of voles and wood mouse (Noyes *et al*. 2002). Such a study, that includes about 100 isolates of avian trypanosomes, is under way (J.V., M.S. and P.V., unpublished results).

Is there a single avian trypanosome or every bird species (genus, family, order) has its own parasite? Sehgal *et al*. (2001) found no evidence for host specificity of trypanosomes from African birds. On the other hand, our repeated attempts to infect quails, chickens and canaries with raptor trypanosomes were unsuccessful (data not shown). Moreover, in spite of similar SSU rDNA sequences, significant differences in the structure of kDNA exist between isolates originating from Falconiformes and Passeriformes (Yurchenko *et al*. 1999; Lukeš & Votýpka, 2000). Our preliminary data also suggest that, although commonly found in the nests of raptors, infected mosquitoes and hippoboscid flies do not transmit their trypanosomes to the birds of prey. So far, out of tested raptor and insect trypanosomes, only those isolated from mosquitoes were infective for the laboratory-reared females of *Culex pipiens* (J.V. and M.S., unpublished results). These findings indicate that, to a certain degree, avian trypanosomes are both vector and host specific.

Using the isoenzyme analysis, Dirie *et al*. (1990) identified *Eusimulium latipes* as the vector of a trypanosome parasitizing sparrowhawk (*Accipiter nisus*) in Britain. The ornithophilic simuliids *Eusimulium aureum*, *E. quebecense*, *E. latipes*, *E. croxtoni* and *Prosimulium decemarticulatum* were also described as natural transmitters of trypanosomes of the *T. avium* complex in different bird orders in Canada (Bennett & Fallis, 1960). Moreover, it was proposed that *Simulium* spp. could serve as a vector of *Trypanosoma numidae*, a parasite of the African guinea fowl (*Numida mitrata*) and domestic chicken (Fallis, Jacobson & Raybould, 1973). Our results correspond with these findings, since the isolate from *Eusimulium* matched the avian trypanosomes in

the 18S rRNA sequences as well as in the size of minicircles and kDNA structure.

The situation is, however, not as clear for the other potential vectors. The hippoboscid flies are mentioned as vectors of *Trypanosoma corvi* (the *T. avium* complex), a parasite of corvids in Britain (Baker, 1956*a*, *b*; Mungomba *et al*. 1989). In our study, the kDNA of isolates obtained from the hippoboscid fly and mosquito is composed of relatively small minicircles, and consequently, their kDNA has a typical disc-shaped structure. These features are in good correlation with the position of both isolates in phylogenetic trees, where they constitute a separate branch within the *T. avium* clade. We conclude that both isolates are most likely also avian trypanosomes, however, they may parasitize birds other than raptors.

In this paper we demonstrated, using molecular approaches, that among the blood-sucking insects attacking birds, the ornithophilic simuliids such as *E. securiforme* are the most probable vectors of raptor trypanosomes. Kinetoplastid flagellates found in the hippoboscid flies and mosquitoes may be to bird trypanosomes as well, but they supposedly do not infect raptors.

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