Available online at www.sciencedirect.com
SCIENCE DIRECT®
Veterinary Parasitology 128 (2005) 11–21
veterinary parasitology
www.elsevier.com/locate/vetpar

Cryptic species within the *Tetratrichomonas gallinarum* species complex revealed by molecular polymorphism☆

Ivan Cepicka*, Kateřina Kutišová, Jan Tachezy, Jaroslav Kulda, Jaroslav Flegr

Department of Parasitology, Faculty of Science, Charles University, Vinicna 7, Prague 2 128 44, Czech Republic

Received 31 December 2003; received in revised form 11 October 2004; accepted 1 November 2004

Abstract

Tetratrichomonas gallinarum is a widespread intestinal parasite of galliform and anseriform birds. The pathogenicity of this species is controversial, presenting an unsettled problem as yet. We analysed the polymorphism and genetic relationship among 29 isolates of *T. gallinarum* obtained from eight bird species and five *T. gallinarum*-like isolates from the oral cavity and lower respiratory tract of human patients. Two methods were used for the analyses: RAPD and sequencing of 16S rRNA, 5.8S rRNA, ITS1 and ITS2 genes, both producing consistent and well-supported results. The isolates were divided into five groups, A–E, with eleven subgroups. The distance between groups E, D and the cluster A–B–C considerably exceeded usual intraspecific polymorphism seen in trichomonads. Moreover, the largest subgroup, A2 (containing 18 isolates), was divided into three branches according to the host specificity. All isolates from humans were placed into avian subgroups A2 and B2. We conclude that our isolates represent, at least, three morphospecies or rather complexes of several cryptic species. Since certain species of the *T. gallinarum* complex can differ in their biological characteristics and some of them can infect humans, the problem of *T. gallinarum* pathogenicity should be re-examined with regard to specific genetic groups and zoonotic potential of some of these lineages should be considered.

Keywords: Phylogeny; RAPD; rRNA; Pathogenicity; Zoonotic potential

1. Introduction

Tetratrichomonas gallinarum is a common intestinal flagellate of fowl and other galliform and anseriform birds, both domesticated and living in natural environments. This species, once thought to be an economically important pathogen, is at present generally considered as a harmless commensal. Early authors suspected *T. gallinarum* of causing fatal enterohepatitis in turkey (Hadley, 1916), but the idea was abandoned after discovery of the true etiologic agent, *Histomonas meleagridis* (Tyzzer, 1919). Still, some authors insisted that trichomonads could induce lesions in caecum and liver of domestic fowl and

☆ Nucleotide sequence data reported in this paper are available in the GenBank™ under the accession numbers AY245106–AY245163.

* Corresponding author. Tel.: +42 022 1951821; fax: +42 022 4919704.
E-mail address: cepicka@natur.cuni.cz (I. Cepicka).

0304-4017/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2004.11.003

turkey (Allen, 1941) and case reports on pathological manifestations of *T. gallinarum* infection continue to appear (Mohan et al., 1966; Patton and Patton, 1996; Norton, 1997; Crespo et al., 2001). In many of reported cases, the presence of trichomonads in lesions or at unusual extraintestinal sites apparently was secondary to pathological conditions of another etiology. Also, majority of experimental infections did not bring evidence on a pathogenic potential of *T. gallinarum* (Delappe, 1957; Goedbloed and Bool, 1962; Kemp and Reid, 1965; Kulda et al., 1974; Pecka, 1991), the report of Lee (1971) being an exception. Despite of prevailing standpoint, the question of *T. gallinarum* pathogenicity has not been settled definitely as the strain diversity or even the presence of several species within the *T. gallinarum* complex has not been taken into account.

Tetratrichomonas gallinarum was originally described from chickens (Martin and Robertson, 1911), but similar organisms have also been found in intestines of other bird species from more than ten orders. Several of these trichomonads were described as independent species mainly on the basis of assumed host specificity (Kotlán, 1923; Tanabe, 1926; Hegner, 1929; Travis, 1932; Richardson, 1934). However, due to an incompleteness of the descriptions, variability of *T. gallinarum* with respect to size and shape (McDowell, 1953; Friedhoff et al., 1991) and successful experimental transmissions between anseriform and galliform birds (Hegner, 1929; Kulda et al., 1974; Friedhoff et al., 1991), the validity of most of these species is questionable. Interestingly, tetratrichomonads of *T. gallinarum* morphology have been recently identified (Kutisova et al., sent to press) in a sample of isolates isolated from the bronchi and oral cavity of human patients suffering from serious lung disorders (Teras et al., 1980). *T. gallinarum* relationships of these organisms misinterpreted as *Trichomonas tenax* has been confirmed by molecular typing, thus indicating that the host spectrum of *T. gallinarum*-like tetratrichomonads might be wider than expected.

Until the taxonomy of the *T. gallinarum*-like species is definitely settled, pathogenicity studies performed with poorly characterised *T. gallinarum* isolates should be interpreted with caution. The presence of *T. gallinarum*-like organisms in humans also opens exciting questions, whether the strains or cryptic species with zoonotic potential do exist among the *T. gallinarum*-like trichomonads. To this end, we performed a detailed analysis of 5.8S rRNA, 16S rRNA, ITS1 and ITS2 genes as well as RAPD analysis with a total of 52 isolates, including 34 *T. gallinarum*-like isolates obtained from eight bird species (29 isolates) and humans (5 isolates), and 18 isolates representing species of seven trichomonad genera from other vertebrate and invertebrate hosts.

