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Phylogenetic Relationship of *Trypanosoma corvi* with Other Avian Trypanosomes

Jan VOTÝPKA[1,2], Julius LUKEŠ[2,3] and Miroslav OBORNÍK[2,3]

[1]Department of Parasitology, Faculty of Science, Charles University, Prague; [2]Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice; [3]Faculty of Biological Sciences, University of South Bohemia, České Budějovice, Czech Republic

Summary. The phylogenetic relationships of avian trypanosomes, common parasites of birds, remain ambiguous and validity of many species is questionable. Analyses based on 18S rRNA sequences, dimensions of the kinetoplast disc and the size of kinetoplast (k) DNA minicircles were used to differentiate among large trypanosomes parasitizing birds of the Old World. These trypanosomes with typical striated appearance formed two well-supported groups - the "*Trypanosoma avium*" clade and the "*Trypanosoma corvi*" clade. Interestingly, the isolate derived from the Central European hipposcid fly (*Ornithomyia avicularia*) is closely related to *T. corvi* from a raven captured in the U.K., whereas a trypanosome obtained from the blood of a raven of the Central Europe origin (Czech Republic) is a typical member of the "*T. avium*" clade.

Key words: 18S rRNA, hipposcid fly, insect vectors, kinetoplast DNA, phylogeny, *Trypanosoma avium*, *T. corvi*.

INTRODUCTION

Avian trypanosomes, transmitted by various blood-sucking invertebrates, are widespread parasites of birds (Apanius 1991). About one hundred species of bird trypanosomes have been described worldwide, mostly on the basis of "one host - one species" paradigm, according to which a new species was assigned for every "new" bird host (Bishop and Bennett 1992). However, clear-cut evidence for strict host specificity of bird trypanosomes is lacking, while several experimental transmissions of trypanosomes from one host

Address for correspondence: Jan Votýpka, Department of Parasitology, Faculty of Science, Charles University, Viničná 7, 128 44 Prague, Czech Republic; Tel: +42 2 21951818; Fax: +44 2 24919704; E-mail: vapid@natur.cuni.cz

species to another (sometimes across a family or an order) have been described (Baker 1956a, c; Bennett 1970; Woo and Bartlett 1982; Chatterjee 1983). Since the validity of most species has been questioned, many taxa are considered *nomina dubia* and the taxonomic status of avian trypanosomes remains generally controversial.

In 1885 Danilewsky described *T. avium* from birds of the families Corvidae, Accipitridae and Laniidae, and in 1889 included *Asio otus* (Strigiformes) and *Coracias garrulus* (Coraciiformes) as additional hosts. Later, *Strix aluco* obtained from a street market in Paris enlarged the list of *T. avium* hosts, while Laveran proposed that trypanosomes from *C. garrulus* belong to a different species (Laveran 1903). Since these early works, avian trypanosomes from many bird species

captured in Europe and Asia have been labelled as *T. avium*, but the descriptions and illustrations are (mostly) inadequate. According to Novy and McNeal (1905), *T. avium* also parasitizes a wide range of hosts in North America (*Agelaius phoeniceus*, *Colaptes auratus*, *Cyanocitta cristata*, *Icterus galbula*, *Lophotrix* sp., *Melopiza melodia*, *Passer domesticus*, *Sialia sialis*, *Turdus migratorius*, *Zenaidura macroura*). A more recent example of the concept that *T. avium* is a widespread parasite of birds is exemplified by its description from a fish crow (*Corons ossifragus*) in Florida (Dusek and Forrester 2002). Quite early, Lühe (1906) used the name *Trypanosoma confusum* for what he considered a "species mix". Many authors accepted this taxonomy of the New Word avian trypanosomes, while according to others *T. confusum* is regarded as a junior synonym of *T. avium* (Baker 1976).

Based on several studies (Bennett 1961, Baker 1976, Bishop and Bennett 1992), large trypanosomes with typical striated appearance (so-called myonemes) have been grouped together as species of the *T. avium* complex or the "*avium*" group (Baker 1976, Woo and Bartlett 1982, Apanius 1991, Bennett *et al.* 1994, Sehgal *et al.* 2001). The flag species of this group are *T. avium* and *T. corvi* from the Old World and *T. confusum* from the New World avian hosts. Baker (1976) proposed a concept according to which there are no more than 12 valid species of trypanosomes in birds world-wide, with *T. avium* restricted to trypanosomes of the Old World owls (Strigiformes), and morphologically similar protozoans parasitizing birds of the New World belonging to *T. confusum*.

