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Characterization of Trichomonad Species and Strains by PCR Fingerprinting

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ABSTRACT. The random amplified polymorphic DNA (RAPD) technique was used for phylogenetic analysis of trichomonads, for intraspecies genealogical study of *Trichomonas vaginalis* strains, and for assessment of intrastrain polymorphism in *Trichomonas vaginalis*. The phylogenetic tree for 12 trichomonad species showed certain discrepancies with current models of trichomonad evolution. However, it shows that RAPD traits retain phylogenetically relevant information. The results of intraspecies analyses of 18 *Trichomonas vaginalis* strains suggested some concordance between the genetic relationship of strains and their geographic origin. They also suggested a concordance between the strain genetic relationships and the resistance to metronidazole. A concordance was also found with respect to the severity of disease observed in donor patients but not with the results of laboratory virulence assays. No concordance was found between genetic relationship of strains and strain infection with a dsRNA *Trichomonas vaginalis* virus (TVV). The latter suggests that TVV might be transmitted horizontally among *Trichomonas vaginalis* populations. The identity of RAPD patterns of clones isolated from in vitro cultures and those of the cultures reisolated independently from the same patient within a period of six weeks suggests that individual *Trichomonas vaginalis* strains are not polymorphic and that the RAPD patterns are stable. Therefore, the RAPD technique seems useful for addressing various clinically relevant issues.

Supplementary key words. Drug resistance, dsRNA virus, phylogeny, polymorphism, RAPD, *Trichomonas*, Trichomonas Vaginalis Virus, *Tritrichomonas*, virulence.

TRICHOMONADS, flagellated amitochondrial anaerobic protists, branch very early in the eukaryotic phylogeny. The group includes several important parasitic species of humans and animals. Despite the medical and veterinary importance of these flagellates and their key role in the understanding of the eukaryotic evolution processes, relatively little effort has been invested into molecular studies of trichomonad diversity. Sequencing of large subunit rRNA genes has been used to construct a phylogenetic tree for nine trichomonad species [43]. Small subunit rRNA sequences have been used to infer the phylogenetic position of trichomonads among the eukaryotes [18], and to infer relationships of species within the group; including three species of symbiotic trichomonads from termites [3, 4] and the morphologically simple human pathogen, *Dientamoeba fragilis* [36]. For more extensive phylogenetic studies and for the studies of intraspecies diversity, a cheaper and high-capacity methods like RFLP (restriction fragment length polymorphism) and RAPD should be developed. The high content of repetitive elements in the genome of many of trichomonad species [32] offers the possibility to use whole-genome-RFLP analysis. However, the evolution of repetitive elements may be influenced by molecular drive, which may obscure the results of phylogenetic studies. The number of random primer-matches correlates with a genetic complexity of the target DNA and is much higher for an unique fraction than for a repetitive fraction of a genome. Therefore, RAPD data reflect mainly the evolution of unique sequences. It is not clear, however, whether rapidly evolving RAPD traits contain phylogenetically relevant information in this ancient and highly divergent taxon of clonal or semiclonal organisms. In this communication we analyze interspecies, intraspecies and intrastrain polymorphism of trichomonads by PCR-fingerprinting with random primers.

MATERIALS AND METHODS

Organisms. Eighteen representatives of the family Trichomonadidae, including members in the subfamilies Trichomonadinae and Tritrichomonadinae, were used for interspecies phylogenetic analysis (Table 1). Table 2 provides information on 18 strains of *Trichomonas vaginalis* selected for intraspecies genealogical analysis. All organisms used are deposited in the culture collection of the Department of Parasitology, Charles University, Prague.

Cultivation of trichomonads. Cultures of all organisms, except that of HC 138 (an unidentified trichomonad from *Hylobates concolor*) were initiated from frozen stabilates cryoprotected with 5% dimethyl sulfoxide and grown in Diamond's TYM medium [8] supplemented with 5% heat inactivated horse serum. The pH of the medium was adjusted to pH 6 for *Trichomonas vaginalis* or pH 7 for all other organisms. Trichomonads from mammals and birds were maintained at 37° C, those from amphibians and reptiles at 26° C. The last three subcultures before harvesting were grown in TYM medium without agar. Cells were harvested in the late logarithmic phase at an approximate density of 1–3 × 10^6 cells/ml. All these cultures were axenic.