2. Materials and methods

2.1. Organisms and culture conditions

Information on the origin of isolates included in the study is summarized in Table 1. Fresh material was obtained from faeces, gut contents or cloaca of both wild and domesticated animals or animals kept in zoological gardens. Trichomonads were isolated in modified medium TYM (Diamond, 1957) supplemented with 10% inactivated horse serum, agar (0.5 g/l) and antibiotics (penicillin 3 mg/ml, amikacin 0.25 mg/ml), in modified medium TYSGM (Diamond, 1982) without mucin and supplemented with rice starch or in Dobell and Leidlaw's biphasic medium (Dobell and Leidlaw, 1926). All cultures except of isolates 20-9-1 and LMA were axenised after 2–10 passages in xenic conditions by a migration technique. Axenic isolates were cultivated in medium TYM without antibiotics. Trichomonads isolated from birds and mammals were cultivated at 37 °C and were subcultured every second or third day. Isolates obtained from amphibians, reptiles and molluscs were cultivated at 26 °C and were transferred into the fresh medium once or twice a week. For DNA isolation, axenic cultures were maintained in medium TYM without agar for five passages. All axenic isolates were cryopreserved and deposited in the cultures collection of the Department of Parasitology, Charles University in Prague.

2.2. rDNA amplification, cloning and sequencing

DNA from approximately 10^8 cells for axenic isolates or 10^5 cells for polyxenic isolates was extracted using the guanidiumhydrochloride method (Pramanick et al., 1976) modified according to

Table 1
List of trichomonad isolates included in the study

Species	Isolates	Host, localization	Origin	Isolations
Tetratrichomonas gallinarum	1-11-M2	*Meleagris gallopavo*	Troupsko, CZ	Kulda (1971)
	12-6-7	*Gallus gallus*	Brno, CZ	Kulda (1972)
	12-7	n.a.[a]	Brno, CZ	Kulda (1972)
	20-9-1	*Anas platyrhynchos*	Volyně, CZ	Čepička (1999)
	26-7	*Gallus gallus*	Brno, CZ	Kulda (1972)
	A6	*Anas platyrhynchos*	Uhlířské Janovice, CZ	Suchánková (1970)
	AA9-8	*Anser anser*	Brno, CZ	Kulda (1973)
	AF[b]	*Anas formosa*	Dvůr Králové nad Labem, CZ	Čepička (2000)
	CYG	*Cygnus olor*	Týnec nad Sázavou, CZ	Čepička (2001)
	CYG2	*Cygnus olor*	Stříbrná Skalice, CZ	Čepička (2001)
	CYG4	*Cygnus olor*	Pyšely, CZ	Čepička (2001)
	GPO	*Gallus gallus*	Přelovice, CZ	Čepička, 1999)
	HD	*Gallus gallus*	Planá, CZ	Čepička (2000)
	KMV	*Anas platyrhynchos*	České Budějovice	Pecka (1991)
	KRA-1	*Anas platyrhynchos*	Volyně, CZ	Čepička (1999)
	KROC	*Meleagris gallopavo*	Libečov, CZ	Čepička (2001)
	M3	*Meleagris gallopavo*	Uhlířské Janovice, CZ	Suchánková and Kulda (1970)
	OAM5	*Anas platyrhynchos*	Hostinné, CZ	Čepička (1999)
	PH18-9	*Phasianus colchicus*	Židlochovice, CZ	Kulda (1973)
	Q7[b]	*Numida meleagris*	Olomouc, CZ	Hampl (1999)
	Q9[b]	*Numida meleagris*	Olomouc, CZ	Hampl (1999)
	TGR8	*Gallus gallus*	Říčany u Prahy, CZ	Čepička (1999)
	TGR9	*Gallus gallus*	Říčany u Prahy, CZ	Čepička (1999)
	TUMK2	*Anas platyrhynchos*	Brno, CZ	Čepička (1999)
	TUMS1	*Gallus gallus*	Brno, CZ	Čepička (1999)
	Z26-7	*Gallus gallus*	Brno, CZ	Kulda (1972)
	ZP-A2	*Anas platyrhynchos*	České Budějovice CZ	Pecka (1985)
	ZP-E2	*Anser anser*	České Budějovice CZ	Pecka (1988)
	ZP-H3	*Anser anser*	České Budějovice CZ	Pecka (1985)
Tetratrichomonas limacis	LMA	*Limax maximus*	Kamberk, CZ	Čepička (1999)
Tetratrichomonas prowazeki	SL	*Anguis fragilis*	Vrchlabí, CZ	Tachezy (1996)
Tetratrichomonas sp.	TX-B1	*Homo sapiens*	Tallin, Estonia	Teras (1975)
	TX-B3	*Homo sapiens*	Tallin, Estonia	Teras (1976)
	TX-B5	*Homo sapiens*	Tallin, Estonia	Teras (1977)
	TX-O6	*Homo sapiens*	Tallin, Estonia	Teras (1964)
	TX-O10	*Homo sapiens*	Tallin, Estonia	Teras, 1977)
	KAJ[b]	*Macroclemys temminckii*	Prague, CZ	Čepička (1999)
Trichomonas vaginalis	TV10-02	*Homo sapiens*	Prague, CZ	Kulda (1973)
Trichomonas gallinae	TGK	*Columba livia*	Prague, CZ	Tachezy (1994)
Pentatrichomonas hominis	KUDU22[b]	*Tragelaphus imberbis*	Dvůr Králové nad Labem, CZ	Čepička (2000)
	PDZB2	*Sus scrofa*	Zbraslav u Brna, CZ	Hampl (2000)
	PH-KT	*Homo sapiens*	Velká Ida, SK	Giboda (1976)
Tritrichomonas augusta	DALM	*Rana dalmatina*	Štěkře, CZ	Čepička (2001)
	HYLA	*Hyla arborea*	Štěkře, CZ	Čepička (2001)
	T-37 (ATCC 30077)	*Bufo* sp.	Stillwater, OK, USA	Twohy (1959)
Tritrichomonas nonconforma	ABI	*Anolis bartschi*	Vinales, Cuba	Tachezy (2000)
	R114	*Anolis bartschi*	San Vincente, Cuba	Kulda (1966)
Tritrichomonas foetus	KVc-1[c]	*Bos taurus*	Žalmanov, CZ	Lípová and Kulda (1962)

Table 1 (*Continued*)

Species	Isolates	Host, localization	Origin	Isolations
Tritrichomonas sp.	LV2	*Lacerta vivipara*	Olešník, CZ	Kulda (1992)
Monocercomonas colubrorum	EUMM[b]	*Eumeces* sp.	Prague, CZ	Čepička (1999)
Hypotrichomonas acosta	L3 (ATCC 30069)	*Drymarchon corais couperi*	Unknown	Honigberg (1948)
Trichomitus batrachorum	BUB	*Bufo bufo*	Veselí nad Lužnicí, CZ	Kulda (1982)

[a] Primary culture inoculated by content of ceca was obtained from Department of Avian Diseases, School of Veterinary Medicine, Brno. Host record not available.

