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Multilocus analysis reveals further genetic differences between *Tritrichomonas foetus* from cats and cattle

Susana Pedraza-Díaz[a,1,2], David Arranz-Solís[a,1,3], Hipólito Gómez-Couso[a,4], Lumila Fuschs[b], Marcelo Fort[b], Claudia Rengifo-Herrera[a,5], Vanesa Navarro-Lozano[a], Luis M. Ortega-Mora[a], Esther Collantes-Fernández[a,*]

[a] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040, Madrid, Spain
[b] Instituto Nacional de Tecnología Agropecuaria (INTA), Anguil, La Pampa, Argentina

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ABSTRACT

Tritrichomonas foetus isolates from feline and bovine origin has been previously shown to carry a certain degree of genetic heterogeneity. Here, novel candidate molecular markers were developed by means of multilocus sequence typing of the gap2 gene (encoding for *T. foetus* glyceraldehyde-3-phosphate dehydrogenase), ITS region, the TR7/TR8 variable-length repeat and microsatellite genotyping. These markers were used to characterize *T. foetus* field isolates from bulls and domestic cats and to compare phylogenetically with the following ATCC isolates: *T. foetus* isolated from cattle and pig (syn. Tritrichomonas suis), *Tritrichomonas mobilensis*, *Tetratrichomonas gallinarum* and *Pentatrichomonas hominis*. Among them, TFMS10 and TFMS7 were found to be the most polymorphic markers. Moreover, an 809 bp fragment of the *gap2* gene was successfully amplified from all the trichomonads included in this study and the sequence analysis revealed differences between *T. foetus* porcine and feline genotypes and *T. mobilensis* in comparison to the bovine *T. foetus* ATCC isolate. The TR7/TR8 repeat pattern was not reproducible, being only consistent the fragments of approximately 110 and 217 bp. Sequence analysis of the latter revealed the existence of 3 SNPs resulting in 98.6 % homology between bovine and feline isolates. A search for similar sequences was carried out to develop a Restriction Length Fragment Polymorphism analysis. A 503 bp region, named TF1, revealed the existence of two *BbvI* restriction enzyme sites that were able to generate different length fragments for *T. foetus* feline and bovine isolates. Finally, the neighbour-joining analyses showed that *T. foetus* porcine genotype clusters together with bovine genotype, whereas *T. mobilensis* and the feline genotype form a separate cluster.

1. Introduction

Tritrichomonas foetus is a trichomonad protozoan parasite and the etiological agent of bovine trichomonosis, a sexually transmitted disease considered an important cause of early reproductive failure in cattle. The parasite colonizes the preputial cavity of bulls and the urogenital tract of cows and is transmitted during coitus (reviewed in BonDurant, 1997). In the last two decades, the same trichomonad species has been recognized as an important enteric pathogen of domestic cats, causing chronic diarrhoea (Gookin et al., 1999; Levy et al., 2003). Transmission occurs by the faecal-oral route through direct contact among animals, being more common in breeding facilities, shelters or catteries (reviewed in Yao and Köster, 2015). On the other hand, *T. foetus* has been also observed as a commensal parasite in the

* Corresponding author at: SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040, Madrid, Spain.
E-mail address: esthercf@ucm.es (E. Collantes-Fernández).
[1] These authors contributed equally to the work.
[2] Current affiliation: Environmental Toxicology, National Center for Environmental Health (CNSA), Instituto de Salud Carlos III, 28220-Majadahonda, Spain.
[3] Current affiliation: Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California Davis, One Shields Ave., Davis, 95616, California, USA.
[4] Current affiliation: Laboratory of Parasitology, Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Santiago de Compostela, Campus Vida, 15782 Santiago de Compostela, A Coruña, Spain.
[5] Current affiliation: Faculty of Veterinary Medicine, University of Panama. Campus Harmodio Árias Madrid (Curundu). Apartado 3366, Panama 4, Panama.

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nasal cavity, stomach, colon and caecum of domestic pigs, usually without causing clinical signs. This parasite, which was formerly known as *Tritrichomonas suis* but currently considered synonymous with *T. foetus* (Tachezy et al., 2002; Lun et al., 2005; Šlapeta et al., 2012), can be transmitted directly from an infected animal to other hosts by direct nasal contact (Lun et al., 2005).

