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Biodiversity of avian trypanosomes

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ABSTRACT

We have studied the biodiversity of trypanosomes from birds and bloodsucking Diptera on a large number of isolates. We used two molecular approaches, random amplification of polymorphic DNA (RAPD) method, and sequence analysis of the small subunit ribosomal RNA (SSU rRNA) gene. RAPD method divided the isolates into 11 separate lineages. Phylogenetic analysis of the SSU rRNA gene was congruent with the RAPD. Morphometric analysis of kinetoplast width and cell length was in agreement with molecular data. Avian trypanosomes appeared polyphyletic on SSU rDNA tree; thus, they do not represent a taxonomic group. We propose that all lineages recovered by SSU analysis probably represent distinct species of avian trypanosomes. We discuss possible transmission ways and geographical distribution of new avian trypanosome lineages. Finally, we recommend methods that should be used for species determination of avian trypanosomes.

1. Introduction

The genus *Trypanosoma* (Kinetoplastida: Trypanosomatidae) was described by David Gruby more than 150 years ago (Gruby, 1843). It is the most abundant and most important genus among kinetoplastids. Trypanosomes are extensively studied because they cause serious diseases of humans (Chagas disease, sleeping sickness) and domestic animals (nagana, surra, mal de caderas, dourine) (Balakrishnan and Zumla, 2001; Kirchhoff, 2001; Taylor et al., 2007). In contrast to their mammalian relatives, avian trypanosomes are in most cases harmless to their hosts (Macfie and Thomson, 1929; Baker, 1976); thus, they remain understudied although they are not less interesting.

Avian trypanosomes occur in all continents except for the polar regions (Baker, 1976; Apanius, 1991; Bennett et al., 1992; Allander and Bennett, 1994). Similarly to other members of the genus, their life cycle is digenetic. However, complete life cycles have been elucidated only in a few cases. Suggested vectors represent different bloodsucking arthropods (black flies, hippoboscids, mosquitoes, biting midges or mites) (Baker, 1976; Molyneux, 1977) but, their vectorial status has not always been proven (Baker, 1956b; Votypka and Svobodova, 2004; Votypka et al., 2011). Many bird orders are parasitized, songbirds and raptors most often (Baker, 1976; Kucera, 1983; Apanius, 1991), while ducks, geese, and sparrows are only rarely infected (Kucera, 1983). Prevalence ranges between less than 1% and more than 40% depending on the methods used for their detection; cultivation or PCR diagnosis being the most reliable (Kucera, 1983; Kirkpatrick and Lauer, 1985; Kirkpatrick and Suthers, 1988; Sehgal et al., 2001).

The majority of known trypanosome species infects mammals. However, approximately one fifth of the total species number has been described from birds (Podlipaev, 1990). The first description of an avian trypanosome with measurements and drawings was given by Danilewsky (1885, 1889). Although almost 100 species have been described since (Podlipaev, 1990; Sehgal et al., 2006), only a few reliable species descriptions are available nowadays. Large number of descriptions was based solely on supposed host specificity and provided no morphological or molecular data for comparison (see Sehgal et al., 2001; Votypka and Svobodova, 2004; Valkiunas et al., 2011). In addition, only three type cultures (*Trypanosoma bennetti*, *Trypanosoma corvi* and *Trypanosoma culicavium*) are currently available. Gene sequences (SSU rRNA, glycosomal glyceraldehyde-3-phosphate dehydrogenase, minicircle kinetoplast DNA) have been assigned to seven species names (*Trypanosoma anguiformis*, *Trypanosoma avium*, *T. bennetti*, *T. corvi*, *T. culicavium*, *Trypanosoma gallinarum*, and *Trypanosoma polygranularis*). Thus, a big discrepancy exists between the number of described species and the number of well-characterized ones.

We have studied 60 new isolates of avian trypanosomes obtained from birds and insects, employing RAPD method and the SSU rRNA gene phylogenetic analysis. Results of the analyses were supported by morphological data (kinetoplast width, cell type and length). By the combination of three different approaches

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we tried to resolve the number of avian trypanosome species and in some cases their putative life-cycles.

2. Materials and methods

2.1. Isolation of parasites

Three hundred and fifty five isolates from birds (285) and insects (70) were obtained during 1997–2007, mainly in the Czech Republic. Sixty five isolates of avian trypanosomes and three isolates of other typanosomatids as outgroups were used in the study (Table 1). Isolates from raptors were obtained from nestlings or adults in breeding seasons as described previously (Votypka et al., 2002). Isolates from passerines were obtained in breeding seasons from birds caught at watering places. The isolates from collared flycatchers (*Ficedula albicollis*) originated from nesting females caught in their nest boxes. Male warblers (*Phylloscopus* spp.) were mistnetted using song playback. Isolation of trypanosomes from bird blood was described elsewhere (Votypka et al., 2002). Vector isolates were obtained from insects caught overnight to sucking CDC (Centre for Disease Control) traps placed on nests of buzzards (*Buteo buteo*), sparrowhawks (*Accipiter nisus*) or marsh harriers (*Circus aeruginosus*) as described elsewhere (Votypka et al., 2002). Hippoboscid flies were caught directly on hosts. Insects were dissected and trypanosomatids were isolated as described previously (Votypka et al., 2002; Votypka and Svobodova, 2004).

2.2. DNA extraction for RAPD method

Isolates of avian trypanosomes (Table 1) were cultivated for at least 3 days until the cell concentration reached approximately 10^6 per ml. Total DNA from all strains used in the RAPD analysis was isolated using the DNA tissue isolation kit (Roche) according to the manufacturer's instructions with the following modification: before adding proteinase K, each sample was treated with lysis buffer and RNase A at 37 °C for 1 h. After this step, the isolation continued as described in the manual for the isolation of nucleic acids from mammalian tissue.

2.3. RAPD analysis

All insect isolates (70) from our trypanosomatid collection (26 from mosquitoes, 28 from black flies and 16 from hippoboscid flies) were used for preliminary RAPD analyses, as well as selected avian isolates (49 from raptors and 26 from songbirds). The isolates were chosen according to the host species, isolation locality, host age, to represent the highest possible diversity. Analyses, performed with 145 isolates using nine primers, preliminarily sorted the trypanosomatids (data not shown). Isolates representing distinct groups were used for the final RAPD analysis.