[b] Isolates obtained from animals kept in zoological gardens.

[c] Isolate KVc-1 was obtained by re-cloning the KV-1 isolate (ATCC 30924), the clone derived from the original KV stock isolated by Lípová in 1962.

Vaňáčová et al. (1997). The 5.8S rRNA region, i.e. 5.8S rRNA with the flanking areas ITS1 and ITS2 and with adjacent parts of 16S rRNA and 28S rRNA, was amplified using trichomonad specific primers ITSF (TTCAGTTCAGCGGGTCTTCC) and ITSR (GTAGGTGGACCTGCCGTTGG) that are similar to the primers TFR1 and TFR2 designed by Felleisen (1997). 16S rRNA was amplified using trichomonad specific primers 16Sl (TACTTGGTTGATCCTGCC) and 16Sr (TCACCTACCGTTACCTTG). Most of the genes were bidirectionally sequenced directly from purified PCR products which prevented manifestations of Taq polymerase errors during the PCR cycle (Innis et al., 1988). Genes for the 5.8S rRNA region of isolates BUB, 20-9-1 and KAJ were T–A cloned into the pGEM®-T EASY vector using the pGEM®-T EASY VECTOR SYSTEM I (Promega Corp.) and at least two clones obtained from two independent PCR amplifications were bidirectionally sequenced. The external primers used for direct sequencing from PCR product were ITSF, ITSR, 16Sl and 16Sr described above. SP6 (ATTTAGGTGACACTATA) and T7 (TAATACGACTCACTATA) primers were used for sequencing from the vector. The primers used for sequencing of internal regions of PCR fragment of 16S rRNA were 295R (AGTCCGACGGTAACCGC), 665R (ATACWCTAAGCGTCCTG), 514F (GTGCCAGCMGCCGCGG), 1055R (CGGCCATGCACCACC), 1055F (CGGCCATGCACCACC) and 1385R (GATCCTAACATTGTAGC).

2.3. Analyses of sequences

The length of newly obtained sequences varied from 293 bp (isolate PH-KT) to 352 bp (isolate BUB) for the 5.8S rRNA region and from 1507 bp (isolate GPO) to 1518 bp (isolate KAJ) for 16S rRNA. Besides 58 newly determined sequences, following sequences obtained from GenBank™ were used in phylogenetic analyses: AF124609 (16S rRNA of *Pentatrichomonas hominis*), AF124608 (16S rRNA of *Tetratrichomonas gallinarum*), AF236105 (5.8S rRNA region of *Tetratrichomonas* sp. 'duck salpingitis'), AY244647, AY244648 and AY244649 (5.8S rRNA region of *Tetratrichomonas gallinarum*), AY244641, AY244642, AY244643, AY244644, AY244645 and AY244646 (5.8S rRNA region of *Tetratrichomonas* sp. from humans), AF076958 (16S rRNA of *Trichomitus batrachorum*), AY244650 (5.8S rRNA region of *Tetratrichomonas prowazeki*), AY244651 (5.8S rRNA region of *Tetratrichomonas limacis*), U86614 (5.8S rRNA region of *Trichomonas gallinae*), D49495 (5.8S rRNA region of *Trichomonas tenax*), U86615 (5.8S rRNA region of *Trichomonas tenax*), U17510 (16S rRNA of *Trichomonas vaginalis*), AY055802 (16S rRNA of *Tritrichomonas* sp., isolate LV2; the isolates LV2 does not represent the species *Tritrichomonas augusta* but some other, probably undescribed *Tritrichomonas* species from *Lacerta vivipara*. The description of this species will be the subject of another study.), AY055799 (16S rRNA of *Tritrichomonas foetus*), U85967 (5.8S rRNA region of *Tritrichomonas foetus*), AY055801 (16S rRNA of *Tritrichomonas mobilensis*), U86612 (5.8S rRNA region of *Tritrichomonas mobilensis*) and AY055803 (16S rRNA of *Tritrichomonas nonconforma*).

Three data sets, containing 21 sequences of 5.8S rRNA region, 20 sequences of 16S rRNA and 22 concatenated sequences of the 5.8S rRNA region and 16S rRNA, respectively, were created. Sequences from each data set were aligned using ClustalX 1.81 (Thompson et al., 1997) and alignments were manually edited using the BioEdit sequence editor (Hall, 1999). Phylogenetic trees were constructed

using distance, maximum parsimony and maximum likelihood methods implemented in PAUP 4.0 beta10 (Swofford, 1998). The Fitch–Margoliash method with logdet distances computed after the exclusion of constant positions was used to construct distance trees by ten replicates of a heuristic search. The starting tree was obtained via stepwise addition procedure with random taxon addition and swapped by the tree bisection–reconnection algorithm. Bootstrapping was performed with 1000 replicates. Similarly, in the parsimony analyses, the starting tree was obtained by stepwise addition of taxa and swapped. Bootstrappings with 1000 replicates each with ten replicates of random taxa addition were performed. The best substitution model for maximum likelihood, i.e. the general time reversible model with among-site rate variation and proportion of invariant sites (GTR + Γ + I) with gamma shape parameter 0.5342 and estimated proportion of invariable sites 0.4967 for the data set 3, was determined by hierarchical nested likelihood ratio test implemented in Modeltest 3.06 (Posada and Crandall, 1998). A heuristic search with ten replicates of starting tree construction by random taxa addition followed by branch swapping was used. Bootstrapping was performed with 100 replicates. Alignments are available upon requests.