At present, *T. foetus* from bovine, feline and pig origin are considered to be different host-adapted genotypes of the same *Tritrichomonas* species, *T. foetus* (Šlapeta et al., 2012). Comparisons carried out between these isolates revealed a lack of compelling genetic and morphological differences (Tachezy et al., 2002; Lun et al., 2005; Frey and Müller, 2012; Šlapeta et al., 2012). However, a degree of genetic heterogeneity in *T. foetus* isolates from feline and bovine origin has been previously observed (reviewed in Yao and Köster, 2015), such as a conserved single nucleotide polymorphism (SNP) in the internal transcribed spacer-2 (ITS-2) region, as well as other differences between polymorphisms elongation factor 1 alpha, in the TR7/TR8 variable-length repeat and cysteine protease (Šlapeta et al., 2012; Yao and Ketzis, 2018) among others. However, there is a need for more informative genetic markers.

The aim of the present study was to development new genetic markers to molecularly characterize *T. foetus* field isolates obtained from bulls and domestic cats. The candidate molecular markers were the gap2 gene (encoding for *T. foetus* glyceraldehyde-3-phosphate dehydrogenase), ITS region, the TR7/TR8 variable-length repeat (Šlapeta et al., 2010) and microsatellite genotyping. These markers were also applied to the following ATCC isolates: *T. foetus* isolated from cattle and pig (syn. Tritrichomonas suis), *T. mobilensis* (affecting squirrel monkeys), *Tetratrichomonas gallinarum* (affecting poultry) and *Pentatrichomonas hominis* (affecting a variety of vertebrate species) to compare phylogenetically these related species of trichomonads. The microsatellite panel (especially TFMS10 and TFMS7) showed to be the most robust polymorphic markers. We describe here a novel set of molecular markers that can be used to study the genetic diversity of *T. foetus*.

2. Materials and methods

2.1. Trichomonad isolates and in vitro cultivation

Origins of the trichomonad isolates used for genomic analysis are summarized in Table 1. *T. foetus* field isolates from bovine (n = 17) and feline (n = 9) origin were analysed from previous studies carried out in Spain. Field bovine isolates were primarily obtained from smegma samples collected from bulls (Mendoza-Ibarra et al., 2012; Collantes-Fernández et al., 2019) and feline isolates from faecal samples collected from domestic cats (Arranz-Solís et al., 2016). For the establishment of protozoan cultures, positive samples were grown in Diamond's medium as previously described (Sager et al., 2007) and cryopreserved in liquid nitrogen by using freezing media containing 90 % foetal bovine serum and 10 % DMSO. In addition, the isolates *T. foetus* MT TFC-5-1 isolated from cattle (ATCC-PRA-164) and C 19 F isolated from pig (ATCC-30169) (syn. T. suis), *T. mobilensis* USA:M776 isolated from squirrel monkey (ATCC-50116), *T. gallinarum* TP-79 isolated from domestic turkey (ATCC-30097) and *P. hominis* R-51 isolated from cattle (ATCC-30098) were obtained from the American Type Culture Collection (ATCC) (Table 1). Trichomonads were cultivated *in vitro* according to the ATCC recommended protocols.

2.2. Identification and selection of molecular markers

The *gap2* gene, encoding for *T. foetus* glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), was evaluated in this study as a candidate molecular marker. Sequences of the *gap2* gene from *T. foetus* and *Trichomonas vaginalis* were retrieved from the GenBank database and analysed using BLAST® v.2.2.31 (https://blast.ncbi.nlm.nih.gov/Blast.cgi, National Center for Biotechnology Information, Bethesda, MD, USA) and BioEdit Sequence Alignment Editor v.7.2.5 (Copyright© 1997–2013 Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA).

The genetic characterization was also investigated by DNA sequencing of ITS region and the TR7/TR8 variable-length repeat (Šlapeta et al., 2010). A restriction fragment length polymorphism (RFLP) analysis was developed after searching for sequences in GenBank® that were similar to the 217 bp obtained in the amplification of the TR7/TR8 variable-length DNA repeat. Three *T. foetus* expressed sequence tags (ESTs) were retrieved and primers were designed to amplify a 503 bp region named TF1 region.