The final RAPD tree was obtained using 41 avian isolates; three trypanosomatids served as outgroups (Table 1). Twenty one primers were from Operon technologies: OPA3 (AGTCAGCCAC), OPA9 (GGGTAACGCC), OPA10 (GTGATCGCAG), OPD3 (GTCGCCGTCA), OPD5 (TGAGCGGACA), OPD8 (GTGTGCCCCA), OPD13 (GGGGTGACGA), OPE14 (TGCGGCTGAG), OPF1 (ACGGATCCTG), OPI6 (AAGGC GGCAG), OPI12 (AGAGGGCACA), OPI13 (CTGGGGCTGA), OPI14 (TGACGGCGGT), OPL3 (CCAGCAGCTT), OPL4 (GACTGCACAC), OPL5 (ACGCAGGCAC), OPL7 (AGGCGGGAAC), OPL8 (AGCAGGTGGA), OPL16 (AGGTTGCAGG), OPL20 (TGGTGGACCA) and OPO12 (CAGTGCTGTG). Eight other primers were used (Kolarik et al., 2004; Zemanova et al., 2004), 10R (GGCCAGTGTGAATATGC), 8F (GCTCTGAGATTGTTCCGGCT), A03 (AGTCAGCCAC), B18 (CCACAGCAGT), PLID2-9 (CAAAAGTCCCCACCAATCCC), TA150 (ATGCGATGA GTGGTTGAG), TA610 (TCAACCGATTACAAACCA) and TAF300 (CACCTCAAAACATACCCC), and primer 30F (GAGGACGATTCATCAACC) which was obtained from Dr. Kolarik. The RAPD reactions were performed as described (Svobodova et al., 2007). Briefly, the annealing temperature was 38 °C, the PCR products of each reaction were electrophoresed on 2% agarose gels which were processed manually. The final matrix included 1505 lines where every visible band was evaluated as 1 and missing band as 0. The tree was constructed using FreeTree 0.9.1.50 program (Pavlicek et al., 1999) with the neighbor-joining algorithm (Nei-Li/Dice distances) and the bootstrap supports were calculated from 5000 replicates.

2.4. DNA extraction and sequencing

Genomic DNA was extracted using the tissue isolation kit (Roche) according to manufacturer's manual. SSU rDNA was amplified using eukaryote-specific primers MedlinA (CTGGTTGATCCT GCCAG) and MedlinB (TGATCCTTCTGCAGGTTCACCTAC) (Medlin et al., 1988). PCR products were bidirectionally sequenced directly from purified PCR products. The external primers used for direct sequencing from PCR product were MedlinA and MedlinB. The primers used for sequencing of internal regions of PCR fragment of 18S rRNA were kin577F (GCCAGCACCCGCGGT), kin577R (ACCGCGGGTGCTGGC), kin1510F (CAGGTCTGTGAYGCTG), and kin1510R (CAGCRTCACAGACCTG). Thirty nine new isolates were sequenced. Additionally, six following isolates were re-sequenced: A1412 (U39578), APO1 (AF416559), BUT15 (AY099320), FT2 (AY099319), ITMAP180795 = LUM-LSHTM (AY461665) and SIM3 (AF416563). GenBank accession numbers are listed in the Table 1. The corrected sequences were submitted to GenBank as new versions under the same accession numbers.

2.5. Construction of phylogenetic trees

A data set containing 118 SSU rDNA sequences of trypanosomatids was created: 56 sequences belonged to avian trypanosomes, 44 to other trypanosomes, and 18 sequences of other kinetoplastids. The sequences were aligned using the MAFFT (Multiple Alignment using Fast Fourier Transform) method (Katoh et al., 2002) with the help of the MAFFT 6 server http://align.bmr.kyushu-u.ac.jp/mafft/online/server/ with G-INS-i algorithm at default settings. The alignment was manually edited using BioEdit 7.0.9.0 (Hall, 1999). The final data set consisted of 1990 positions. Phylogenetic trees were constructed by maximum likelihood and Bayesian methods. Maximum likelihood analysis was performed in Phyml 3.0 (Guindon and Gascuel, 2003) under the GTR + I + Γ model which was selected by AKAIKE criterion implemented in Modeltest 3.7 (Posada and Crandall, 1998). The tree was bootstrapped with 1000 replicates. Bayesian analysis was performed using MrBayes 3.1.2. (Ronquist and Huelsenbeck, 2003) using the GTR + I + Γ + covarion model. Four MCMCs (Markov chain Monte Carlo) were run for 210^6 generations and trees were sampled every 100th generation. First 25% of trees were removed as burn-in.

2.6. Light and transmission electron microscopy (TEM)

Light and electron microscopy were made using cells maintained in culture for 3–15 days. The preparation of smears for light microscopy and the ultrathin sections for TEM were already described (Zidkova et al., 2010). Isolates for morphometry were selected according to results of molecular analyses. If available, at least one bird and one vector isolate from every lineage found in RAPD and SSU trees were selected. Cells of 39 isolates were smeared on microscopic slides, Giemsa-stained, and their cell lengths were measured (Table 1). Twenty of these isolates were

Table 1
Isolates used in the study and their GenBank accession numbers.