2.4. RAPD analysis

RAPD analysis was performed with 26 *T. gallinarum* isolates and 19 other trichomonad isolates according to Hampl et al. (2001). The effects of magnesium, template DNA, dNTP and primers concentrations were examined. A total of 45 primers were initially screened against five trichomonad isolates from different genera. 18 primers (Operon technologies) which produced strong and most reproducible products were chosen: OPA-03 (AGTCAGCCAC), OPA-04 (AATCGGGCTG), OPA-10 (GTGATCGCAG), OPA-11 (CAATCGCCGT), OPA-12 (TCGGCGATAG), OPA-13 (CAGCACCCAC), OPA-20 (GTTGCGATCC), OPF-01 (ACGGATCCTG), OPF-02 (GAGGATCCCT), OPF-03 (CCTGATCACC), OPF-04 (GGTGATCAGG), OPF-06 (GGGAATTCGG), OPF-09 (CCAAGCTTCC), OPF-10 (GGAAGCTTGG), OPF-12 (ACGGTACCAG), OPF-13 (GGCTGCAGAA), OPF-14 (TGCTGCAGGT) and OPF-16 (GGAGTACTGG). The DNA samples from all isolates used in RAPD analysis were amplified with the particular primer in the same experiment using the same master mix. Products were electrophoresed in 2% agarose gel. All visible bands in electrophoretic patterns were included in the analysis. At first, a binary matrix coding the presence and absence of DNA fragments at the same positions in the respective gel lanes was created. Then FreeTree 0.9.1.50 (Pavlíček et al., 1999) was used for computation of genetic distances (d) of samples from Nei and Li's coefficient of similarity, as $d = 1 - s$ (Nei and Li, 1979), construction the dendrogram by the Neighbor-joining method and bootstrap analysis with 5000 replicates.

3. Results

3.1. Phylogenetic analyses of sequence data

On the basis of sequence data, *T. gallinarum* isolates split into five groups (A–E) and eleven subgroups (A1, A2, B1, B2, B3, C1, C2, C3, D1, D2 and E) each subgroup having identical 5.8S rRNA region and 16S rRNA sequences inside (Table 2). Subgroups A1 and A2 had identical 5.8S rRNA region, but they differed in one nucleotide in 16S rRNA. The subgroups B2 and B3 had identical 16S rRNA and they differed from the subgroup B1 in two nucleotides. The subgroups B1 and B2 had identical 5.8S rRNA and they differed from the subgroup B3 by insertion of one nucleotide. The subgroups B1 and C2 and the subgroups B3 and C3, respectively, had identical 16S rRNA, but they differed in the 5.8S rRNA region considerably. All human isolates were identical with isolates of either subgroup A2 or B2.

The average nucleotide distance between the group E and cluster A–B–C–D was 8.9% for the 5.8S rRNA region and 2.6% for 16S rRNA. The average distance between the group D and cluster A–B–C was 4.7% for the 5.8S rRNA region and 1.8% for 16S rRNA. The average distance among three *Trichomonas* species was 7.0% for the 5.8S rRNA region. The distance between two *Trichomonas* species was 2.4% for 16S rRNA. The average distance among five *Tritrichomonas* species was 6.7% for the 5.8S rRNA region. The average distance among four *Tritrichomonas* species was 1.2% for 16S rRNA. The distance between *Tetratrichomonas limacis* and *T. prowazeki*

Table 2
Division of *T. gallinarum* isolates into the groups and subgroups on the basis of sequences of 5.8S rRNA region and 16S rRNA

Groups	Subgroups	Both 5.8S rRNA region and 16S rRNA available	Only 5.8S rRNA region available
A	A1	CYG2	12-7, 26-7, KMV, PH18-9, TGR8, TGR9,
	A2	1-11-M2, A6, CYG4, HD, TX-O10	TUMK2, Z26-7, ZP-A2, ZP-E2, ZP-H3, TX-B1, TX-B3, TX-B5[a]
B	B1	CYG	–
	B2	TUMS1, TX-O6	–
	B3	Q7, KRA-1, 12-6-7	Q9
C	C1	M3	*Tetratrichomonas* sp. 'duck salpingitis'
	C2	OAM5	KROC
	C3	AA9-8	–
D	D1	AF	–
	D2	20-9-1	–
E	E	GPO	–

[a] Subgroups A1 and A2 do not differ in the 5.8S rRNA region, therefore, we can place isolates 12-7–TX-B5 only into the group A on the basis of sequence data. These isolates were placed into the subgroup A2 on the basis of RAPD analysis.

was 16.3% for the 5.8S rRNA region and 2.0% for 16S rRNA.

The alignment from 22 concatenated sequences of the 5.8S rRNA region and 16S rRNA (data set 3) contained 1870 positions, including those with gaps. Fig. 1 shows the optimal maximum likelihood topology. Trees constructed using distance and maximum parsimony methods (not shown) had topology similar to the maximum likelihood best tree. They differed in position of the chelonian isolate KAJ (sister position to the *Tetratrichomonas prowazeki–Tetratrichomonas limacis* branch in the distance tree), in position of *Tritrichomonas nonconforma* (basal branch of the genus *Tritrichomonas* in the maximum parsimony tree) and in the relationship between *T. gallinarum* groups A–B–C (sister position of groups B and C in the distance tree).

The genus *Tetratrichomonas* formed one clade with three branches (the *T. gallinarum* branch, the *T. prowazeki–T. limacis* branch and the isolate KAJ branch), but its monophyly had only moderate statistical support. Low bootstrap values of the clade were caused by the unstable position of the isolate KAJ. *T. gallinarum* and human tetratrichomonad isolates formed a robust clade with three well-supported branches (E, D and A–B–C). The relationship among the groups A–B–C was not resolved, as it varied with different tree construction methods and was not supported by high bootstrap values.