In addition, *T. foetus* sequences deposited in GenBank were screened for putative microsatellite sequences (TFMS) with Tandem Repeat Occurrence Locator (TROLL, Castelo et al., 2002) and Tandem Repeats Finder (TRF) (Benson, 1999) software. These sequences included both ESTs derived from a *T. foetus* cDNA library as well as nucleotide sequences.

2.3. DNA extraction and PCR amplification

DNA was isolated from 200 to 300 µl of each parasite culture from cryopreserved stocks with the Real Pure Genomic DNA Extraction kit (Durviz, Valencia, Spain) according to the manufacturer's instructions.

The rDNA ITS and the TR7/TR8 variable-length DNA repeat were amplified according to the protocols described by Felleisen et al. (1998) and Šlapeta et al. (2010), respectively.

For each marker selected in this study, primers were designed from GenBank sequences using Primer3 software (Rozen and Skaletsky, 2000) and checked with Primer-BLAST (Table 2). Primers were synthesised by Sigma Aldrich Quimica SA (Madrid, Spain). PCR reactions were performed in 50 µl volumes of molecular grade water containing 1 × PCR buffer, 1.5 mM (GAPDH2 and TF1) or 2 mM (TFMSs) $MgCl_2$, 200 µM dNTPs, 0.4 µM of each primer, 1 unit (TFMSs) or 1.25 units (GAPDH2 and TF1) of EcoTaq DNA polymerase (Ecogen, Madrid, Spain) and 5 µl (approximately 50 ng) of template DNA. Reaction conditions for GAPDH2 were initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 45 s and 72 °C for 45 s, and a final extension at 72 °C for 10 min. For amplification of the TF1 region and TFMSs, the reaction conditions were initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min.

Negative controls (distilled water) were included in each batch of amplifications. To avoid carryover of contaminating nucleic acids, each step of the procedure was performed in separate rooms using aerosol-resistant pipette tips and dedicated pipettes.

Aliquots of 5 µl of the PCR products were examined by electrophoresis in 1.5 % agarose/ethidium bromide gels and visualised under UV light.

2.4. DNA sequencing

PCR products were purified using the GENECLEAN Turbo kit (QBiogene, California, USA) according to the manufacturer's instructions and directly sequenced in both directions with the primers used for PCR amplification using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, Foster City, CA, USA) and a 3730 DNA analyser (Applied Biosystems) at the Genomic Unit of the Madrid Science Park.

Sequences were analysed using BioEdit Sequence Alignment Editor v.7.2.5. Multiple alignments were performed using the ClustalW program with further manual refinements and neighbour-joining trees were constructed from the aligned sequences using the MEGA X software (Kumar et al., 2018).

Nucleotide sequence data reported in this paper are available in the GenBank® database under the accession numbers **MK770832-MK771087**.

Table 1
List of the *Tritrichomonas foetus* field isolates and *T. foetus*, *T. mobilensis, Tetratrichomonas gallinarum* and *Pentatrichomonas hominis* ATCC isolates used in the present study.

Isolate	Host	Sample name	Reference
Tritrichomonas foetus	Bull	Bull 1	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 2	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 3	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 4	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 5	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 6	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 7	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 8	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 9	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 10	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 11	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 12	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 13	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 14	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 15	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 16	Mendoza-Ibarra et al., 2012
Tritrichomonas foetus	Bull	Bull 17	Arranz-Solís et al., 2016
Tritrichomonas foetus	Cat	Cat 6	Arranz-Solís et al., 2016
Tritrichomonas foetus	Cat	Cat 9	Arranz-Solís et al., 2016
Tritrichomonas foetus	Cat	Cat 18	Arranz-Solís et al., 2016
Tritrichomonas foetus	Cat	Cat 64	Arranz-Solís et al., 2016
Tritrichomonas foetus	Cat	Cat 120	Arranz-Solís et al., 2016
Tritrichomonas foetus	Cat	Cat 176	Arranz-Solís et al., 2016
Tritrichomonas foetus	Cat	Cat 180	Arranz-Solís et al., 2016
Tritrichomonas foetus	Cat	Cat 181	Arranz-Solís et al., 2016
Tritrichomonas foetus	Cat	Cat 213	Arranz-Solís et al., 2016
Tritrichomonas foetus	Bovine	*T. foetus* MT TFC-5-1	ATCC-PARA-164
Tritrichomonas foetus	Pig	*T. suis* C19 F	ATCC-30169
Tritrichomonas mobilensis	Squirrel Monkey	*T. mobilensis* USA M776	ATCC-50116
Tetratrichomonas gallinarum	Domestic Turkey	*T. gallinarum* TP-79	ATCC-30097
Pentatrichomonas hominis	Bovine	*P. hominis* R-51	ATCC-30098

Table 2
List of primers used in the present study.