The HC 138 strain was maintained in xenic culture in TYSGM medium [9] without mucin. Before harvesting, the organisms were introduced into agar-free TYM medium containing 2,000 IU/ml penicillin and 500 μg/ml streptomycin sulfate. Two transfers under these conditions reduced the number of accompanying bacteria such that no bacteria were observed microscopically in cell suspensions used for DNA isolation.

DNA isolation. Nucleic acids were isolated using a modified guanidium chloride method [34]. Briefly, 2–8 × 10^7 cells were resuspended in 200 μl of isotonic saline and mixed with an equal volume of 8 M guanidium chloride. To remove proteins, two volumes of chloroform-isoamyl alcohol (24:1) were added. The mixture was vortexed for 5 min followed by centrifugation at 10,000 *g* for 5 min. The aqueous phase was recovered and DNA precipitated with the addition of 0.8 volumes of isopropanol (−20° C overnight). The DNA pellet, obtained after 15 min centrifugation at 12,000 *g*, was dried and resuspended in 30 μl of 0.01 M Tris buffer with 0.001 M EDTA, pH 8.0.

Virulence. Clinical and laboratory data allowing the assessment of strain virulence were available for ten *T. vaginalis* strains (Table 3).

Pathogenic effects on donor female patients were assessed by clinical and histopathological findings and rated according to increasing severity by four arbitrary units characterized in reference [22].

Virulence for mice was evaluated by the following methods: (a) Subcutaneous mouse assay [19] based on measurement of six day abscesses resulting from subcutaneous administration with 8 × 10^5 trichomonads to males of inbred C57BL/6 mice (18–20 g) as described in reference [24]. Abscesses were measured on day six after inoculation; (b) Mortality of mice expressed as percentage of mice that died within three weeks after intraperitoneal inoculation of 10^6 trichomonads; (c) The mean virulence index of Cavier [5] based on rating lesions in abdominal organs and quantity of ascites fluid after intraperitoneal inoculation of 10^6 trichomonads. Male outbred "H" mice (18–20 g) with the genetic background of the A strain produced by SEVAC (Prague) were used for intraperitoneal assays.

All mouse assays are described in detail in [22] (page 148).

Metronidazole resistance. The susceptibility of *T. vaginalis* strains to metronidazole was determined in vitro using a microtitre plate assay [40]. The trichomonads were exposed to two-fold serial dilutions of metronidazole in the presence of air for 48 h at 37° C. Two strains not surviving air exposure in plates were tested by a tube assay [39]. The minimal lethal concentration (MLC) was determined microscopically as the lowest dilution of metronidazole in which no motile organisms were observed.

Presence of dsRNA. The presence of dsRNA virus was assayed by 1% agarose gel electrophoresis of total nucleic acid extracts. The double stranded RNA nature of the satellite band was confirmed by RNase treatment of the extracts in low and high ionic strength buffers before electrophoresis [15].

RAPD analysis. DNA was amplified in 25 μl of buffer with 5 pmol primer, 2.5 mM $MgCl_2$ (MBI, Fermentas), 200 μM of each dNTP, PCR reaction buffer for Taq polymerase (MBI, Fermentas), and 1.75 units of Taq DNA polymerase (MBI, Fermentas). The reactions were performed in 0.5 ml microtubes overlaid with mineral oil (Perkin Elmer) in a PHC 3 thermocycler (TECHNE). The amplification profile consisted of an initial denaturation step at 94° C for 5 min followed by 40 repetitions of 1 min at 94° C, 1 min at 36° C (for random primers) or 38° C (for TV primers) and 2 min at 72° C (15 min at 72° C after the last cycle). PCR products were analyzed by electro-

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Table 1. List of trichomonad strains.