Trypanosoma corvi Stephens et Christophers, 1908, originally described from a house crow (*Corvus splendens*) in India, was re-described from the material obtained from a raven (*Corvus frugilegus*) captured in the U.K., and emended for the large striated trypanosomes of corvids and blackbirds (*Turdus merula*) (Baker 1956a, c, 1976). Finally, flagellates obtained from the blood of a tree pic (*Dendrocitta vagabunda*) captured in India served as material for another morphology-based re-description of *T. corvi* (Nandi and Bennett 1994). At present, *C. splendens* is the type host of *T. corvi*, with *C. frugilegus, C. monedula spermologus, D. vagabunda*, and *Turdus* spp. being additional hosts. Generally, all large trypanosomes found in Corvidae and other families (e.g. Turdidae) of the Old World birds are considered to be conspecific into this species (Baker 1976).

The classification summarised above proved helpful for distinguishing trypanosomes from different bird hosts and continents; however, several taxonomic problems with the avian trypanosomes remain unsettled. The lack of morphological features among different isolates is out weighted by biochemical and molecular data. Biochemical characterization of trypanosome strains obtained from the American raptors (*Buteo jamaicensis*, *Accipiter striatus*) and owls (*Aegolius acadicus*) led Kirkpatrick and Terway-Thompson (1986) to conclude that they are closely related. The electrophoretograms of *T. corvi* from ravens were similar to those of trypanosomes isolated in the U.K. from sparrowhawks (*Accipiter nisus*) and black flies (*Eusimulium latipes*) (Dirie *et al.* 1990) indicating that this blood-sucking insect is the vector of *T. corvi*. However, according to Baker (1956b) and Mungomba *et al.* (1989), *T. corvi* is transmitted by the hippoboscid fly (*Ornithomyia avicularia*).

The 18S rRNA gene sequences are available for dozens of trypanosome species and are widely used to infer their relationship. Our previous study based on the sequences of the 18S rRNA genes showed that isolates from four raptor species (*Buteo buteo*, *Accipiter nisus*, *Falco tinnunculus*, *Aquila pomarina*), a chaffinch (*Fringilla coelebs*), a raven (*Corvus frugilegus*), and a black fly (*Eusimulium securiforme*), all originating from Central Europe, belong to a single robustly monophyletic "*T. avium*" clade (Votýpka *et al.* 2002). Furthermore, we have shown that the different bird trypanosomes are diverse in the size of kinetoplast (k) DNA minicircles and that this feature is reflected in a unique and characteristic shape of their kinetoplast (Lukeš and Votýpka 2000, Votýpka *et al.* 2002). In this study we have used molecular techniques (sequencing of the 18S rRNA gene and determination of minicircle size) and electron microscopy (measurement of kinetoplast thickness) to compare trypanosomes originating from a raven captured in the U.K. and described as *T. corvi* with the other Old World avian trypanosomes including isolates from a raven (*C. frugilegus*) captured in the Czech Republic and a hippoboscid fly (*Ornithomyia avicularia*).

MATERIALS AND METHODS

Strain origin and cultivation of trypanosomes. The *Trypanosoma corvi* strain "LUM-LSHTM" (ITMAP 180795) was isolated by J. R. Baker from the blood of an adult raven (*C. frugilegus*)

captured in Fordingbridge, Hampshire, U.K. in June 1970. Cultivation was performed on blood agar with RPMI 1640 medium (Sigma) and supplemented with 10 % (v/v) foetal calf serum (Sigma) and gentamicin (80 µg/ml) at 23°C.

DNA processing. Isolation of total cellular DNA, PCR amplification of the 18S rRNA gene using primers S762 and S763, cloning, and sequencing with a set of conserved primers were performed as described previously (Maslov *et al.* 1996). Isolation of the kDNA network and enzyme digestion of minicircles was done according to Votýpka *et al.* (2002).

Electron microscopy. For transmission electron microscopy, cells collected from culture in the exponential phase (6-day-old culture) were washed in 0.1 M phosphate-buffered saline solution and fixed in 2.5 % glutaraldehyde in the same buffer at 4°C. Samples were further processed and the kDNA structure was analyzed as described elsewhere (Lukeš and Votýpka 2000).