Isolate	Host species (scientific name)	Host species (English typical name)	Year	Origin	GenBank Acc. no.	Isolation
A1412*	*Corvus frugilegus*	Rook	1978	Krkonoše mountains (CZ)	U39578	Bedrník
AGE3**	*Accipiter gentilis*	Goshawk/adult	2002	Milovice game preserve (CZ)	JN006829	Votýpka et al.
ANI14A*	*Accipiter nisus*	Sparrowhawk/nestling	1999	Prague (CZ)	AY099318	Votýpka et al.
ANI14B*	*Accipiter nisus*	Sparrowhawk/nestling	1999	Prague (CZ)	FJ649483	Votýpka et al.
ANI21	*Accipiter nisus*	Sparrowhawk/nestling	2001	Prague (CZ)	RAPD only	Votýpka et al.
ANI36	*Accipiter nisus*	Sparrowhawk/nestling	2001	Prague (CZ)	RAPD only	Votýpka et al.
ANI54	*Accipiter nisus*	Sparrowhawk/nestling	2001	Prague (CZ)	JN006849	Votýpka et al.
APO1*	*Aquila pomarina*	Lesser spotted eagle/nestling	1997	Vyšné Ružbachy (SK)	AF416559	Votýpka et al.
APO7**	*Aquila pomarina*	Lesser spotted eagle/nestling	2000	Vyšné Ružbachy (SK)	JF778738	Votýpka et al.
T. carrassi BD	*Cyprinus carpio*	Common carp	1974	Českobudějovicko (CZ)	RAPD only	Lom
BUT15*	*Buteo buteo*	Buzzard/nestling	1999	Milovice game preserve (CZ)	AY099320	Votýpka et al.
BUT16	*Buteo buteo*	Buzzard/nestling	1999	Milovice game preserve (CZ)	RAPD only	Votýpka et al.
BUT17	*Buteo buteo*	Buzzard/nestling	1999	Prague (CZ)	JN006854	Votýpka et al.
BUT19**	*Buteo buteo*	Buzzard/nestling	1999	Prague (CZ)	JN006828	Votýpka et al.
BUT23	*Buteo buteo*	Buzzard/nestling	2000	Prague (CZ)	RAPD only	Votýpka et al.
BUT26	*Buteo buteo*	Buzzard/nestling	2000	Prague (CZ)	RAPD only	Votýpka et al.
BUT50	*Buteo buteo*	Buzzard/nestling	2001	Prague (CZ)	JN006825	Votýpka et al.
CUL1*	*Culex pipiens*	Northern house mosquito	1998	Milovice game preserve (CZ)	AF416561	Votýpka et al.
CUL2*	*Culex pipiens*	Northern house mosquito	1999	Milovice game preserve (CZ)	JN006834	Votýpka et al.
CUL5**	*Culex pipiens*	Northern house mosquito	2000	Milovice game preserve (CZ)	JN006838	Votýpka et al.
CUL6**	*Culex pipiens*	Northern house mosquito	2000	Milovice game preserve (CZ)	HQ107970	Votýpka et al.
CUL15**	*Culex pipiens*	Northern house mosquito	2000	Prague (CZ)	JN006830	Votýpka et al.
CUL24	*Culex pipiens*	Northern house mosquito	2001	Prague (CZ)	RAPD only	Votýpka et al.
CUL28	*Culex pipiens*	Northern house mosquito	2002	Třeboňsko (CZ)	HQ107967	Votýpka et al.
CUL30**	*Culex modestus*	mosquito	2002	Třeboňsko (CZ)	HQ909084	Votýpka et al.
CUL31	*Culex pipiens*	Northern house mosquito	2006	Třeboňsko - Ruda (CZ)	HQ107968	Votýpka
FT2*	*Falco tinnunculus*	Kestrel/adult	1999	Slatina nad Zdobnicí (CZ)	AY099319	Votýpka et al.
T. corvi ITMAP180795**	*Corvus frugilegus*	Rook	1970	Fordingbridge, Hants (GB)	AY461665	Baker
OA06*	*Ornithomyia avicularia*	Hippoboscid fly	1999	Prague (CZ)	AF416562	Votýpka et al.
OA08*	*Ornithomyia avicularia*	Hippoboscid fly	2000	Prague (CZ)	JN006844	Votýpka et al.
OA11**	*Ornithomyia avicularia*	Hippoboscid fly	2001	Prague (CZ)	JN006824	Votýpka et al.
OA12	*Ornithomyia avicularia*	Hippoboscid fly	2001	Prague (CZ)	RAPD only	Votýpka et al.
PAS21**	*Fringilla coelebs*	Chaffinch/1st year juvenile	2002	Milovice game preserve (CZ)	JN006826	Svobodová et al.
PAS23**	*Emberiza citrinella*	Yellowhammer/adult	2002	Milovice game preserve (CZ)	JN006850	Svobodová et al.
PAS44	*Sitta europaea*	Nuthatch/1st year juvenile	2003	Milovice game preserve (CZ)	JN006837	Svobodová et al.
PAS48**	*Sylvia atricapilla*	Blackcap/1st year juvenile	2003	Milovice game preserve (CZ)	JN006845	Svobodová et al.
PAS56	*Fringilla coelebs*	Chaffinch/adult	2004	Milovice game preserve (CZ)	JN006827	Svobodová et al.
PAS64	*Emberiza citrinella*	Yellowhammer/adult	2004	Milovice game preserve (CZ)	JN006851	Svobodová et al.
PAS71*	*Turdus philomelos*	Song thrush/adult	2004	Milovice game preserve (CZ)	JN006847	Svobodová et al.
PAS72	*Parus caeruleus*	Blue tit/yearling	2004	Milovice game preserve (CZ)	JN006846	Svobodová et al.
PAS93*	*Ficedula albicollis*	Collared flycatcher/adult	2006	Pohansko (CZ)	JN006852	Svobodová et al.
PAS94**	*Ficedula albicollis*	Collared flycatcher/adult	2006	Pohansko (CZ)	JN006841	Svobodová et al.
PAS95**	*Ficedula albicollis*	Collared flycatcher/adult	2006	Milovice game preserve (CZ)	JN006842	Svobodová et al.
PAS96*	*Ficedula albicollis*	Collared flycatcher/adult	2006	Milovice game preserve (CZ)	JN006848	Svobodová et al.
PAS99**	*Ficedula albicollis*	Collared flycatcher/adult	2006	Milovice game preserve (CZ)	HQ107969	Svobodová et al.
PAS105*	*Phylloscopus collybita*	Chiffchaff/adult	2007	Milovice game preserve (CZ)	JN006831	Svobodová et al.
PAS106*	*Phylloscopus collybita*	Chiffchaff/adult	2007	Milovice game preserve (CZ)	JN006833	Svobodová et al.
PAS107*	*Ficedula albicollis*	Collared flycatcher/adult	2007	Pohansko (CZ)	JN006835	Svobodová et al.
PAS108*	*Ficedula albicollis*	Collared flycatcher/adult	2007	Pohansko (CZ)	JN006840	Svobodová et al.
PAS109*	*Ficedula albicollis*	Collared flycatcher/adult	2007	Pohansko (CZ)	HQ107966	Svobodová et al.
PAS110**	*Phylloscopus collybita*	Chiffchaff/adult	2007	Milovice game preserve (CZ)	JN006836	Svobodová et al.

Table 1 (*continued*)

Isolate	Host species (scientific name)	Host species (English typical name)	Year	Origin	GenBank Acc. no.	Isolation
PAS111**	*Ficedula albicollis*	Collared flycatcher/adult	2007	Pohansko (CZ)	JN006843	Svobodová et al.
PAS112**	*Phylloscopus collybita*	Collared flycatcher/adult	2007	Milovice game preserve (CZ)	JN006832	Svobodová et al.
PAS113*	*Phylloscopus collybita*	Chiffchaff/adult	2007	Milovice game preserve (CZ)	JN006839	Svobodová et al.
PAS114**	*Phylloscopus sibilatrix*	Wood warbler/adult	2007	Milovice game preserve (CZ)	JN006853	Svobodová et al.
SIM1	*Eusimulium latipes*	Black fly	1999	Milovice game preserve (CZ)	RAPD only	Votýpka et al.
SIM3*	*Eusimulium securiforme*	Black fly	1999	Milovice game preserve (CZ)	AF416563	Votýpka et al.
SIM4	*Eusimulium securiforme*	Black fly	1999	Prague (CZ)	RAPD only	Votýpka et al.
SIM6	*Eusimulium securiforme*	Black fly	1999	Prague (CZ)	RAPD only	Votýpka et al.
SIM8	*Eusimulium securiforme*	Black fly	2000	Milovice game preserve (CZ)	RAPD only	Votýpka et al.
SIM13	*Eusimulium securiforme*	Black fly	2001	Milovice game preserve (CZ)	RAPD only	Votýpka et al.
SIM17	*Eusimulium securiforme*	Black fly	2001	Milovice game preserve (CZ)	RAPD only	Votýpka et al.
SIM28	*Eusimulium securiforme*	Black fly	2002	Prague (CZ)	RAPD only	Votýpka et al.
T.brucei STIB247	*Alcelaphus buselaphus cokii*	Coke's hartebeest	1971	Serengeti Area (TZ)	RAPD only	Geigy and Kauffman
Wallaceina inconstans	*Grypocoris sexguttatus*	Bug	1986	Pskov region (RS)	RAPD only	Podlipaev et al.

* Cell length of isolate was measured.
** Cell length and kinetoplast width of isolate was measured.

also processed for electron-microscopy and their kinetoplast widths were measured (Table 1). Each cell type and kinetoplast was measured at least 25 or 30 times, respectively. Measurements presented in the phylogenetic tree (Fig. 2) and in Table 2 are arithmetic means of the obtained values.