Species and strain	Host-localization	Locality	Isolation	Reference
Trichomonas vaginalis Tv10-02	Human—vagina	Prague, Czech Republic	Kulda 1973	[22]
Trichomonas vaginalis Tv17-48	Human—vagina	Prague, Czech Republic	Kulda 1973	[22]
Trichomonas vaginalis RU-375	Human—vagina	USA, unspecified	Obtained from Alderete 1987	[33]
Trichomonas vaginalis CP-1	Human—vagina	Peking, China	Tachezy 1987	
Trichomonas tenax TXO-8	Human—oral cavity	Tallin, Estonia	Teras 1975	[41]
Trichomonas tenax TXB-5	Human—bronchi	Tallin, Estonia	Teras 1977	[41]
Trichomonas gallinae TGK	Pigeon *Columba livia*—crop	Prague, Czech Republic	Tachezy 1995	—
Tetratrichomonas gallinarum A6	Duck *Anas platyrhynchos*—cecum	Uhlířské Janovice, Czech Republic	Kulda 1970	[23]
Pentatrichomonas hominis HOM-V4	Human—large intestine (feces)	Da-Nang, Vietnam	Tolarová, Kulda 1988	—
Pentatrichomonas hominis HOM-V5	Human—Large intestine (feces)	Da-Nang, Vietnam	Tolarová, Kulda 1988	—
Trichomitus batrachorum BUB	Toad *Bufo bufo*—cloaca	Ruda, Czech Republic	Kulda 1983	—
Tritrichomonas foetus Kvc-1 (ATCC 30924)	Bull *Bos taurus*—preputial washing	Žalmanov, Czech Republic	Lípová, Kulda 1957	[23]
Tritrichomonas foetus LUB	Heifer *Bos taurus*—rectum	Lublin, Poland	Stepkowski 1965	—
Tritrichomonas suis SV-113b	Pig *Sus scrofa*—cecum	Halle, Germany	Kulda 1988	—
Tritrichomonas mobilensis M776 (ATCC 50116)	Squirrel monkey *Saimiri boliviensis boliviensis* rectal swabs	Mobile, Alabama, USA	Pindak 1984 obtained from Culberson 1989	[7]
Tritrichomonas augusta LV2	Lizard *Lacerta vivipara*—cloaca	Šumava mt., Czech Republic	Kulda 1992	—
Tritrichomonas nonconforma R114	Lizard *Anolis bartschii*—cloaca	Viñales, San Vincente, Cuba	Kulda 1965	—
Unidentified species HC 138	Gibbon *Hylobates concolor*—feces	ZOO, Ústí n L., Czech Republic	Kadlec 1988	—

Table 2. List of *Trichomonas vaginalis* strains.

Strain	dsRNA	Geographic origin	Isolation	Reference
Tv7-37	–	Prague, Czech Republic	Kulda 1973	[25]
Tv10-02	+	Prague, Czech Republic	Kulda 1973	[25]
Tv14-85	–	Prague, Czech Republic	Kulda 1973	[25]
Tv67-77	–	Prague, Czech Republic	Kulda 1973	[25]
Tv71-96	+	Prague, Czech Republic	Kulda 1973	—
Tv73-87	+	Prague, Czech Republic	Kulda 1973	—
Tv79-49	+	Prague, Czech Republic	Kulda 1973	—
Tv85-08	+	Prague, Czech Republic	Kulda 1973	—
FF 28	+	Bratislava, Slovakia	Demeš 1987	—
HL-4MT	–	Liberec, Czech Republic	Těmín 1986	—
IR-78 (ATCC 50138)	–	Vienna, Austria	Meingassner 1978	[28]
BO	+	Gothenburg, Sweden	Forsgren 1978	[17]
TALL-MT	–	Tallin, Estonia	Tompel 1987	—
CDC-85 (ATCC 50143)	–	Columbus, Ohio, USA	Lossick 1980	[30]
JH-31A (ATCC 30236)	–	Baltimore, Maryland, USA	Hollander 1963	[25]
C-1:NIH (ATCC 30001)	+	Washington, D.C., USA	Jacobs 1956	[35]
CP-1	–	Peking, China	Tachezy 1987	—
JT	–	Rio de Janeiro, Brazil	Silva Filho 1982	[37]

Table 3. Virulence of *Trichomonas vaginalis* strains as assessed on the basis of clinical findings, histopathological examination of ectocervical biopsies and results of mouse assays after intraperitoneal and subcutaneous inoculation.