Sequence availability. 18S rRNA sequences of *T. corvi* and *T. "avium"* FT2 were deposited in GenBank under the accession numbers AY461665 and AY099319, respectively. Nucleotide sequences used in phylogenetic analysis are available from the GenBank/EMBL databases under the following accession numbers: *T. cruzi* PERU (X53917), *T. cruzi marinkellei* (AJ009150), *T. dionisii* PJ (AJ009152), *T. dionisii* P3 (AJ009151), *T. rangeli* (AJ012416), *T. vespertilionis* (AJ009166), *Trypanosoma* sp. "kangaroo" (AJ009168), *T. lewisi* (AJ223566), *T. microti* (AJ009158), *Trypanosoma* sp. OA6 (AF416562), *Trypanosoma* sp. CUL1 (AF416561), *T. bennetti* (American kestrel), *T. grayi* (AJ223565), *T. "avium"* A1412 (U39578), *T. "avium"* SIM3 (AF416563), *T. "avium"* APO1 (AF416559), *T. "avium"* LSHTM144B (AJ009140), *T.* cf. *paddae* N335 (AJ223570), *T. varani* (AJ223572), *T. scelopori* (U67182), *Trypanosoma* sp. "wombat" (AJ009169), *T. pestanai* (AJ009159), *T. theileri* (AJ009164), *Trypanosoma* sp. D30 (AJ009165), *T. theileri* (AJ009163), *T. cobitis* (AJ009143), *Trypanosoma* sp. "fish" (L14841), *Trypanosoma* sp. "leech" (AJ009167), *T. boissoni* (U39580), *T. triglae* (U39584), *T. rotatorium* B2-II (AJ009161), *T. rotatorium* B21(U39583), and *T. mega* (AJ009157). Information on avian trypanosomes (for which molecular data are available) has been summarized in Table 1.

Phylogenetic analyses. The entire 18S rRNA gene sequences from 35 trypanosomes were aligned using the ClustalX program (Thompson *et al.* 1997), alignment was manually corrected, and gaps and ambiguously aligned regions were excluded from phylogenetic analyses. Maximum parsimony (MP) and maximum likelihood (ML) trees, as well as trees based on LogDet-distances (D) were constructed. MP trees were constructed using PAUP* program (Swofford 2000) with TBR as a branch swapping method and random addition of sequences with 10 replicates. MP bootstrap support was computed out of 1000 replicates with random addition of sequences with 10 replicates. ML tree was computed using the DNAml program in Phylip package (Felsenstein 2001) version 3.6a3, using HKY85 model with discrete gamma distribution in 8+1 categories. Gamma distribution parameter α and portion of invariable sites were estimated from dataset using Tree-Puzzle program (Strimmer and von Haeseler 1996), version 5.0. Maximum-likelihood puzzle support was computed in 10,000 puzzling steps using Tree-Puzzle 5.0 with discrete gamma distribution in 8+1 categories. Distance tree was constructed using LogDet/paralinear distances as implemented in PAUP* version 4b10, with constant positions excluded from the analysis. Consistent with previously published phylogenies of trypanosomes (Stevens and Gibson 1999, Votýpka *et al.* 2002), all constructed trees were rooted using *T. mega* as an outgroup.

RESULTS AND DISCUSSION

Alignment used for phylogenetic analysis contained 35 taxa and 2031 characters and is available from the authors upon request. In this alignment, 441 characters are variable, with 281 characters parsimony informative. Maximum parsimony analysis resulted in construction of 22 equally parsimonious trees with TL (tree length) = 1039 steps; CI (consistency index) = 0.5987; RI (retention index) = 0.6936; RC (rescaled consistency index) = 0.4152. Although the general topology of the MP tree shown in Fig. 1 is unstable, the cluster composed of *T. varani*, *T. scelopori, T. pestanai*, and *Trypanosoma* sp. "wombat" branches together with the "*T. avium*" clade in 20 out of 22 equally parsimonious trees constructed. These 20 trees differ only in the internal

Table 1. Summary details of avian trypanosomes analysed.