3.2. RAPD analysis

In addition to analysis of sequence data, we performed an analysis based on RAPD data. Fig. 2 shows an example of RAPD amplification. The data matrix for 45 trichomonad isolates contained 1386 characters. Unrooted dendrogram was constructed using Neighbor-joining method (Fig. 3). The intergeneric relationship was not reliable, but species belonging to the same genus always formed one branch. The position of *T. gallinarum* and human tetratrichomonad isolates was in agreement with the results of sequence analysis. The unidentified tetratrichomonad KAJ was placed into the same branch joined with groups D and E, but its position was not statistically well supported. The distances between groups D, E and the isolate KAJ were too high to obtain reliable information about their position by RAPD method. Groups A–B–C formed a robust cluster with well-resolved internal topology. The subgroup A1 formed a separate branch in a sister position to the subgroup A2. The subgroup A2 (18 isolates) divided with high statistical support into three branches A21, A22 and A23 with unresolved internal topology. These branches could not be detected on the basis of less polymorphic sequence data. The presence of the isolate in particular branch reflected the taxonomy of the host. The branch A21 formed by seven isolates from gallinaceous birds was

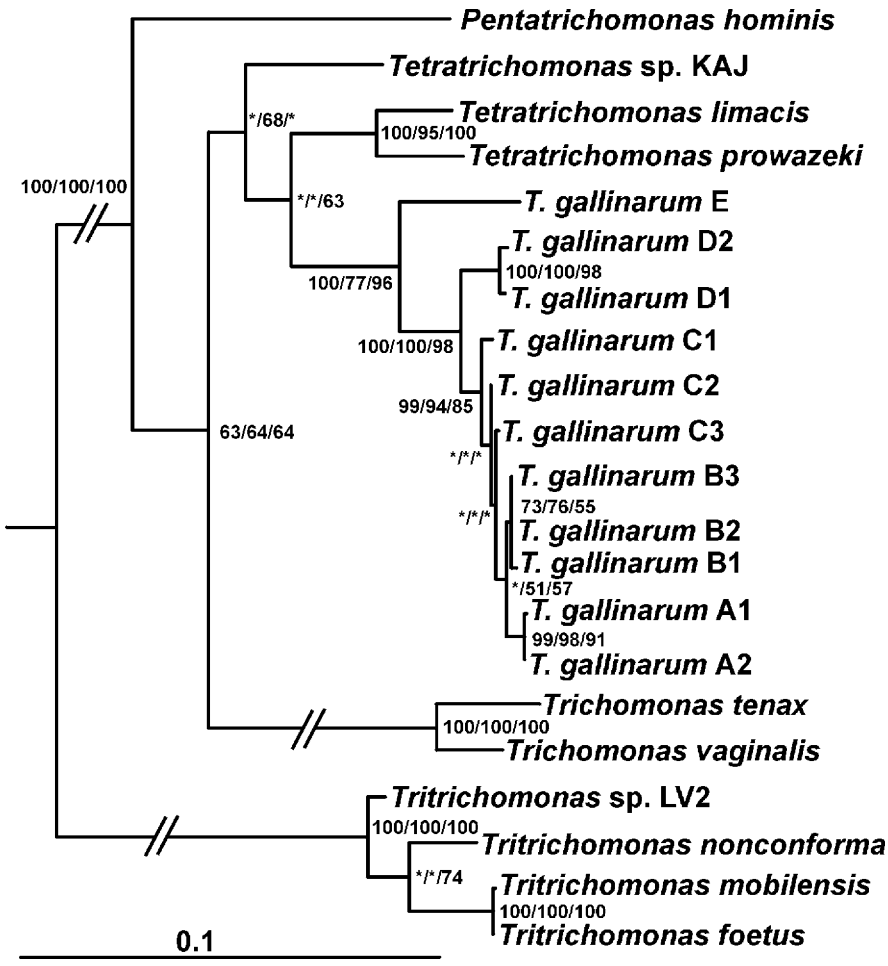

Fig. 1. Phylogenetic tree of trichomonads. The tree was constructed on the basis of concatenated 5.8S rRNA region and 16S rRNA (1870 aligned nucleotide positions) using the maximum likelihood under the GTR + Γ + I model with *Trichomitus batrachorum* as an outgroup. Bootstrap values from logdet/maximum parsimony/maximum likelihood are shown at the nodes. Asterisks indicate nodes with bootstrap values below 50%. The scale bar represents 10 changes per 100 positions.

in a sister position to the branch A22 formed by four isolates from humans, but this clade was not well supported by bootstrap values. Six of seven isolates of the branch A23 for which the host origin was known were all obtained from anseriform birds. The probability of such division of isolates according to their host specificity by chance can be calculated by exact test as $p = (7!\ 4!\ 6!\ 3!)/17! = 1.5 \times 10^{-6}$. The groups B and C were in a sister position and the topology inside was well-resolved. The separation of isolates into particular branches did not correspond with their host specificity.

4. Discussion

4.1. Molecular polymorphism of Tetratrichomonas gallinarum

Sequence analysis of 16S rRNA, 5.8S rRNA, ITS1 and ITS2 genes suggested that the distance between some *T. gallinarum* isolates considerably exceeded the usual intraspecific polymorphism seen in trichomonads. Actually, the distance between the clusters D, E and A–B–C was comparable or higher than the distance between different species of genera