3. Results

3.1. RAPD analysis

RAPD tree was constructed using 41 isolates of avian trypanosomes chosen by preliminary RAPD analyses (data not shown). Additionally, three other trypanosomatids were used as outgroups. Avian trypanosomes split into 11 lineages (I–XI), see Fig. 1. The interrelationships among the lineages were generally unresolved with few exceptions: lineages II and III clustered robustly, as did lineages IV and V, and lineages VI and VII clustered with moderate support. Four lineages were represented by one isolate only (I, II, VI, and IX). Six lineages were formed exclusively either by vector (I, II, and V) or avian (VI, VII, and IX) isolates. Lineages III, IV, VIII, X, and XI consisted of both avian and vector isolates. Mosquito isolates were placed in lineages II, III, and V; isolates from hippoboscid flies belonged to lineages I, IV, and X while black fly isolates were placed in lineages X and XI. The lineage X was the only one which consisted of isolates from two different vector families (black flies and hippoboscid fly). Songbird isolates fell into lineages III, VI, VII, VIII, and X; isolates from raptors were placed in lineages IV, VIII, IX, and X. The type isolate of *T. culicavium* (CUL1) was placed within the lineage V together with two other mosquito isolates. OA6 isolate that clustered with *T. corvi* in the previous analyses (Votypka et al., 2004, 2011) formed lineage IV together with one isolate from hippoboscid fly (OA12) and one from buzzard (BUT17). Isolates APO1, ANI14B, FT2, and SIM3 representing *T.* confer[1] *avium* (*T.* cf. *avium*) in the previous analyses (Votypka et al., 2002, 2004) were all placed within the lineage X. This lineage was additionally formed by two black fly isolates, one isolate from a chaffinch and seven isolates from different raptors.

3.2. Phylogenetic analysis of the SSU rRNA gene

The interrelationships among the lineages formed by the RAPD method were investigated further by sequence analysis. Fig. 2 shows the maximum-likelihood phylogenetic tree of 89 representatives of the genus *Trypanosoma* based on SSU rDNA sequences. The tree was rooted with 28 other kinetoplastids (not shown). The genus *Trypanosoma* appeared to be monophyletic with a high support in both maximum-likelihood and Bayesian topologies. The interrelationships within the genus *Trypanosoma* were only poorly resolved. Clades of fish, reptile, and "African" trypanosomes were well supported. Avian trypanosomes did not form a monophyletic group and split into three groups, here designated as A, B, and C. The groups B and C were robustly monophyletic, whereas the monophyly of the group A was only poorly supported (bootstrap 63 and posterior probability less than 0.5). Nevertheless, the group A clustered robustly with *T. irwini* from koalas. Groups B and C did not show affinity to any particular *Trypanosoma* group. Lineages I–IX formed in the RAPD were recovered by the SSU rDNA sequence analysis as well. Isolates of lineages X and XI possessed identical SSU rDNA sequences. New lineage XII was formed by two isolates (PAS108 and PAS 111 from collared flycatchers) which were not included in the RAPD analysis.

The group A was formed by lineages VI, VII, VIII, and IX, and by four sequences not included in the RAPD analysis (three isolates from songbirds and one sequence of the type strain of *T. bennetti* from American kestrel). Lineages VII, VIII, and IX were robustly monophyletic and isolates of the lineage VIII had identical sequences. The isolate APO7 from lesser spotted eagle was sister to *T. bennetti*. All 11 isolates in the group A were obtained from birds only (eight from songbirds and three from raptors). Lineages I, IV, V, XII, and eight isolates not included in the RAPD analysis (six from collared flycatchers, one from currawong, and one sequence of the type strain of *T. corvi* from rook) formed the group B. The lineages I, V, and XII were robustly monophyletic, isolates of the

[1] Confer [Latin] = compare with. This term is used for specimen that looks like "type strain", but has not been well determined due to various reasons. The name *T. avium* has been used for more than one species and its taxonomical status should be revised in the future (see Discussion).

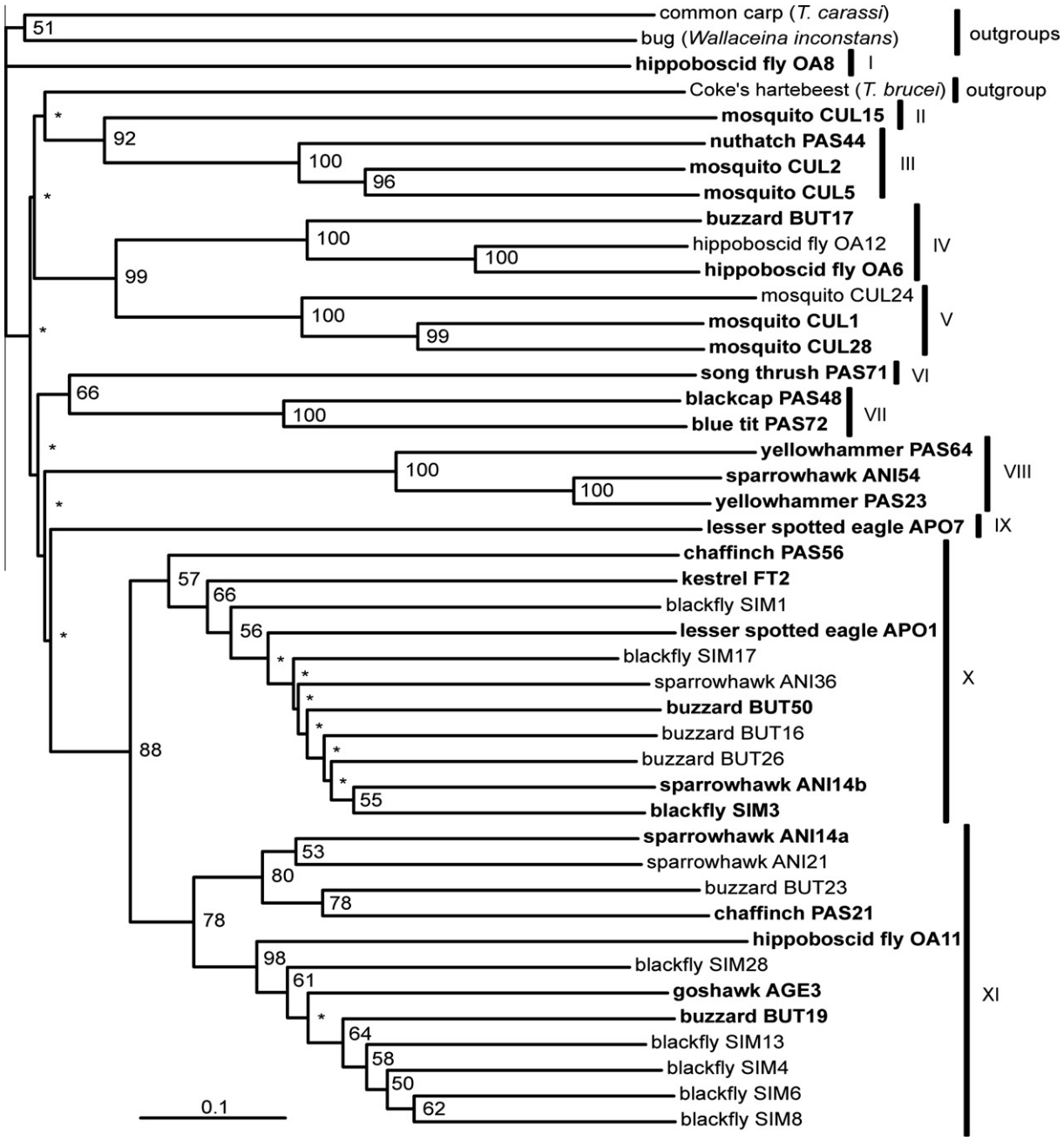

Fig. 1. Unrooted tree based on the dataset of 1505 scored RAPD amplicons of selected avian trypanosomes isolates (41) and other trypanosomatids (3) (see Table 1). The tree was constructed using the neighbor-joining method with Nei-Li-Dice distances. Bootstrap values shown at the nodes were counted with 5000 repetitions, asterisks indicating the values lower than 50. Isolates shown in bold were included also in the sequence analysis. Vertical lines and roman numerals denote individual lineages of avian trypanosomes. Bar represents 0.1 substitutions per site.