Strain	Intraperitoneal mouse test			Subcutaneous mouse test		Patients[d]	
	Mortality (%)[a]	Virulence[b]	n	Abscess (mm³)[c]	n	Symptoms	Histopathology
Tv85-08	50	7.7	20	132	38	2	2
Tv73-87	43	10.4	30	66	31	2	2
Tv71-96	24	6.0	37	121	38	2	3
Tv79-49	73	10.7	30	176	65	1	1
Tv14-85	86	10.8	30	127	29	4	3
Tv67-77	47	8.7	30	63	40	2	2
Tv10-02	0	3.7	20	79	37	1	1
Tv7-37	90	13.4	30	230	41	4	4
FF28	75	7.6	12	117	28	0	nd
JH-31A	nd	nd	nd	78.6	49	0	0

[a] Percent of inoculated mice that died within three weeks after inoculation.

[b] Virulence index of Cavier et al. [5] ranges from 0 (avirulent) to 16 (maximum virulence).

[c] Mean volumes of subcutaneous abscesses that developed six days after inoculation.

[d] Pathological changes found at gynaecological and histopathological examinations rated according to increasing severity from 1 to 4 as indicated in [22].

phoresis in 1.2% agarose and visualized with UV light after ethidium bromide staining. All primers tried (up to now 20 random primers OPD1-OPD20 and four Tv specific primers have been used in different trichomonads studies) provided distinct patterns of amplified DNA fragments. The number of fragments and their interspecies and intraspecies polymorphism varied. In our study we used five random primers from Operon Technologies Inc. (OPD1–OPD5, ACCGCGAAGG, GGACCCAACC, GTCGCCGTCA, TCTGGTGAGG, TGAGCGGACA) and two primers complementary to the *T. vaginalis* repetitive element TV E-650 [32] (TV1 GAAGATCTAATGTTTGATGTGAA and TV3 GTATAATAAGCTTTATTATCTAGAAG) that provided moderate numbers of DNA bands with a high amount of polymorphism. DNA from all 18 strains or all 12 trichomonad species was amplified with a particular primer in one experiment (aliquots of reaction mixture without the DNA were distributed into tubes containing either DNA samples or TE buffer (negative control)). The amplification with one primer was repeated several times with different concentrations of the DNA (20–100 ng). The PCR fragment was scored as absent in the given OTU (operational taxonomical unit) if the band of the same electrophoretic mobility did not appear after the amplification with any concentration of DNA. The relative amount of amplified DNA (intensity of electrophoretic bands) was not considered.

Genetic polymorphism analysis. Genetic distances between DNA samples were computed from Nei's coefficients of similarity ($S = 2 \times Mxy / (Mx + My)$, where Mxy, Mx and My are the numbers of common fragments between sample x and y, number of all fragments (common and unique) of sample x and of sample y, respectively) corrected for multiple hits using the iteration method originally designed for analysis of RFLP data [31]. According to our experience, phylogenetic trees constructed on the basis of corrected distances (using the formula $k = -(2 / r) \times \ln G$, where k is the corrected distance, r is the length of a typical random primer (ten nucleotides) and G is a probability that a matching site for a primer remained unaltered (computed by the iteration method)) showed greater support for topological elements than trees constructed from uncorrected Nei's coefficients. The r (the length of a target site) is probably lower than ten nucleotides because the priming can occur at matches comprising as few as five matches out of six bases at the 3′ end of the primer [27]. However, at present, we prefer to use the value ten, the most conservative estimation of the parameter r. The MEGA program was used to construct phylogenetic trees from the distance matrices by the Neighbor Joining (NJ) method [26]. Support for topological elements was estimated by OTU-based Jackknifing (N reduced trees were constructed for N − 1 OTU by sequentially leaving out 1 OTU from the original set, where N = the number of taxa in the data set). The Jackknife value for a branch represents the fraction (in percent) of reduced trees containing that particular branch. Distance matrices, including resampled data-based matrices for OTU-Jackknife analyses were calculated using the RAPDIST program, which is available by anonymous FTP in the folder www.natur.cuni.cz/~flegr/programs..