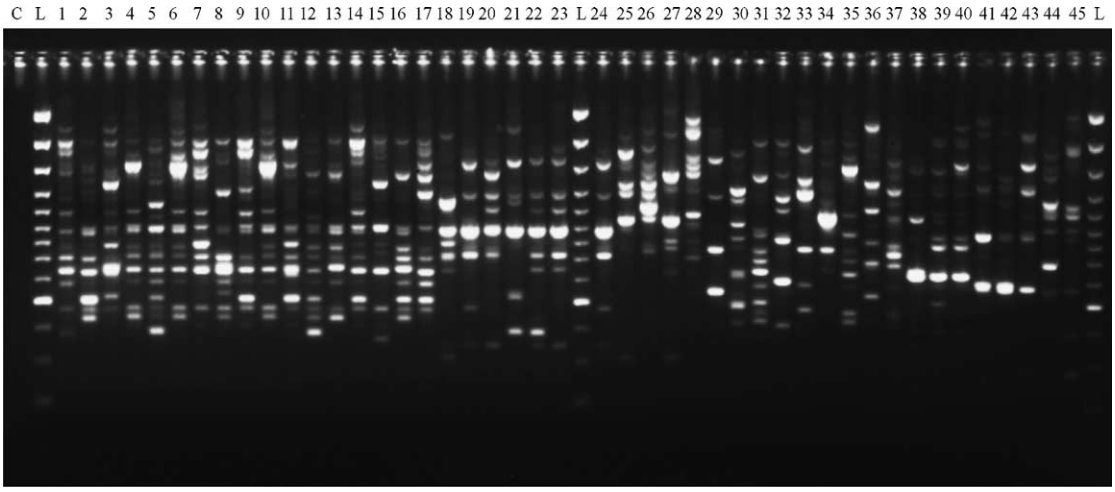

Fig. 2. Products of RAPD amplification, primer OPA3, 2% agarose gel. The particular lanes represent (C) negative control; (L) O'GeneRuler™ 100 bp DNA Ladder Plus (Fermentas, #SM0321); (1) A6; (2) PH18-9; (3) ZP-E2; (4) TX-B5; (5) 26-7; (6) TX-O10; (7) ZP-H3; (8) 12-7; (9) TUMK2; (10) TX-B1; (11) ZP-A2; (12) Z26-7; (13) HD; (14) CYG4; (15) CYG2; (16) TGR8; (17) 1-11-M2; (18) CYG; (19) Q7; (20) 12-6-7; (21) TX-O6; (22) KRA-1; (23) TUMS1; (24) Q9; (25) OAM5; (26) AA9-8; (27) M3; (28) AF; (29) GPO; (30) EUMM; (31) TGR9; (32) KAJ; (33) TV10-02; (34) TGK; (35) TX-B3; (36) KVc-1; (37) ABI; (38) T37; (39) DALM; (40) HYLA; (41) PH-KT; (42) KUDU22; (43) PDZB2; (44) L3; (45) BUB.

Trichomonas, *Tritrichomonas* and *Tetratrichomonas*. Considering such extensive polymorphism in *T. gallinarum* isolates, we suggest that branches D, E and A–B–C represent, at least, three independent species. According to our preliminary observations, representatives of these putative species differ in morphological phenotype and in their cultivation properties. However, the multi-species hypothesis has to be confirmed by a detailed morphological study before the new species are established.

While groups A–B–C include 32 isolates in total, the group D includes just two isolates and the E includes only one isolate. Several possible explanations of this unequal distribution could be suggested. Since our isolates were established at different places during last 30 years and most of them belong to the groups A and B, it is highly probable that these groups persistently predominate in domestic fowls. Possibly, the isolates of groups C, D and E originated from wild hosts and are able to infect domesticated birds only occasionally. Actually, most of the isolates obtained from wild hosts formed separate branches, as in cases of the isolates AF and 20-9-1 (group D), CYG2 (subgroup A1) and CYG (subgroup B1). On the other hand, isolates CYG4 and KRA-1 from wild swan and duck, respectively, fell directly among isolates obtained from domestic fowls. It is possible that even larger polymorphism than we observed exists in *T. gallinarum* populations from wild gallinaceous and anseriform birds. Therefore, there is a strong need to investigate molecular polymorphism of the genus *Tetratrichomonas* also in natural hosts obtained from natural habitats.

Analysis of rRNA is in principle a single locus method and a given gene tree may contradict the species tree. These analyses were also unable to resolve the relationship between groups A, B and C as well as the question about the relationships of isolates inside these groups and the exact position of human oral and bronchial tetratrichomonad isolates. These problems were resolved by the multilocus RAPD analysis. The obtained dendrogram topology was almost in perfect agreement with the topology of phylogenetic trees constructed from sequence data. RAPD analysis showed clearly that the groups B and C form distinct but related clades. The topology inside groups A and B was well supported and, except for the case of subgroups B2 and B3, consistent with results obtained from analyses of sequences.

The subgroup A2 divided into three lineages according to host specificity: A21 (galliform), A22 (human) and A23 (anseriform). The lineages differed