lineage XII had identical sequences. The monophyly of the lineage IV was supported only moderately (bootstrap 63 and posterior probabilities 0.84). Isolates from the group B were from mosquitoes (5), hippoboscid flies (2), songbirds (8) and raptor (1). *T. corvi* type strain clustered with moderate support with isolates from hippoboscid fly (OA6) and buzzard (BUT17), the only raptor isolate in the group B. Lineages I and XII were formed by isolates from vector or avian hosts. Lineages IV and V consisted of both vectors and avian hosts. All five mosquito isolates were placed within the robust lineage V together with two isolates from collared flycatchers. This lineage represented *T. culicavium*, a recently described species with the type isolate CUL1 from mosquito (Votypka et al., 2011). The group C was the largest among the avian groups and consisted of 29 isolates. It was formed by lineages II, III, X, and XI, and 14 more sequences not included in the RAPD analysis (10 of isolates from songbirds, three from raptors, and one from sand fly). Strains of lineages X and XI had identical sequences. The lineage III was robustly monophyletic; monophyly of the lineage II was unsupported. The group C was formed by isolates from songbirds (13), raptors (10), mosquitoes (3), black fly (1), hippoboscid fly (1), and sand fly (1). All three lineages (II, III, and X + XI) consisted of isolates from vectors and avian hosts. Lineages II and III were formed by isolates from mosquitoes and insectivorous songbirds. Ten isolates from raptors, two from chaffinches, one from black fly and one from hippoboscid fly formed lineages X and XI all of them sharing 100% sequence similarity.

Table 2
Cell length and kinetoplast width of selected trypanosome isolates.

Lineage (SSU & RAPD analyses)	Isolate	Epimastigote		Smaller epimastigote/Trypomastigote		Kinetoplast	
		Length [μm] ± SE	Range	Length [μm] ± SE	Range	Width [nm] ± SE/SD[†]	Range
A group	PAS114	**23.0** ± 0.8 (25)	9.4–5.1	–	–	**364** ± 6 (40)	304–446
A–VI	PAS71	**28.6** ± 0.7 (40)	19.2–39.3	–	–	–	–
A–VII	PAS48	**14.9** ± 0.4 (39)	11.2–20.3	–	–	**299** ± 7 (39)	226–398
A group	PAS96	**24.6** ± 0.9 (25)	17.1–36.5	–	–	–	–
A group	PAS93	**16.7** ± 0.7 (25)	11.7–24.3	–	–	–	–
A–IX	APO7	**16.5** ± 0.4 (51)	10.9–25.1	–	–	**456** ± 6 (37)	400–532
A–VIII	PAS23	**37.1** ± 0.9 (40)	27.5–48.0	**14.6** ± 0.4 (40)	11.0–21.6	**403** ± 8 (34)	332–488
B–I	OA8	**20.4** ± 0.6 (30)	14.2–27.8	**9.6** ± 0.2 (30)	7.0–12.4	–	–
B–XII	PAS111	**28.1** ± 0.9 (25)	21.5–40.1	**12.9** ± 0.3 (25)[*]	10.6–14.9	**354** ± 7 (40)	275–492
B–XII	PAS108	**29.1** ± 1.0 (25)	21.4–37.0	**12.9** ± 0.4 (25)[*]	8.7–16.4	–	–
B group	PAS94	**19.1** ± 0.6 (25)	13.7–25.5	–	–	**319** ± 4 (41)	268–387
B group	PAS95	**17.4** ± 0.6 (25)	12.2–22.5	–	–	**317** ± 4 (40)	285–415
B–IV	ITMAP180795	**18.2** ± 0.4 (35)	13.5–22.1	–	–	**373** ± 6 (44)	301–466
B–IV	OA6	**21.8** ± 0.4 (30)	17.0–24.9	–	–	**368** ± 15 (30) [†]	
B–V	CUL6	**27.8** ± 1.2 (25)	18.5–40.3	**13.3** ± 0.2 (25)[*]	10.9–15.1	**299** ± 5 (40)	231–379
B–V	CUL1	**30.8** ± 1.0 (25)	22.4–42.9	**13.5** ± 0.2 (25)[*]	10.7–15.9	**310** ± 31 (35) [†]	
B–V	PAS99	**28.5** ± 0.9 (30)	19.3–39.5	**13.7** ± 0.3 (30)[*]	11.0–16.7	**341** ± 7 (37)	240–415
B–V	PAS109	**28.2** ± 1.4 (25)	19.1–45.6	**13.3** ± 0.2 (25)[*]	11.5–14.9	–	–
B–V	CUL30	**29.7** ± 0.9 (50)	19.2–50.6	**13.1** ± 0.2 (49)[*]	9.8–15.5	**295** ± 7 (55)	219–423
C group	A1412	**22.3** ± 0.5 (35)	17.3–27.9	–	–	**1063** ± 11(30)	934–1164
C–II	PAS105	**20.4** ± 0.9 (24)	13.0–29.9	**10.1** ± 0.2 (26)	7.3–12.0	–	–
C–II	CUL15	**18.5** ± 0.3 (25)	15.6–20.8	**10.7** ± 0.2 (25)	9.2–12.6	**546** ± 8 (53)	458–668
C–II	PAS112	**17.8** ± 0.4 (25)	13.6–21.5	–	–	**527** ± 9 (40)	446–676
C–II	PAS106	**19.4** ± 0.8 (25)	13.5–25.9	**10.5** ± 0.3 (25)	8.0–13.9	–	–
C–III	PAS113	**32.0** ± 1.3 (25)	20.1–44.1	–	–	–	–
C–III	PAS110	**35.6** ± 1.4 (25)	23.4–50.9	–	–	**859** ± 9 (41)	685–966
C–III	PAS107	**31.5** ± 1.0 (25)	24.4–43.0	**12.7** ± 0.3 (25)[*]	9.4–15.5	–	–
C–III	CUL5	**33.6** ± 1.3 (25)	22.7–48.9	**13.3** ± 0.5 (25)[*]	9.3–18.5	**749** ± 14 (36)	579–882
C–III	CUL2	**35.1** ± 1.5 (25)	21.8–49.1	**13.2** ± 0.3 (25)[*]	10.1–15.8	–	–
C–X	FT2	**20.4** ± 0.4 (41)	12.9–25.5	–	–	**748** ± ± 58 (34) [†]	
C–X	APO1	**23.1** ± 0.5 (30)	16.7–28.1	–	–	**848** ± 67 (35) [†]	
C–X	ANI14b	**19.6** ± 0.4 (50)	14.1–28.3	–	–	**782** ± 55 (40) [†]	
C–X	SIM3	**21.7** ± ± 0.7 (30)	15.6–29.3	–	–	**888** ± 48 (40) [†]	
C–XI	ANI14a	**23.3** ± 0.5 (30)	17.3–27.8	–	–	–	–
C–XI	PAS21	**19.4** ± 0.6 (32)	13.8–25.1	–	–	**585** ± 8 (46)	470–702
C–XI	OA11	**18.7** ± 0.5 (30)	12.9–25.4	–	–	**565** ± 7 (56)	440–697
C–XI	AGE3	**20.5** ± 0.6 (76)	12.0–31.5	–	–	**637** ± 7 (48)	544–762
C–XI	BUT19	**22,8** ± 0.6 (55)	14.5–29.4	–	–	**625** ± 7 (47)	505–756
C group	BUT15	**19.9** ± 0.4 (35)	15.5–25.7	–	–	**691** ± 80 (38) [†]	

[*] Trypomastigote morphotypes.
[†] From Lukes and Votypka (2000) and Votypka et al. (2002).