Species	Acc. No.	Strain	Host species		Origin
T. "avium"	AF416559	AAQU/SK/97/APO1	lesser-spotted eagle	*Aquila pomarina*	Slovakia
T. "avium"	AF416563	IEUS/CZ/99/SIM3	black fly	*Eusimulium securiforme*	Czech Rep.
T. "avium"	AY099319	AFAL/CZ/99/FT2	European kestrel	*Falco tinnunculus*	Czech Rep.
T. "avium"	AJ009140	LSHTM144B	chaffinch	*Fringilla coelebs*	Czech Rep.
T. "avium"	U39578	A1412	raven	*Corvus frugilegus*	Czech Rep.
T. cf. *paddae*	AJ223570	N335	Java sparrow	*Padda oryzivora*	Asia (?)
T. corvi	AY461665	ITMAP 180795	raven	*Corvus frugilegus*	England (U.K.)
Trypanosoma sp.	AF416562	IORN/CZ/99/OA6	hippoboscid fly	*Ornithomyia avicularia*	Czech Rep.
Trypanosoma sp.	AF416561	ICUL/CZ/98/CUL1	mosquito	*Culex pipiens*	Czech Rep.
T. bennetti	AJ223562	KT-2 (ATCC 50102)	American kestrel	*Falco sparverius*	USA, New Jersey
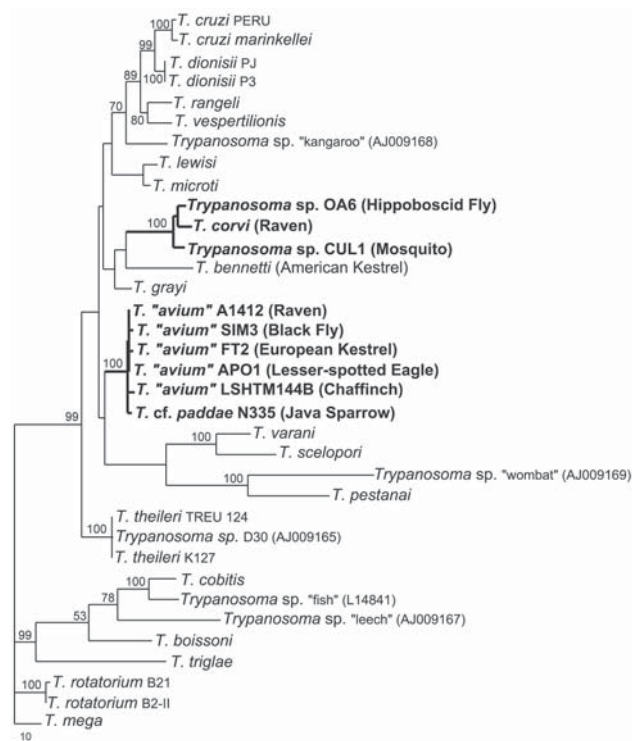
topologies of particular clusters. In the remaining two trees, this cluster does not form a sister group to the "*T. avium*" clade, which is more closely related to *T. theileri*. Moreover, in these two trees, *T. grayi* does not appear on the root of the "*T. corvi*" cluster, as it does in most MP trees, but becomes affiliated to *T. theileri*. However, bootstrap support for these topological variations is low (data not shown). In ML tree (Fig. 2), the *T. varani, T. scelopori*, *T. pestanai* and *Trypanosoma* sp. "wombat" cluster appears between the "*T. corvi*" and "*T. avium*" clades, but similarly to the MP bootstrap analysis, this position is not supported by the ML-puzzle tree. Finally, in phylogenetic trees based on the LogDet/paralinear distances, position of the above-mentioned cluster differs from that found in the ML and MP trees, since it is related to *Trypanosoma* sp. "kangaroo" (Fig. 3), while in the same tree, *T. theileri* appears on the
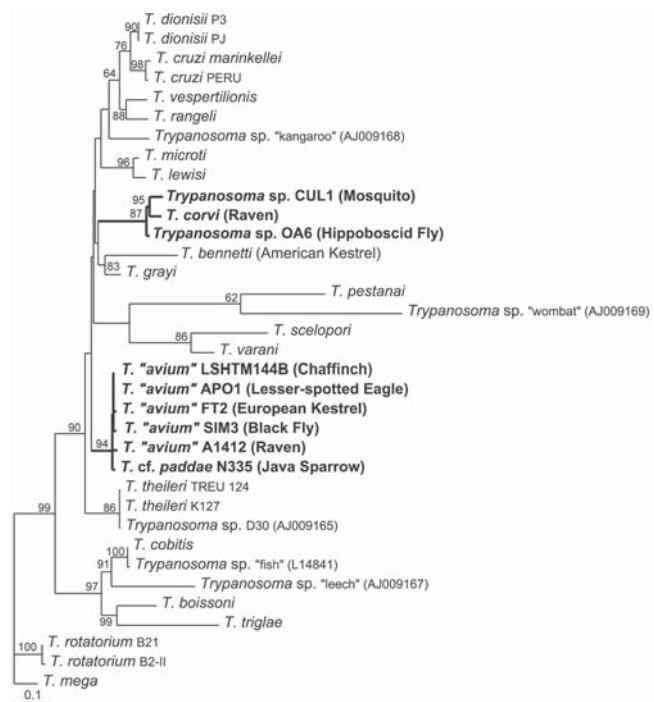
root of the clade composed of *T. cruzi*, *T. dionisii*, *T. rangeli*, and *T. vespertilionis*. The four equal LogDet trees constructed differed only in the internal topology of the *T. avium* cluster. Importantly, in all constructed trees, *T. corvi* and *T. avium* formed highly supported separate clusters (see Figs 1-3), however, their mutual relation has not been resolved. In all trees, except the two MP trees, *T. grayi* and *T. bennetti* appear on the root of the *T. avium* cluster, although this relationship is not supported by bootstrap or puzzle analyses.

