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Short communication

Determination of internal transcribed spacer regions (ITS) in *Trichomonas vaginalis* isolates and differentiation among *Trichomonas* species

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ABSTRACT

The nucleotide sequence of the 5.8S rRNA gene and the flanked internal transcribed spacer (ITS) regions of six *Trichomonas vaginalis* isolates with different metronidazole sensitivity and geographic origin were genotyped. A multiple sequence alignment was performed with different sequences of other isolates available at the GenBank/EMBL/DDBJ databases, which revealed 5 different sequence patterns. Although a stable mutation in position 66 of the ITS1 (C66T) was observed in 26% (9/34) of the *T. vaginalis* sequences analyzed, there was 99.7% ITS nucleotide sequence identity among isolates for this sequence. The nucleotide sequence variation among other species of the genus *Trichomonas* ranged from 3.4% to 9.1%. Surprisingly, the % identity between *T. vaginalis* and *Pentatrichomonas hominis* was ~83%. There was >40% divergence in the ITS sequence between *T. vaginalis* and *Tritrichomonas* spp., including *Tritrichomonas augusta*, *Tritrichomonas muris*, and *Tritrichomonas nonconforma* and with *Tetratrichomonas prowazeki*. Dendrograms grouped the trichomonadid sequences in robust clades according to their genera. The absence of nucleotide divergence in the hypervariable ITS regions between *T. vaginalis* isolates suggests the early divergence of the parasite. Importantly, these data show this ITS1-5.8S rRNA-ITS2 region suitable for inter-species differentiation.

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1. Introduction

Trichomonas vaginalis is the causative agent of trichomonosis, the number one, nonviral sexually transmitted infection (STI) worldwide with an annual incidence of more than 170 million cases [1]. Infection by this protist produces a wide range of adverse clinical outcomes, such as cervical [2] and prostate cancer [3], and infertility or atypical pelvic inflammatory disease [4]. Additionally, trichomonosis also increases the predisposition to HIV [5,6]. In pregnant women, this STI is significantly associated with premature labor, low-birth-weight infants and premature rupture of the placental membranes [7].

The *T. vaginalis* isolate G3 has a genome of ~160 Mb with 65% of repeated and transposable elements as well as different proteins with domains homologous with those of bacteria, viruses, and protozoa [8]. The ribosomal genes, in particular the DNA region encoding for the 18S ribosomal RNA gene (18S rRNA), are considered one of the main genetic markers used for phylogenetic analysis due to its slow evolution and conserved nature. The rRNA genes are essential for protein synthesis and ribosome generation and are more conserved within species than the non-transcribed spacers [9]. Internal transcribed spacers (ITS) are more variable and, therefore, may have utility for species differentiation [10,11] or even for isolate differentiation among some parasites [12,13] as ITS regions are removed via splicing during transcript processing [9]. ITS1 separates 18S rRNA from 5.8S rRNA, while ITS2 separates 5.8S rRNA from 28S rRNA. Many reports have been focused on improving the sensitivity of diagnostic methods targeting the 18S rRNA gene of *T. vaginalis* [14]. Some reports have analyzed the nucleotide sequences of the ITS1-5.8S rRNA-ITS2 to characterize phylogenetically the parabasalids [14–16]. Nonetheless, few studies have been conducted to determine the utility of the ITS region as a molecular tool for *T. vaginalis* inter- and intra-species differentiation. There is a paucity of reports studying the ITS1 region to search the intragenomic variation of parabasalid protozoa like *T. vaginalis*, as evidenced by a report on *Dientamoeba fragilis* [17].