3.3. Cell type and length

Morphology of 39 isolates was observed and their cell lengths were measured (Figs. 2 and 3, Table 2). Parasites occurred in cultures usually in the epimastigote form and 10 isolates were also observed in the trypomastigote form, both morphotypes being present concurrently. These 10 isolates were from mosquitoes or insectivorous songbirds and formed three different lineages (III, V, and XII). In case of lineage III only 3 out of 5 isolates formed both morphotypes. Isolates belonging into a particular lineage usually resembled each other in their cell lengths that ranged around the same average size independently of the host species. Average cell lengths of trypomastigotes were about 14 μm. Epimastigote cells were more variable. In case of five isolates epimastigotes occurred in two different cell lengths concurrently (lineages I, II, and VIII). The length of small epimastigotes averaged 10–15 μm and that of large epimastigotes was around 19 μm (lineages I and II) or 37 μm (lineage VIII). In the group A, three different sizes of epimastigotes occurred (lineage VIII > VI > IX = VII) while two were present in each of the groups B and C, respectively.

3.4. Kinetoplast width

Average kinetoplast widths of 20 measured isolates (Fig. 2 and Table 2) ranged between 295 and 1063 nm. Isolates of group A (299–456 nm) and B (295–373 nm) had thinner, while group C (527–1063 nm) had wider kinetoplasts. Kinetoplasts of the groups A and B were variable. In case of the group C kinetoplast widths differed considerably among lineages. Isolates of lineages II and XI had thinner kinetoplast (527–691 nm) than isolates in lineages III and X (748–888 nm). The widest kinetoplast was found in the isolate A1412 (from rook) that was placed separately of all recovered lineages of the group C.

4. Discussion

Phylogeny of avian trypanosomes was assessed using two different methods. Results of both methods were congruent. The 11 separate lineages recognized by the RAPD analysis (Fig. 1) were recovered also in the SSU rRNA gene analysis, with the exception of lineages X and XI, which had identical sequences, and thus undistinguishable from each other in the SSU rRNA gene analysis. The new lineage XII recognized in the SSU rRNA gene analysis consisted of isolates not included in the RAPD dataset. Avian trypanosomes formed three separate major groups, A–C. Each established type strain (*T. bennetti*, *T. corvi* and *T. culicavium*) belonged to a different lineage (IV, V, and IX). We are convinced that at least 7 out of 9 remaining lineages (I–III, VI–VIII and XII) represent new species. Isolates of lineages X and XI recovered by the RAPD analysis did not show any sequence variability in the SSU rRNA gene. We suppose that analysis of genes more variable than SSU rRNA would reveal

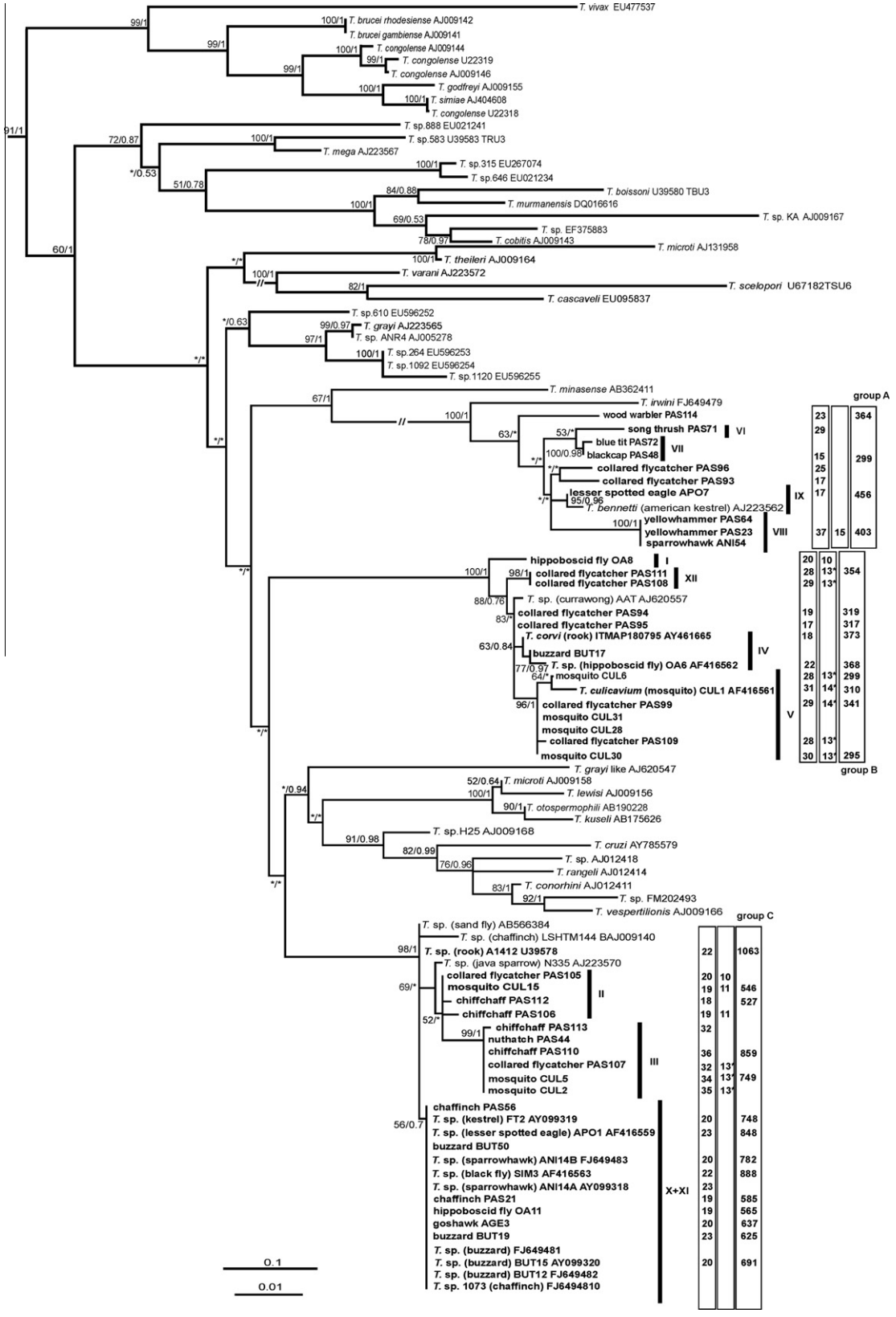

Fig. 2. Phylogenetic tree of avian (56), other trypanosomes (44) and other kinetoplastids based on SSU rRNA gene sequences and constructed by the maximum-likelihood method. The tree was rooted with eight bodonid sequences (not shown) together with 10 sequences of non-trypanosoma kinetoplastids. Bootstrap values from maximum-likelihood and Bayesian posterior probabilities are shown at nodes. Asterisks indicate nodes with bootstrap support lower than 50%. Sequences shown in bold represent isolates deposited in cryostabilate collection of Department of Parasitology, Charles University in Prague, Czech Republic. Vertical lines indicate trypanosomatid lineages as found in RAPD analysis, three major groups of avian trypanosomes are marked by side boxes. Bold branches were shortened and two bar lengths were used: bold represents 0.1, and thin 0.01 substitutions per site, resp. Numbers in first box show average cell lengths [μm] of (bigger) epimastigotes, in the second box, average cell lengths [μm] of smaller epimastigotes or trypomastigotes (∗) while the right box summarizes average kinetoplast widths [nm] of selected isolates.