Several strains of *T. corvi* isolated from ravens in the U.K. have been studied so far: (i) unnamed strains isolated by J. R. Baker from ravens in Hertfordshire (England) between the years 1953 and 1955 were used to reveal the incidence of parasites in birds and transmission experiments using hippoboscid flies (Baker 1956a, b, c) and, (ii) together with our model strain ITMAP
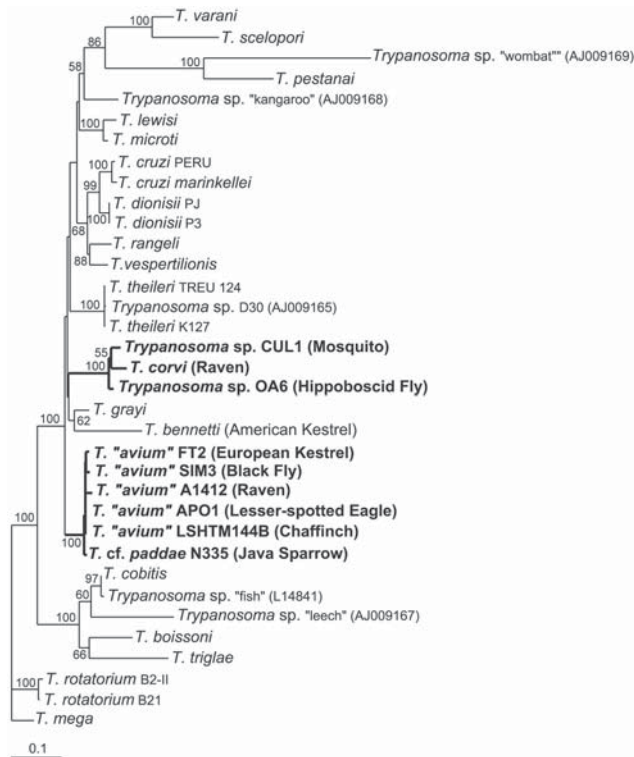
Fig. 3. Phylogenetic tree (one of four trees) based on LogDet distances, as inferred from 18S rRNA gene sequences. Tree was constructed using PAUP* program (Swofford 2000) and alignment containing 441 variable positions (constant positions excluded). Minimum evolution score was 2.57308. Numbers above branches indicate bootstrap support (higher than 50%) computed with 1000 replicates. Tree was rooted using *T. mega* as an outgroup.

180795, have been used for species re-description of *T. corvi* (Baker 1976); (iii) strain no. 85 from a bird captured in 1973 in the same locality (Hertfordshire) as the above-mentioned strains was used for immunity studies (Molyneux and Gordon 1975); and (iv) strain from Cheshire (England) was subjected to electron microscope observation of development in the gut of its insect vector (Mungomba *et al.* 1989). We assume that all these strains belong to the same species, of which the one available to us (ITMAP 180795) is considered to be most representative.