As there have been few studies overall on the *T. vaginalis* ITS loci, we felt it to be important to determine whether the ITS1-5.8S rRNA-ITS2 genomic region is a useful tool for differentiation among *T. vaginalis* isolates themselves and for distinguishing *T. vaginalis* from other *Trichomonas* species. We, therefore, conducted a comparative analysis of the nucleotide sequences obtained from *T. vaginalis* isolates with those of other related species obtained from the GenBank/EMBL/DDBJ

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databases. A parallel sequencing using the conserved 18S region was performed in order to compare and support the phylogenetic inferences obtained with the ITS results. The *T. vaginalis* alignments showed 5 different sequence types with only one nucleotide divergence in the ITS regions consistent with other studies [18]. However, a constant point mutation was observed at nucleotide position 66 (C/T) of the ITS1 region in 26% (9/34) of the sequences analyzed, which was identified in earlier studies [18–21], indicating stability of this mutation. Moreover, the phylogenetic studies show the ITS1-5.8S rRNA-ITS2 locus is suitable for the study of variability at an inter-species level. The significance of our results is discussed.

2. Materials and methods

2.1. Parasites

T. vaginalis isolates used for DNA extraction were cultivated at 37 °C and 5% CO_2 in TYM medium supplemented with 10% heat-inactivated bovine serum and antibiotics. The *T. vaginalis* isolates 1807, 1232, and 11 were obtained from women patients attending health centers of Madrid. *T. vaginalis* isolates C-1:NIH (Ref. no. 30001) and JH31A#4 (Ref. no. 30236) were sensitive to metronidazole and tinidazole when tested in our laboratory. The isolate IR 78 (Ref. no. 50138) is resistant to metronidazole [22]. Except for the Spanish isolates, the others were obtained from the American Type Culture Collection (ATCC) and are considered as reference strains [22].

2.2. DNA extraction

The *T. vaginalis* isolates were grown to mid- to late-logarithmic phase for extraction of genomic DNA using the Speedtools DNA Extraction Kit (Biotools, Spain) using the manufacturer's recommendations.

2.3. PCR amplification and sequencing

The 18S rRNA region was amplified using the primers 18SF (forward primer 5′-ACG CCG TAG TCT GAA TTG GC-3′) and 18SR (reverse primer 5′-AGA CAG GTC AAC CCA CGC AC-3′) that were designed after analysis of published sequences of the 18S rRNA encoding region of *T. vaginalis* (GenBank ID, U17510). The published primers Tv1 and Tv2 were also used in the amplification of the 18S rRNA contig [14]. On the other hand, the 5.8S rRNA gene flanked by the internal transcribed sequences ITS1 and ITS2 was sequenced using primers and TFR2 [15].

The PCR reactions were carried out in a final volume of 25 μl using the PureTaq Ready-to-Go kit (GE Healthcare, UK) using the manufacturer's specifications. Amplification of ribosomal genes was performed by using established protocols [14,15]. The PCR products were purified by using the Illustra GFX PCR DNA and Band Purification Kit (GE Healthcare, UK), and DNA sequencing was made in an ABI PRISM DNA 3730 DNA Sequencer (Applied Biosystem) using the primers described previously.

2.4. Bioinformatic analysis

The sequences of both 18S rDNA and ITS region were compared with different trichomonad sequences obtained from the GenBank and included the following genus: *Trichomonas* spp., *Tritrichomonas* spp. and *Tetratrichomonas* spp. were from different hosts, and, finally, the human intestinal protist *Pentatrichomonas hominis* (for more detail see Electronic Supplementary Material 1).

The chromatograms were processed by using the Chromas Pro v. 1.5 software (Technelysium Pty. Ltd., Australia). Comparisons and alignments with other trichomonad sequences were done by using the MegAlign program of the Laser gene package. The percent sequence divergence was calculated by comparing nucleotide pairs in relation to the phylogeny reconstructed by MegAlign, and the program calculates the percent nucleotide identity by comparing sequences directly without accounting for any phylogenetic relationship. The length of 5.8S rRNA regions was determined by the database Rfam 11.0 while the ITS regions were defined according to published data [15]. Phylogenetic analyses were conducted by using Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 [23]. The dendrograms were inferred by using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method [24]. The evolutionary distances were computed by using the Maximum Composite Likelihood method [25], and the boostrap statistical support was carried out by using 1000 replicates [26].