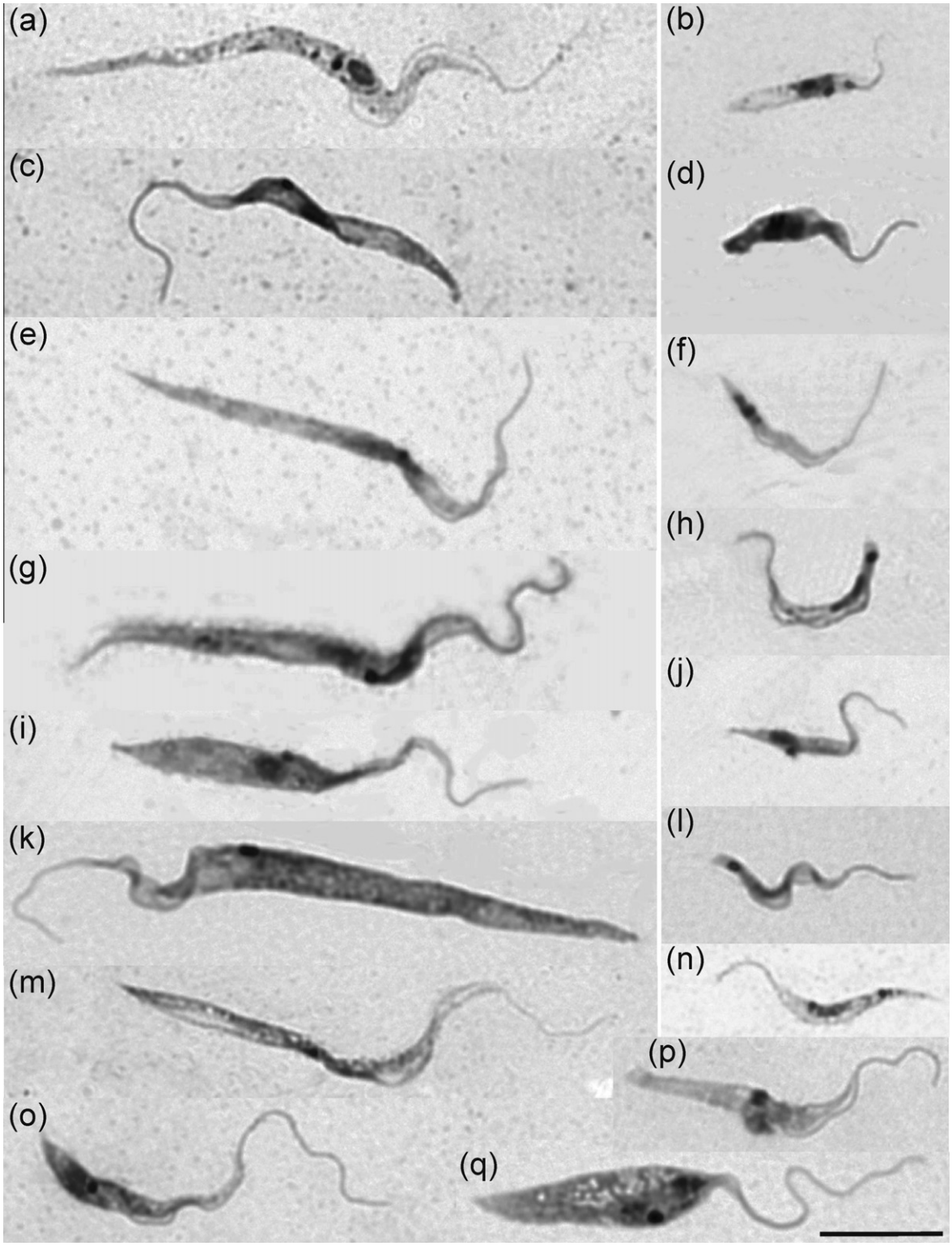

Fig. 3. Light microscopy of different cell types of avian trypanosomes from culture: (a) large epimastigote of PAS23, (b) small epimastigote of PAS23, (c) large epimastigote of OA8, (d) small epimastigote of OA8, (e) epimastigote of PAS111, (f) trypomastigote of PAS111, (g) epimastigote of CUL1, (h) trypomastigote of CUL1, (i) large epimastigote of PAS105, (j) small epimastigote of PAS105, (k) epimastigote of CUL2, (l) trypomastigote of CUL2, (m) epimastigote of PAS71, (n) epimastigote of PAS48, (o) epimastigote of *T. corvi* ITMAP 180795, (p) epimastigote of FT2 and (q) epimastigote of PAS21. Bar, 10 μm.

more genetic variability since the lineages not only were distinct in the RAPD analysis but they also differed morphologically in their kinetoplast widths and/or estimated minicircle size (Votypka et al., 2002).

The name most frequently used for avian trypanosomes is *T. avium*. Several isolates of the lineages X and XI, and also some other sequences branching separately in the group C were formerly assigned to this species (Votypka et al., 2002, 2004, 2011). In the

SSU rRNA gene analysis these putative *T. avium* sequences appeared to be polyphyletic. Considerable differences were found between lineages X and XI, and isolate A1412 in their kinetoplast widths. The exceptionally wide kinetoplast of the isolate A1412, originally described in Lukes and Votypka (2000), was remeasured with a similar result. As suggested earlier (Votypka et al., 2004), this isolate does not belong to *T.* cf. *avium*.

T. avium was originally described from Accipitridae, Laniidae and Corvidae in 1885 by Danilewsky, but neither slides with the type material nor the reference strain were preserved. In 1903 Laveran proposed to restrict this species name for parasites of owls however, few followed his recommendation. On the contrary, the name *T. avium* was often used to designate any bird trypanosome (Baker, 1956a,b,c; Stabler et al., 1966; Bennett, 1970; Tarello, 2005). Sequences designated as *T. avium*, isolates established in 1984, now deposited in ATCC, or blood films of three avian species on microscopic slides deposited in the International Reference Centre for Avian Hematozoa (Bennett et al., 1980) do not represent the type strain since they were not connected to any formal redescription or species revision. Clearly, redescription and establishment of neotype slides is needed.