Unexpectedly, based on the 18S rRNA sequence, the trypanosome strain obtained from the blood of a raven captured in the Czech Republic (strain A1412) branched within the well-supported "*T. avium*" clade, in the company of strains parasitizing a chaffinch, lesser-spotted eagle, java sparrow and black fly (Votýpka *et al.* 2002). However, when features like minicircle size (10 kb) and

kinetoplast thickness (1.2 µm) are considered, the strain A1412 is clearly different from other avian trypanosomes (Lukeš and Votýpka 2000, Votýpka *et al.* 2002). These results imply that the studied European populations of ravens are infected by at least two different trypanosome species, which may possibly be transmitted by different vectors.

Previous studies identified the hippoboscid fly *Ornithomyia avicularia* as the vector of *T. corvi* (Baker 1956b, Mungomba *et al.* 1989). When we included the newly obtained 18S rRNA gene sequence of *T. corvi* ITMAP 180795 into the available 18S rRNA dataset, it clustered together with the trypanosomes isolated from a hippoboscid fly (strain OA6) and a mosquito (CUL1) collected on raptor nestlings in the Czech Republic, forming a highly supported clade (Figs 1–3). Analyses of the kinetoplast structure and the kDNA minicircle size further supported this relationship. In fact, the low-pitched and elongated kinetoplast observed by electron microscopy in the *T. corvi* cells (thickness 0.39 ± 0.35 µm; n = 38) was almost indistinguishable from the kinetoplast disc of the strain OA6 obtained from a hippoboscid fly (Fig. 4; see also Votýpka *et al.* 2002). This is in contrast to the cylindrical-shaped kinetoplasts characteristic of trypanosomes from Central European raptors, raven, black bird, and black fly (Lukeš and Votýpka 2000, Votýpka *et al.* 2002).

In our recent work (Votýpka and Svobodová 2004) we show that kinetoplast thickness is a character stable for a particular trypanosome species in its host, vector and derived culture stages. Moreover, the thickness of the kinetoplast disc correlates with the size of minicircles that constitute the kDNA network (Lukeš and Votýpka 2000). The thin disc of *T. corvi* is composed of 3.0 kb long minicircles (data not shown) similar in size to minicircles of the strain OA6 from the hippoboscid fly (Votýpka *et al.* 2002). In addition, the hindgut and rectum of infected hippoboscid flies harbour a heavy infection of slender epimastigotes (data not shown), the localization and morphology of which is reminiscent of flagellates described from vectors by Baker (1956b) and Mungomba *et al.* (1989). Molecular and morphological data thus provide evidence that *T. corvi* from a raven captured in the U.K. is either identical with, or very closely related to, the trypanosome isolated from a hippoboscid fly in central Europe three decades later.

Close relatedness of *T. corvi* isolated from a raven with trypanosomes originating from a hippoboscid fly (strain OA6) and a mosquito (strain CUL1), based on the 18S rRNA sequences as well as morphological
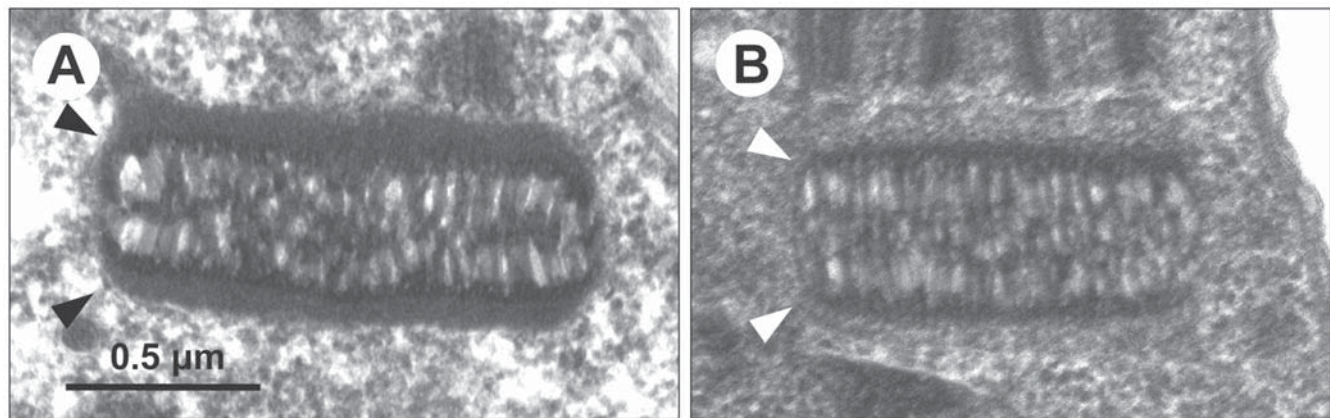
Fig. 4. Transmission electron microscopy of low-pitched and elongated kinetoplasts from *Trypanosoma corvi* (strain ITMAP 180795) (**A**) and trypanosome isolated from the hippoboscid fly (*Ornithomyia avicularia*) (strain OA6) (**B**). Arrowheads indicate thickness of the kinetoplasts. Both micrographs are to the same scale.