The ITS1-5.8S rRNA-ITS2 sequences of the six *T. vaginalis* isolates have been deposited in the GenBank/EMBL/DDBJ databases under the accession numbers KC513774 through KC513779.

3. Results and discussion

3.1. 18S rRNA amplifications and alignments

The 18S rRNA from six distinct *T. vaginalis* isolates were sequenced and alignment results showed complete identity. Further, ClustalW alignments with other trichomonadid organisms belonging to different genera were studied by using the consensus contig of 644 nucleotides obtained from the two amplified 18S rRNA regions from the 6 different isolates. The comparative analyses showed a 100% identity with other *T. vaginalis* sequences available at the GenBank/EMBL/DDBJ databases. A total of 10 and 50 trichomonadid sequences from the 18S rDNA and ITS1-5.8S rRNA-ITS2 regions, respectively, were initially included in the study; however, repeated data of sequences with 100% homology were removed. Although the 18S rRNA region showed the same length in all the sequences from the same species, a high polymorphism was observed in comparison with other trichomonadids, even between organisms from the same genus. Nonetheless, the small subunit rRNA gene showed the highest homology in terms of nucleotide length. Furthermore, the divergence results observed in the comparative study among distinct species of the related genus *Tritrichomonas*, *Tetratrichomonas*, *Trichomonas* and *Pentatrichomonas* with the consensus sequences obtained from our isolates are summarized in Electronic Supplementary Material 1. The alignment report of the 18S rRNA contig composed of the two amplified regions showed a 97.7% identity with *Trichomonas tenax* while less homology of 85.8% and 86.4% was observed with the *Tritrichomonas* spp. sequences of *Tritrichomonas foetus*, *Tritrichomonas muris*, and *Tritrichomonas augusta*. The high degree of homology between *T. vaginalis* and the human oral parasite *T. tenax* was also observed in the ITS1-5.8S rRNA-ITS2 amplicons and in the phylogenetic studies consistent with earlier work of others [27,28].

3.2. ITS1-5.8S rRNA-ITS2 amplifications and alignments

T. vaginalis has one of the biggest genomes in comparison with other organisms (Electronic Supplementary Material 2). The *T. vaginalis* genome is highly repetitive, with a predicted 254 copies of 18S rRNA and 251 copies of 5.8S rRNA [8]. As the ITS region evolves faster than the rDNA genes, it is conceivable that a possible variation in the hypervariable ITS regions might be detected and which might then be useful for isolate discrimination. Indeed, this has been seen in other parabasalids, like *Dientamoeba* [17]. In this context, the ITS regions have been defined as good intra- and interspecific variability markers for determination of polymorphism in other protozoa alignments in terms of size and sequence differences [12,13,29].

Comparative sequence analysis of the ITS1-5.8S rRNA-ITS2 from the six isolates was conducted. However, the current analysis of this locus showed no intraspecific difference between isolates (only a 0.7% of divergence). Thus, alignments by the ClustalW method within all published *T. vaginalis* sequences showed variable identity rates differing from our sequences by nucleotide mutations in three different positions

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of the ITS1 region and 1 difference in the ITS2 locus, thus revealing 5 sequence types that include the consensus sequence. The 5.8S rRNA gene sequences were 100% identical among the 34 *T. vaginalis* isolates analyzed. The sequences differed in only one nucleotide, which included 1 transversion (A → C) and 2 translations (T → C and C → T) in the ITS1 region, and 1 transversion in the ITS2 region (A → T) (Electronic Supplementary Material 3). The mutation C → T, located at nucleotide position 66 (C/T) of the ITS1 region was observed in 26% of the *T. vaginalis* sequences analyzed in the current study. Although 4 nucleotide mutations were observed in the alignments, only the point mutation in ITS1 (C66T) was identified in more than one sequence derived from different reports by others [18–21]. Interestingly, the majority of the C66T positive mutation sequences were from the Philippines, China and Iran [18,20,21]. Only one group of the sequences with the C66T mutation was obtained from the USA but the geographic origin of the patients was not identified in the study [19].