Statistical testing of trees concordance. The correspondence of geographic origin and biological properties of *T. vaginalis* strains (resistance for metronidazole, virulence, and presence of dsRNA virus) with their position in the tree was estimated by a permutation tail probability test [1]. For any parameter studied, the average distance between sister OTU (i.e. sister strains or sister branches of the tree) was calculated from the genealogical tree obtained from RAPD data by NJ method. Then 20,000 trees were generated by random permutation of apical branches. For every permutated tree the average distance between sister OTU was calculated and these distances were compared with the average distance of the inferred genealogical tree. If the average distance of the inferred genealogical tree fell among the shortest 5% of lowest distances of permutated trees, we considered correspondence of particular biological property with the position of the strain in the genealogical tree statistically significant. In the metronidazole resistance study, the sensitivities of strains were characterized by logarithms of minimal lethal concentration (MLC) of the drug, in the dsRNA study the presence of dsRNA was treated as one and its absence as zero, and in the virulence study five different indexes of pathogenicity (see Table 3) were used. The distances between two OTU in the metronidazole resistance, dsRNA and virulence studies were calculated as the absolute value of the difference of logarithms of MLC or numbers characterizing the presence of dsRNA or virulence in two compared strains (or arithmetic means of these numbers in the cases of composite OTU).

In the geographical origin-study a matrix of nonparametric distances between the sites of origin for every pair of strains was prepared. The distances for strains isolated in the same city, same country, same continent and different continents were considered as 1, 2, 3 and 4, respectively. Our PC program TREEPT for permutation tail probability testing can be obtained at www.natur.cuni.cz/~flegr/programs.

RESULTS

DNA was extracted from 18 isolates of 12 different species of trichomonads and amplified with five different random primers and two *Trichomonas vaginalis* specific primers. All primers provided distinct patterns of amplified DNA fragments. There was no difference in the number and length distribution of the PCR bands or in the amount of revealed polymorphism between the random primers and *T. vaginalis* specific primers. The average number of fragments per sample and the average genetic distance between two species were 9.0 and 0.046, respectively. A dendrogram constructed on the basis of the RAPD data by