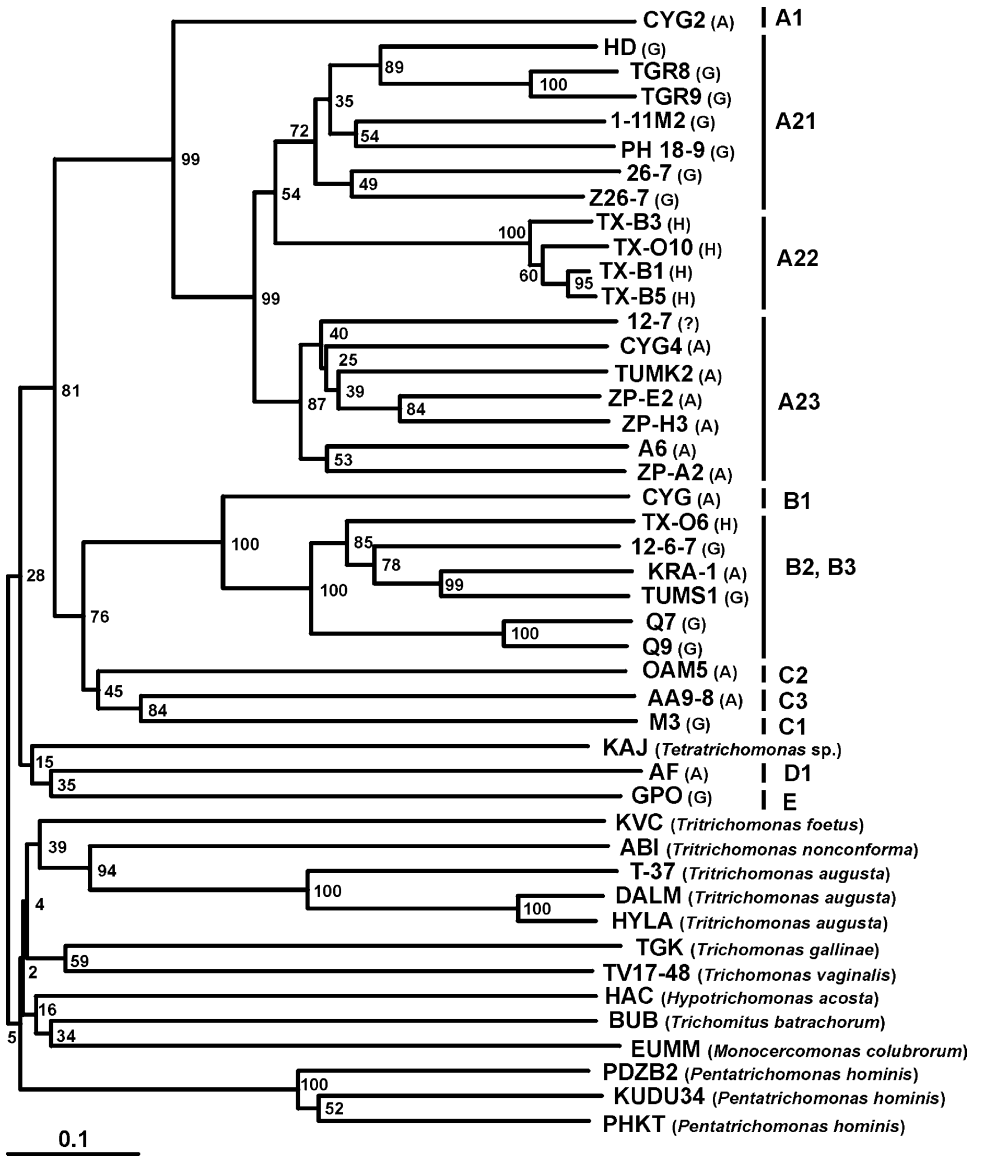

Fig. 3. Unrooted dendrogram based on a RAPD analysis of 45 trichomonad isolates. The dendrogram was constructed using Neighbor-joining method. Bootstrap values are shown at the nodes. For *T. gallinarum* isolates, host taxonomic position is listed: A, anseriform birds; G, galliform birds; H, humans; ?, unspecified domesticated bird. The scale bar represents 0.1 unit of Nei and Li's distance.

in a geographic distribution. Lineages A21 and A23 were sympatric (Czech Republic), while all isolates from the lineage A22 were obtained from a different restricted area (Estonia). As these lineages can be characterised not only by a common history of isolates revealed by our phylogenetic analysis, but also by such an important character as a host origin, they might probably represent three independent species. As a morphological study performed at this department (Kulda, 1965) revealed that isolates of the subgroups A21 and A23 were morphologically indistinguishable, these species are likely cryptic. Although there is a report on successful experimental infection of turkeys with the isolate A6 from the "anseriform" subgroup A23 (Kulda et al., 1974), the "galliform" and "anseriform" subgroups may be, in fact, isolated in

nature. The experimental transmission was achieved under unnatural conditions by intracloacal inoculation of a large inoculum of parasites.

The group B presented a different pattern with respect to host/isolate specificity. Again, the internal topology was well resolved but the branching did not correspond to isolates' origin. Because the group B contains only seven isolates and the group C contains only three isolates, we cannot reject that some host specific branches inside these groups also exist. However, differences in ranges of host spectra of various lineages, similar to those revealed by genotyping of *Cryptosporidium* and *Giardia* (Widmer, 1998; Monis et al., 2003) should be also taken into consideration.

We showed in this molecular study that *T. gallinarum* does not represent the single species, but rather is a species complex consisting of at least three putative ''morphospecies'', some of them possibly, including additional cryptic species. If the pathogenic phenotype are specifically associated with certain members of the complex only, our results will aid in explaining controversies regarding the *T. gallinarum* pathogenicity. Because the heterogeneity of *T. gallinarum* was not known when pathogenicity studies were performed, it is probable that different authors used different members of the *T. gallinarum* complex that might differ in their ability to cause pathologic manifestations. Unfortunately, most of the isolates used in reported experiments are not presently available for molecular characterisation. Thus, studies on *T. gallinarum* pathogenicity probably have to be repeated with better-characterised material.

4.2. Human tetratrichomonad isolates

All human oral and bronchial isolated included in this study were obtained as axenic cultures from professor J. Teras (Institute of Experimental Biology, Estonian Academy of Science, Tallin). All were isolated in Tallin, Estonia, within the period 1964–1977 in course of a study aimed at investigations on etiologic role of *Trichomonas tenax* in human lung pathologies (Teras et al., 1980). Morphological re-examination of these organisms, believed to be *Trichomonas tenax*, revealed that they all belonged to the genus *Tetratrichomonas* (Kutišová et al., sent to press). Our results convincingly showed their pertinence to the *T. gallinarum* complex and their placement inside the groups A and B was strongly supported by all methods. The position of the human tetratrichomonads in the avian groups A and B suggests that the infection of a human host probably occurred at two independent events.

Almost identical human isolates from the subgroup A2 formed an independent branch A22 with an uncertain relationship to the branches A21 from galliform birds and A23 from anseriform birds. The present results do not allow us to determine whether humans are being continuously infected by tetratrichomonads from unknown avian hosts that were not included in our study or whether these isolates represent stable and adapted human parasites. As probably at least two lineages from the *T. gallinarum* complex are able to infect humans' further search for human isolates and critical studies on zoonotic potential of avian tetratrichomonads are of utmost importance.

Acknowledgements

We wish to express our thanks to Z. Pecka for providing four axenic *T. gallinarum* isolates. The work was supported by Grants MSM113100004 and GAUK 140/2002/B.5.

References

Allen, E.A., 1941. Macroscopic differentiation of lesions of histomoniasis and trichomoniasis in turkeys. Am. J. Vet. Res. 2, 214–217.

Crespo, R., Walker, R.L., Nordhausen, R., Sawyer, S.J., Manalac, R.B., 2001. Salpingitis in pekin ducks associated with concurrent infection with *Tetratrichomonas* sp. and *Escherichia coli*. J. Vet. Diagn. Invest. 13, 240–245.