The sequence comparison with other trichomonads revealed significant size (Electronic Supplementary Material 1) and nucleotide dissimilarities (Electronic Supplementary Material 3 and 4). Interestingly, a higher degree of homogeneity was observed with the flagellates sharing the same host and genus, such as the oral parasite *T. tenax*. On the other hand, the homology obtained with *P. hominis*, a resident of human environment and a different genera than the urogenital *T. vaginalis*, was higher than expected (>82.5%). All *T. vaginalis* isolates shared the lowest similarity rates with the *Tritrichomonas* genus, ranging from 66.7% to 72.5%. The *T. foetus* presented an increased divergence (~38%), which was found to be higher than the bovine *Tetratrichomonas buttreyi* (~25%). Furthermore, the differences were higher in comparison with the rest of trichomonads from other genus and hosts. The main divergences (~40%) in comparison with *T. vaginalis* sequences were mainly observed in trichomonads from poikiloterm hosts (amphibians, reptiles, lizards and slugs). A high polymorphism was also noted in the rodent flagellate *T. muris* (Electronic Supplementary Material 4). The early divergence of *T. muris* from the *Tritrichomonas* spp. was confirmed in the phylogenetic studies.

The suggested possible recent genome expansion of *T. vaginalis* [8] could explain the low polymorphism in the hypervariable region in comparison with other protozoa with a minor genome having less repetitive DNA. As *T. vaginalis* is one of the protozoa with a higher number of rRNA encoding genes and is placed among the earliest eukaryotic diverging branches [15], it could be hypothesized that variability in the rapidly evolving ITS loci would be detected. However, the absence of canonical features of mitochondria and peroxisomes in this organism as a sign of early eukaryote lineages is not widely accepted. For example, some consider that the divergent nature of this parasite is caused by a recent evolution due to the low polymorphism detected in diverse genomic families [8,30].

3.3. Phylogenetic studies

Figs. 1 and 2 present the UPGMA tree based on the bioinformatics' analysis of the 18S rRNA and the ITS1-5.8S rRNA-ITS2 sequences, respectively. The phylogenetic studies grouped the trichomonadid protozoa sequences in well-supported branches according to their genera. The first cluster is formed by the *Trichomonas* species that includes the *T. vaginalis* isolates and an emerging branch with *T. tenax*, *T. canistomae* and *T. gallinae*. The *Tetratrichomonas* genus formed the second cluster, while the third cluster is composed of *P. hominis*. The last cluster grouped all the *Tritrichomonas* species, such as *T. foetus*, *T. augusta*, and *T. muris*. The dendrogram based on the ITS sequence data also includes *T. prowazeki* in the second cluster and the *Tritrichomonas* species *T. nonconforma* and *T. mobilensis* in cluster 4 (Fig. 2). Both figures further show the phylogenetic trees based on both genomic markers that indicate that the first divergence occurred between *Tritrichomonas* spp. and other trichomonadid members. Subsequently, *Tetratrichomonas* spp. from amphibians and slugs diverged from the main branch and then *Trichomonas gallinarum* from poultry. Finally, the member with five anterior flagella emerged as a new independent cluster. The latest branch is related to the independent evolution of *T. vaginalis* from *T. tenax*, *Trichomonas canistomae* and *Trichomonas gallinae* as reflected in Fig. 2.

The dendrograms grouped *T. vaginalis* with the rest of *Trichomonas* spp., revealing the ITS regions as a robust genomic marker for intergenic and interspecies discrimination (Fig. 2). As mentioned above, the high 18S and ITS nucleotide sequence identity of *T. vaginalis* with the oral human parasite *T. tenax* (>97.5% and >92% respectively) is noteworthy. This genomic evidence supports the hypothesis that *T. tenax* may be a variant of *T. vaginalis*, as has been suggested [27,28]. The remarkable genetic identity evidenced by the sequences and dendrograms (Figs. 1 and 2) is notable, as this may help explain new information emerging on the existence of both *T. tenax* and *T. vaginalis* in patients with acute respiratory distress syndrome and pulmonary infection [31,32]. In diverse clinical reports, *T. vaginalis* [33] and *T. tenax* [34] were detected in bronchoalveolar lavage fluid (BALF) samples of patients with bronchopulmonary infection. The identity rates of both *Trichomonas* species obtained in the current study could give a phylogenetic explanation to these clinical cases of pulmonary coinfections.