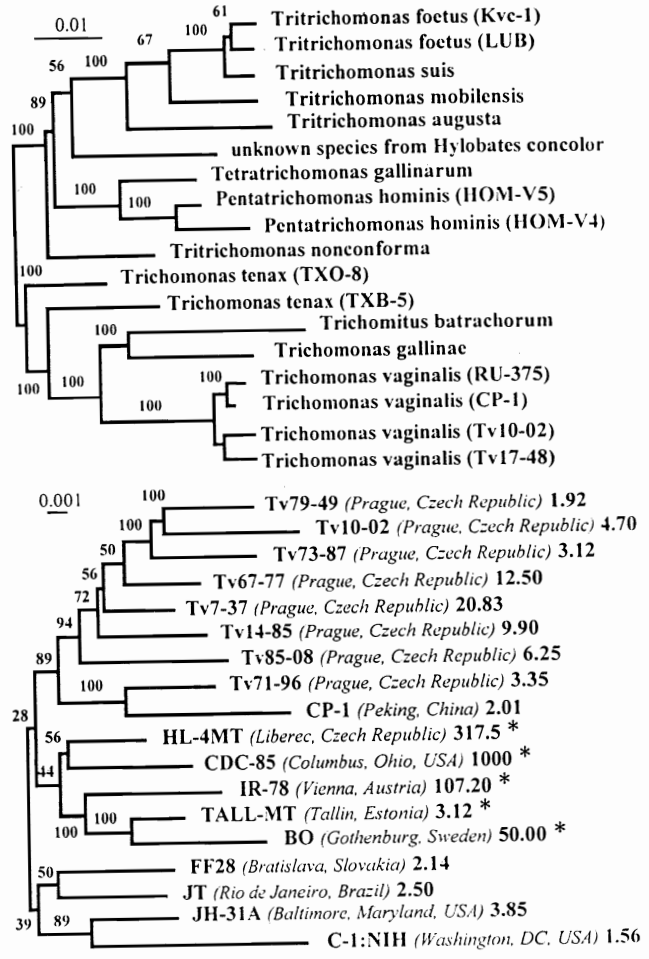

Fig. 1. Phylogenetic trees constructed by Neighbor Joining method. **A**. Phylogenetic tree for 11 species of trichomonads. The branch lengths reflect the genetic distance between the species. The numbers show the OTU based Jackknifing values (in percent) which reflect the statistical support for the existence of particular branches. The strain descriptions are given in the Methods section. **B**. The genealogical tree for 18 strains of *Trichomonas vaginalis*. The branch lengths reflect the genetic distance between the strains. The integer numbers represent the OTU based Jackknifing values (in percent) which reflect the statistical support for the existence of particular branches. At the end of apical branches the geographical origins and the values of aerobic minimal lethal concentration (MLC) for metronidazole are listed. The asterisks designate the strains which were refractory to standard metronidazole treatment.

Fig. 2. Results of PCR-fingerprinting. **A**. RAPD electrophoretic patterns of 18 strains of 12 trichomonad species. DNA isolated from *Trichomitus batrachorum* (BUB), *Trichomonas vaginalis* (RU-375), *Trichomonas vaginalis* (CP-1), *Trichomonas vaginalis* (Tv10-02), *Trichomonas tenax* (TXO-8), *Trichomonas tenax* (TXB-5), *Tritrichomonas foetus* (CB-1), *Trichomonas gallinae* (TGK), *Tritrichomonas foetus* (Kvc-1), *Tritrichomonas foetus* (LUB), *Tritrichomonas suis* (SV-H3b), *Tritrichomonas augusta* (LV2), *Tritrichomonas mobilensis* (M776), *Tritrichomonas nonconforma* (R-114), *Tetratrichomonas gallinarum* (A6), *Pentatrichomonas hominis* (HOM-V5), *Pentatrichomonas hominis* (HOM-V4) and the unknown species isolated from *Hylobates concolor* (HC 138) were amplified with the random primer OPD-04 and electrophoresed in the lanes 2–19. The lanes 1 and 20 contain molecular weight standard (λ phage DNA digested with Eco911 and pBR322 DNA digested with AluI). **B**. RAPD electrophoretic patterns of 18 strains of *Trichomonas vaginalis*. DNA isolated from strains Tv85-08, Tv73-87, Tv71-96, Tv79-49, Tv14-85, Tv67-77, Tv10-02, Tv7-37, CP-1, TALL-MT, BO, HL-4MT, IR-78, FF-28, CDC-85, C-1:NIH, JH-31A, and JT were amplified with the random primer OPD-02 and electrophoresed in the lanes 2–19. The figure only illustrates a general outlook of *T. vaginalis* strains RAPD patterns. The similarities of particular one-primer lane patterns may not correlate with the phylogenetic relationships between the strains estimated on the basis of whole set of primers. The lanes 1 and 20 contain molecular weight standard (λ phage DNA digested with Eco911 and pBR322 DNA digested with AluI). **C**. RAPD electrophoretic patterns of eight clones of *Trichomonas vaginalis* strain TALL-MT. DNA isolated from single-cell clones K1–K7 was amplified with the random primer OPD-03 and electrophoresed in 1.2% agarose (lines 2–8). The lanes 1 and 10 contain molecular weight standard (λ phage DNA digested with PstI). Lane 9 contains amplified DNA from the uncloned strain TALL-MT.

pattern and phenotype of the strains. Therefore it can be assumed that the similarity of RAPD patterns reflects phylogenetic relationship, rather than phenetic similarity between the strains. The similarity of RAPD patterns of strains isolated from proximal areas is evidence for high viscosity of *T. vaginalis* (and human) populations rather than evolutionary convergence due to similar selective pressures.