Most descriptions of avian trypanosomes were made between the years 1900–1921 (Podlipaev, 1990) on the basis of morphology of blood trypomastigotes. Nevertheless, it has been shown that the cell lengths of the blood morphotypes are very variable and that they usually change early in the course of infection (Molyneux, 1973). Moreover, parasitemia in avian hosts tends to be very low (Baker, 1956c; Kirkpatrick and Lauer, 1985); therefore, in some cases only few specimens were measured (Chatterjee, 1983). Several authors (Sehgal et al., 2006; Valkiunas et al., 2011) still use blood trypomastigote morphology as an important criterion for species determination of avian trypanosomes. In our opinion, morphology should serve only as an additional approach to phylogenetic analyses. Moreover, we find morphology of cultured cells more informative than that obtained directly from the host (Votypka et al., 2002, 2004, 2011; Svobodova et al., 2007; Teixeira et al., 2011) since morphology does not change during cultivation *in vitro* (Novy and MacNeal, 1905), even in different culture media (Kirkpatrick and Lauer, 1985). According to our findings, the cell length of cultured cells is stable during the stationary phase; 4–7 day after inoculation (unpublished data).

The kinetoplast width was suggested as a useful character to distinguish lower trypanosomatids as it correlates with the length of minicircles (Wallace et al., 1983). Minicircles differ also between isolates of avian typanosomes (Lukes and Votypka, 2000; Votypka et al., 2002, 2004). Trypanosomes in our study could be divided into two groups on the basis of the kinetoplast width: isolates with kinetoplast narrower than 500 nm (groups A and B) and wider than 500 nm (groups C). It is clear that isolates formerly assigned to one species, but having clearly different kinetoplast width, should not be considered as conspecific. On the other hand, similar kinetoplast width does not prove species identity as trypanosomes from different groups or lineages can resemble each other in this trait (e.g., group A and B, lineage II and XI, or III and X).

Presumed host specificity also used to be a criterion for species designation, although host-parasite relationships among avian trypanosomes are poorly known. Avian host spectrum in our study was restricted to songbirds and raptors; trypanosomes of all the three main groups can infect both bird taxa. From the host perspective, one avian host species can harbor several trypanosome species (Molyneux and Gordon, 1975). For example, at least two species were found in rooks (*T.* cf. *avium* and *T. corvi*) (Votypka et al., 2004). Collared flycatchers are host of trypanosomes of all three groups (A–C). Therefore, the identification of trypanosome species based on the avian host is useless. Considering the vector, isolates of only one vector species appeared in each individual lineage. This supports the hypothesis of higher trypanosome specificity towards the vector (Apanius, 1991). Indeed, *T. culicavium* transmitted by *Culex* mosquitoes rarely infects *Aedes aegypti* (Votypka et al., 2011). The only notable exception is the isolate OA11 from a hippoboscid fly in *T.* cf. *avium* (lineage XI), which is presumably transmitted by blackflies. Hippoboscids feed several times a day and have continuous digestion. Trypanosomes can temporarily survive in the digestive tract of hippoboscids supplemented with fresh blood without a need to outlive defecation as in Nematocera (blackflies), which have simultaneous digestion and parasites can be eliminated during defecation. We suppose that this lineage is specific for blackflies.

Natural bird-trypanosome combinations result not only from intrinsic factors but also from life history characters. Insectivorous birds get culicine trypanosomes in their food (Votypka et al., 2011), raptors can get infected from their prey (Dirie et al., 1990). Nest height influences exposure to diverse bloodsucking insects that serve as vectors; e.g., raptors are exposed to foraging black flies while breeding in the canopy (Cerny et al., 2011).

The way of transmission of three trypanosome species has been unambiguously demonstrated so far. *T. corvi* is transmitted from hippoboscid flies to songbirds by ingestion of the vector while grooming (Baker, 1956b; Votypka et al., 2004). Our analysis adds raptors as new avian hosts for this species. *T. culicavium* is transmitted by ingestion of infected culicine mosquitoes by mosquito-eating birds (Votypka et al., 2011). *T.* cf. *avium* is transmitted from black flies (*Eusimulium* spp.) to experimental songbirds by ingestion of infected vectors and also contaminatively, e.g., through conjunctiva (Votypka and Svobodova, 2004). Suggested hosts were owls, raptors and songbirds (Danilewsky, 1885,1889; Laveran, 1903; Baker, 1976; Podlipaev, 1990; Dirie et al., 1990; Votypka et al., 2002). Indeed, lineages X and XI consisted of isolates from black flies, raptors and chaffinches, so passerines are confirmed as natural hosts.

Two more putative life-cycles can be suggested by our SSU analysis. Lineages II and III were both formed by isolates from mosquitoes and insectivorous passerines. The original localization of parasites in the vector was on stomodeal valve (lineage III) or in hindgut (lineage II). Although bird hosts of the lineage II could eventually get infected contaminatively through prediuresis (Votypka and Svobodova, 2004), we suppose that transmission by ingestion, as in the case of *T. culicavium* (Votypka et al., 2011), is more probable. Six remaining putative species (lineages I, VI–IX, and XII) recovered by our analysis still wait for elucidation of their life cycles. Unfortunately, the whole group A consisting of three putative species (lineages VI, VII, and VIII), and lineage IX of *T. bennetti* contains no vector isolate.

We suppose local transmission in most of the lineages revealed in our study. Seven lineages (I–V, X, and XI) contained insect isolates; insects do not disperse far. The lineage VII contained an isolate from blue tit (PAS72), a resident species; two isolates from raptors (APO7 and ANI54 in lineages VIII and IX, respectively) were obtained from nestlings. On the other hand *T. bennetti* (lineage IX) is probably widespread since the described type isolate from the USA clustered with the isolate APO7 obtained from a nestling in Slovakia. Two lineages (VI and XII) include only isolates from migrants and no vectors; however, the number of sequences in these lineages is very low.

We present here the first study on avian trypanosomes biodiversity based on a large number of isolates obtained from both avian and vector hosts. Two molecular methods (RAPD and phylogenetic analysis of the SSU rRNA gene) and two morphological approaches (measurement of kinetoplast width and cell length) were employed. All obtained results were congruent and we were able to distinguish about 11 putative species of avian trypanosomes, nine of them being probably transmitted in the studied region.

Baker (1976) proposed 12 existing species of avian trypanosomes and it seems from our data that his estimation was very close to reality. Although more species may exist, their number is most probably much lower than the number of described avian trypanosomes (almost 100). Our results were in agreement with previous phylogenetic studies that suggested polyphyly of avian trypanosomes (Haag et al., 1998; Votypka et al., 2004, 2011). Thus, avian trypanosomes are polyphyletic similarly to mammalian trypanosomes (Maslov et al., 1996; Hamilton et al., 2004). Three previously described life-cycles are in accordance to our data. The *T. corvi* lineage was formed by isolates from hippoboscid flies, songbirds and newly raptors, the *T. culicavium* lineage was formed by mosquitoes and insectivorous songbirds, and isolates from black flies, songbirds and raptors were in *T.* cf. *avium* lineage(s). We propose that two more lineages of avian trypanosomes that probably represent distinct species are also transmitted by mosquitoes. We suggest that host specificity is probably influenced not only by physiology or immunology of host but also by life-history traits. Since one bird host can be infected with several trypanosome lineages, and one lineage can infect birds of different orders, host specificity should no longer be used for species determination. On the other hand, we are convinced that morphological characters, especially if obtained on cells kept in culture, can be still useful although molecular data are the most reliable.

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