According to these data, the phylogenetic studies (Figs. 1 and 2) and the alignments of both 18S rRNA (data not shown) and ITS region (Electronic Supplementary Material 4) showed a high homology of

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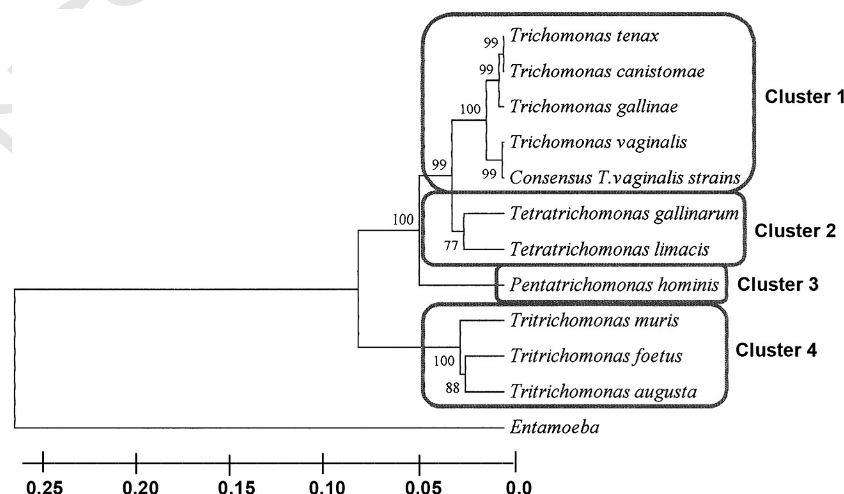

Fig. 1. Dendrograms of trichomonadid species based on the 18S rRNA gene alignment inferred by UPGMA using MEGA4 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. *Entamoeba* was used as external group. Scale bar: 0.05 substitutions per site.

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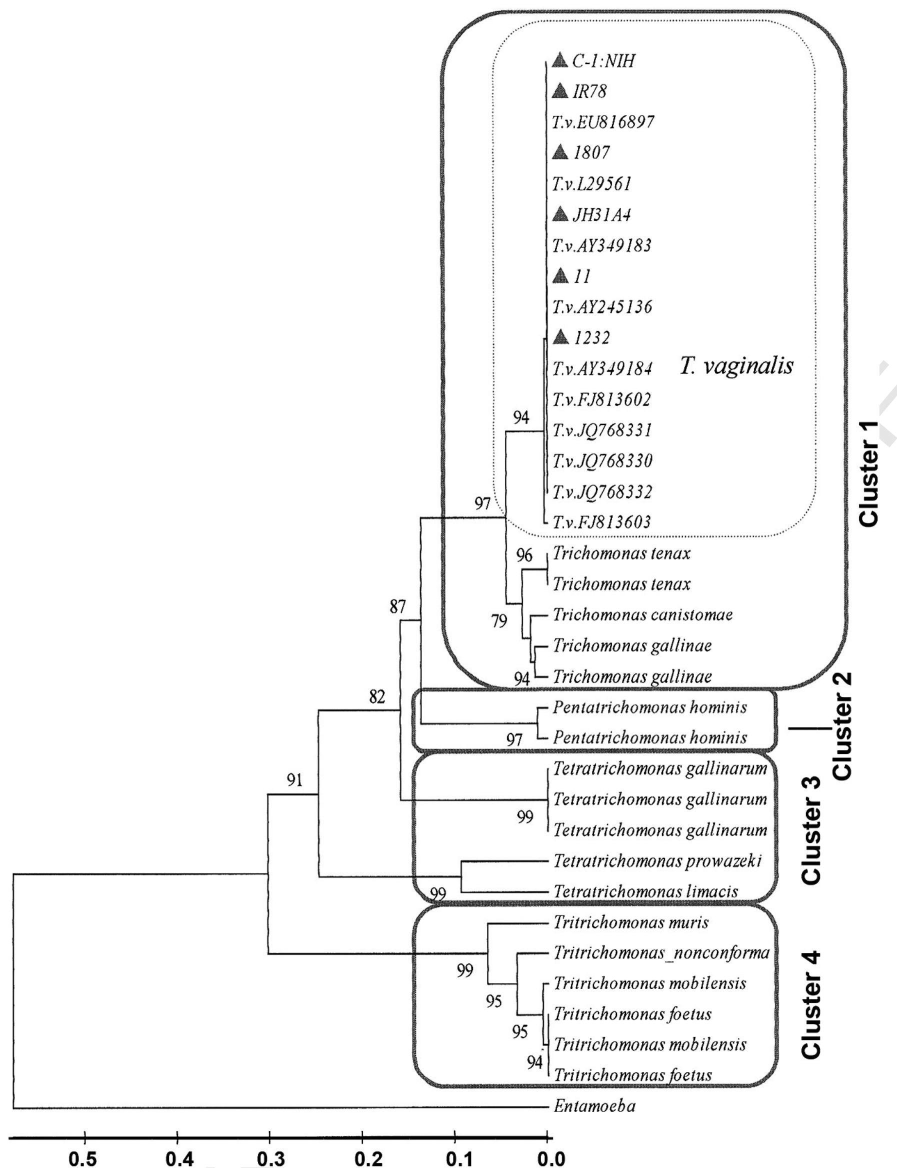