Of special interest is the relatively long branch of *T. vaginalis* strain C-1:NIH. The UPGMA phenetic method separates this strain from all other analyzed strains of *T. vaginalis* (results not shown). This strain is very often used in various biochemical studies (probably because of its position on the top of the ATCC list of *T. vaginalis* strains). It has been observed that the biological properties of this strain differ from typical *T. vaginalis* strains. According to our experience, the generation time of C-1:NIH is unusually short, the organisms are very motile, they grow throughout the volume of cultivation medium and they show poor adherence. The strain also differs in certain enzymatic activities; for example it shows an extremely low activity of NADH oxidase, an important oxygen scavenging enzyme in trichomonads [29]. Considering these observations and the results of our RAPD analyses the *T. vaginalis* strain C-1:NIH may not be the best choice for use as a reference strain.

The lack of correlation between the presence/absence of dsRNA virus (TVV) and the position of the strains in the *T. vaginalis* tree suggests that the distribution of TVV does not reflect the genealogy of *T. vaginalis* strains. It means that the *T. vaginalis* acquired or lost the virus independently several times during its history. Loss of the TVV after prolonged in vitro cultivation has been reported [45]. However, most of our strains were assayed for dsRNA a few passages after axenization. Therefore, our results likely reflect a distribution of TVV in natural populations. The random distribution of TVV within the *T. vaginalis* tree provides an indirect indication that TVV can probably infect virus free strains. Experimental data [6, 44] suggest that horizontal transmission of TVV is not possible. However, the TVV can have a heteroxenous life cycle with a reservoir host different from *T. vaginalis*. In this context the remarkable similarity between TVV and killer factors of yeast *Saccharomyces cerevisiae* [13, 14] is noteworthy.

At present we are reserved in emphasizing the concordance between resistance to metronidazole or severity of clinical and histopathological findings in patients with the strains position of the strains on the *T. vaginalis* tree because of the relatively low number (18) of strains examined. It is impressive, however, that all five strains isolated from patients refractory to metronidazole treatment constitute one branch of the tree. Four of these strains also express in vitro values of aerobic MLC indicative of metronidazole resistance. The fifth strain TALL-MT was susceptible to metronidazole in our in vitro assay. This may be due to the maintenance of this strain in active culture for a long time before testing (the other strains were cryopreserved shortly after they were axenized). Even the TALL-MT strain did show in vitro metronidazole resistance when first isolated (Dr. Teras, Institute of Biology, Estonian Academy of Sciences, personal communication). The results presented here suggest that some genealogical lines of *T. vaginalis* may be genetically predisposed for the development of metronidazole resistance. The same may be true for the capability of strains to cause disease in patients.

The identity of RAPD patterns of clones derived from a common strain indicates that there is very limited, if any, polymorphism within *T. vaginalis* in vitro cultures. Also, the analysis of multiple reisolates obtained from a single patient shows high in vivo stability of RAPD patterns. These results indicate that RAPD analysis may be useful in epidemiological studies. For example RAPD analysis may be used to trace the spread of infection by a particular strain or to distinguish between one-source and multi-source infections.

PCR-fingerprinting methods [46, 47] have predominantly been used for intraspecies or intrageneric studies. Our results confirm that the RAPD method indeed provides valuable genealogical data for intraspecies study of trichomonads. The results also suggest that RAPD traits retain phylogenetically relevant information in this phylum, an ancient and highly divergent group of clonal or semiclonal organisms. Considering the lack of other high capacity multi-locus molecular phylogenetic methods, the usefulness of PCR-fingerprinting techniques should not be underestimated.

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