Delappe, I.P., 1957. Effect of inoculating the chicken and the turkey with a strain of *Trichomonas gallinarum*. Exp. Parasitol. 6, 412–417.

Diamond, L.S., 1957. The establishment of various trichomonads of animals and man in axenic cultures. J. Parasitol. 43, 488–490.

Diamond, L.S., 1982. A new liquid medium for xenic cultivation of *Entamoeba histolytica* and other lumen-dwelling protozoa. J. Parasitol. 68, 958–959.

Dobell, C., Leidlaw, P.P., 1926. On the cultivation of *Entamoeba histolytica* and some other entozoic amoebae. Parasitology 18, 283–318.

Felleisen, R.S., 1997. Comparative sequence analysis of 5.8S rRNA genes and internal transcribed spacer regions (ITS) of trichomonadid protozoa. Parasitology 115, 111–119.

Friedhoff, K.T., Kuhnigk, C., Müller, I., 1991. Experimental infection in chickens with *Chilomastix gallinarum*, *Tetratrichomonas gallinarum*, and *Tritrichomonas eberthi*. Parasitol. Res. 77, 329–334.

Goedbloed, E., Bool, P.H., 1962. The protozoan etiology of blackhead. Avian Dis. 6, 302–315.

Hadley, P.B., 1916. The avenue and development of tissue infection in intestinal trichomoniasis. Rhod. Isl. Agric. Exp. Station Bull. 168, 1–64.

Hampl, V., Vaňáčová, Š., Kulda, J., Flegr, J., 2001. Concordance between genetic relatedness and phenotypic similarities of *Trichomonas vaginalis* strains. BMC Evol. Biol. 1, 11.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Hegner, R., 1929. The infection of parasite-free chicks with intestinal protozoa from birds and other animals. Am. J. Hyg. 10, 33–62.

Innis, M.A., Myambo, K.B., Gelfand, D.H., Brow, M.A.D., 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. Proc. Natl. Acad. Sci. U.S.A. 85, 9436–9440.

Kemp, R.L., Reid, W.M., 1965. Pathogenicity studies on *Trichomonas gallinarum* in domestic poultry. Poult. Sci. 44, 215–221.

Kotlán, A., 1923. Zur Kenntnis der Darmflagellaten aus der Hausente und anderen Waasevögeln. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. Orig. Reihe A 90, 24–28.

Kulda, J., 1965. Study on morphology, cultivation and testing virulence of flagellates of the family Trichomonadidae. PhD Thesis, Charles University in Prague.

Kulda, J., Suchánková, E., Svoboda, S., 1974. Studies on pathogenicity of *Tetratrichomonas gallinarum* in mice and turkey poults. Acta Vet. Brno. 43, 53–64.

Lee, D.L., 1971. Changes in the ultrastructure of the cecum of chickens caused by *Trichomonas gallinarum*. Parasitology 65, 71–76.

Martin, C.H., Robertson, M., 1911. Further observations on the caecal parasites of fowls, with some reference to the rectal fauna of other vertebrates Part 1. Q. J. Microsc. Sci. 57, 53–82.

McDowell Jr., S., 1953. A morphological and taxonomical study of the caecal protozoa of the common fowl *Gallus gallus*. J. Morphol. 92, 337–399.

Mohan, R.N., Sharma, K.N., Mehrotra, M.L., 1966. *Tetratrichomonas gallinarum* in the blood of chickens infected with spirochetes. J. Protozool. 13, 341–344.

Monis, P.T., Andrews, R.H., Mayrhofer, G., Ey, P., 2003. Genetic diversity within the morphological species *Giardia intestinalis* and its relationship to host origin. Infect. Gen. E 3, 29–38.

Nei, M., Li, W.H., 1979. Mathematical model for studying genetic variation of restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. 76, 5269–5273.

Norton, R.A., 1997. Pathogenicity of a strain of *Trichomonas gallinarum* in turkeys and its possible interaction with cecal coccidia. Avian Dis. 41, 670–675.

Patton, C.S., Patton, S., 1996. *Tetratrichomonas gallinarum* encephalitis in a mockingbird (*Mimus polyglottos*). J. Vet. Diagn. Invest. 8, 133–137.

Pavlíček, A., Hrdá, Š., Flegr, J., 1999. FreeTree—freeware program for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of the tree robustness. Application in the RAPD analysis of the genus *Frenkelia*. Folia Biol. (Praha) 45, 97–99.

Pecka, M., 1991. Pathogenicity of *Tetratrichomonas gallinarum*. Vet. Med. (Praha) 36, 183–188.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Pramanick, D.J., Forstová, J., Pivec, L., 1976. Guanidine hydrochloride applied to the isolation DNA from different sources. FEBS Lett. 62, 81–84.

Richardson, F.L., 1934. Studies on experimental epidemiology of intestinal protozoan infections in birds. Am. J. Hyg. 20, 373–403.

Swofford, D.L., 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4, Sinauer Associates, Sunderland, Mass.

Tanabe, M., 1926. Morphological studies on *Trichomonas*. J. Parasitol. 12, 120–130.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows preface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Teras, J., Roigas, E., Kazakova, I., Ranne, H., Trapido, L., Sardis, H., Kaal, V., 1980. Finding of trichomonads in bronchii, sputum and oral cavity of patients with various lung pathologies. Terapeut. Arkhiv. 52, 123–125 (in Russian).

Travis, B.V., 1932. *Trichomonas phasiani*, a new flagellate from the ring-necked pheasant *Phasianus torquatus* Gmelin. J. Parasitol. 18, 285–287.

Tyzzer, E.E., 1919. Developmental phase of the protozoan of the "blackhead" in turkeys. J. Med. Res. 40, 1–30.

Vaňáčová, Š., Tachezy, J., Kulda, J., Flegr, J., 1997. Characterization of trichomonad species and strains by PCR fingerprinting. J. Euk. Microbiol. 44, 545–552.

Widmer, G., 1998. Genetic heterogeneity and PCR detecion of *Cryptosporidium parvum*. Adv. Parasitol. 40, 233–239.