Fig. 2. Phylogenetic tree of trichomonadid protists based on ITS1-5.8S rRNA-ITS2 alignments inferred by UPGMA method. The sequences marked with a gray triangle correspond with the new *T. vaginalis* isolates sequenced in the current study. The evolutionary distances were computed using the Maximum Composite Likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. *Entamoeba* was used as external group. Scale bar: 0.1 substitutions per site.

T. vaginalis and *T. tenax* with *T. canistomae* and *T. gallinae*, both parasites from the same genus. The high homology between sequences from different species of the same genus may suggest a possible recent expansion and divergence of *T. vaginalis* from the rest of the *Trichomonas* spp. that in fact led to different host specificity.

The dendrograms obtained in this study group the distinct flagellates in clusters according to their genera and species. These results suggest that the ribosomal small subunit and the ITS1-5.8S rRNA-ITS2 region are suitable for phylogenetic inference, involving classification at the species level in trichomonadid protozoa. Hence, this feature confirms these loci as suitable tools for genetic identification of new isolates of trichomonads at a specific level. Furthermore, the 5.8S rRNA gene flanked by the ITS regions reveal a higher fidelity within the possible evolution of the distinct trichomonadid species. As shown in Fig. 2, *Trichomonas* and *Pentatrichomonas* could have recently diverged, suggesting the co-evolution of *P. hominis* as the ancestor of *T. vaginalis* [35], both initially sharing the same intestinal environment. Finally our data support the idea that the *Tritrichomonas* and *Tetratrichomonas* genus have evolved and adapted to a wider range of hosts [36–39], including poikilotherms, which shows the evolutionary distance among all of the *Trichomonas* spp.

In conclusion, the current research has studied the ITS1-5.8S rRNA-ITS2 region of *T. vaginalis* isolates. The sequence alignments have shown only one stable mutation in the position 66 of the ITS1 region in 26% of the sequences studied. The low polymorphism in this hypervariable region, the phylogenetic dendrograms obtained with other trichomonadids and the high identity with *T. tenax*, a human oral parasite, and with other *Trichomonas* spp. sequences, suggest the recent diverging evolution of *T. vaginalis.*

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2013.12.017.

4. Uncited references Q3

[40,41,42,43,44,45]

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