features, prompted us to speculate that *T. corvi* is transmitted *via* different vectors among its avian hosts. However, important differences exist between the infection in the hippoboscids, for which a contaminative transmission has been proposed (Mungomba *et al.* 1989), and that in the mosquitoes. In the latter insects, a massive plug of parasites attached to the stomodeal valve in the foregut region was observed, as well as accompanying degenerative changes of the valve, reminiscent of the sand fly infections by *Leishmania* spp. (Hajmová, Votýpka and Volf, unpubl. results). Therefore, we conclude that cycling of trypanosomes between mosquitoes and birds is carried out *via* inoculation or regurgitation rather than by the contaminative way described in the hippoboscids. Such a dissimilar type of transmission is an important feature of the biology of kinetoplastids, and should be considered incompatible with their belonging to one species. On the basis of this difference, we propose that avian trypanosomes isolated from hippoboscids fly and mosquito belong to two different but closely related species.

Questions concerning the host specificity of *T. corvi* still remain open. Baker (1956a) found trypanosomes in ravens (*Corvus frugilegus frugilegus*), jackdaws (*C. monedula spermologus*), and blackbirds (*Turdus merula merula*) and suggested that flagellates from these birds belong to the same species. Furthermore, he transmitted them experimentally to canaries (*Serinus canaries*). After the emendment of *T. corvi* by Stephens and Christophers (1908), Baker restricted the use of the name *T. corvi* to large trypanosomes from non-Ameri-

can corvids and also from other bird families (e.g. Turdidae) (Baker 1976). While our trypanosome strain isolated from a blackbird (strain A493) appears to be a typical member of the '*avian*' group (Lukeš and Votýpka 2000; this work), we could not exclude the possibility that *T. corvi* is able to infect other bird species than corvids.

With the exclusion of *T. bennetti*, all other trypanosomes isolated from birds, for which the 18S rRNA sequence is available (Table 1), constitute a homogeneous group, the monophyly of which is supported by 100% bootstraps. Moreover, this compact group contains one trypanosome isolated from a black fly (strain SIM3). It is hard to find support in our dataset for the existence of more than one species - *T. avium*.

Trypanosoma avium is apparently able to invade many species of birds belonging to distant orders (Bennett 1970, Chatterjee and Ray 1971). While for *T. paddae* (*T.* cf. *paddae*; strain N335) a feature that distinguishes this species from other avian trypanosomes has been described (kinetoplast situated closer to the nucleus) and since it constitutes the earliest branch within the "*avian*" clade, its separate status should be retained until more sequence data is available. *T. paddae* was originally described from the Asian Java sparrow (*Padda oryzivora*) and Baker (1976) speculated that this name is valid for most of the trypanosomes of Fringillidae recorded from Europe and Asia. Our results do not support this view, since trypanosome strain LSHTM144B from chaffinch (*F. coelebs*) has the 18S rRNA sequence more related to *T. avium* from birds of prey and the black fly (Figs 1-3). The inclusion of strains isolated

from small passerine birds into the phylogenetic analysis is necessary to settle the taxonomic status of *T. brimonti*, *T. bakeri* (Baker 1976, Chatterjee 1983) and other avian trypanosomes.

Based on a combined analysis of molecular data and morphologic features, we provide the evidence that avian trypanosomes form two well-supported monophyletic clades: the "*T. avium*" clade and the "*T. corvi*" clade. While no significant differences have been found in their 18S rRNA sequences, avian trypanosomes of the "*T. corvi*" clade isolated from a mosquito and a hippoboscid fly undergo such a different development in the vector that their provisional placement into two different species is unavoidable. The data available for flagellates belonging to the "*T. avium*" clade is consistent with the notion that trypanosomes parasitizing unrelated bird hosts may indeed belong to one species with a low host specificity that is transmitted by black flies.

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