Region	Primer	Sequence (5´→ 3´)	Amplicon (bp)	Reference
ITS	TRF1	TGCTTCAGTTCAGCGGGTCTTCC	372	Šlapeta et al., 2010
	TRF2	CGGTAGGTGAACCTGCCGTTGG		
	TFR3	CGGGTCTTCCTATATGAGACAGAACC	347	
	TFR4	CCTGCCGTTGGATCAGTTTCGTTAA		
VLR	TR7	CTGTTGTCGACGTTTATCCA	NA	Riley et al., 1992
	TR8	GATCACCAGTGGAGGGTGTC		
TF1	TF1F	AGCTGACATCTGCCTTTTGG	503	This study
	TF1R	GATGGCTTTCAGCAACGAAG		
GAPDH2	TFGAPDH2F	GTCGCTGTTCACGATCTTTG	809	This study
	TFGAPDH2R	ATTGGCTCGTCAGTGTAGCC		
TFMS1	TFMS1F	CGAGGAAAGAACGAAGAAGG	282	This study
	TFMS1R	TCTGTCTCTGTTCTGATGAAGAGG		
TFMS2	TFMS2F	GCTGCTGCAAAGAAGAAGAAG	300	This study
	TFMS2R	AGGATTCGTCGGATGATGAG		
TFMS3	TFMS3F	AACCACGGTGGATACTTTGG	291	This study
	TFMS3R	TTGCTGAACTTGTTGCTGTTG		
TFMS4	TFMS4F	CGATCCAAACAATGGAAAGC	327	This study
	TFMS4R	AGTCGTCATCGTCATCATCG		
TFMS5	TFMS5F	TTGGAGGTTGAATTGGTTCG	297	This study
	TFMS5R	ATTGTGGATCGGGGTATGG		
TFMS6	TFMS6F	TGCCTTCACTTTTTGAATCC	329	This study
	TFMS6R	AGGCTGTGATGCAATTCTTG		
TFMS7	TFMS7F	TGTTGTTGAACCAATTGATGG	302	This study
	TFMS7R	TTTGGTTCTGGCAAATTGAAC		
TFMS8	TFMS8F	CGGCACGAGGCATTTATTG	296	This study
	TFMS8R	AAATCCAAGACATCCCGAAG		
TFMS9	TFMS9F	CCAACTGCTACAGCATCACC	345	This study
	TFMS9R	CTGTTTGGGTTTACCGCTTC		
TFMS10	TFMS10F	AACGACAAAGGGATCTTCATC	425	This study
	TFMS10R	CTGTTGACGCTTTCTTACGC		

ITS, internal transcribed spacer. VLR, Variable Length DNA repeats. TF, *Trichomonas foetus*. GADPH, Glyceraldehyde-3-Phosphate Dehydrogenase. MS, Microsatellite.

2.5. Restriction Fragment Length Polymorphism (RFLP) of the TF1 region

A total of 20 µl of the PCR products obtained from the amplification of the TF1 region were digested in final volumes of 25 µl with 2 U of *Bbv*I (New England Biolabs, Izasa, Barcelona, Spain) for 1.5 h at 37 °C. Restriction fragments were resolved on 3 % typing grade agarose/ethidium bromide gels and visualised under UV light.

3. Results

3.1. Gap2 gene

An 809 bp fragment of the *gap2* gene encoding for GAPDH2 was successfully amplified from all the trichomonads included in the study (Table 1), except for *T. gallinarum* and *P. hominis*. Sequence analysis showed that all the 9 *T. foetus* feline isolates were 100 % identical to each other, and all the 17 *T. foetus* bovine isolates were also 100 % identical to each other. However, a SNP at position 180 (A/C) was observed between the *T. foetus* sequences from bull isolates and the *T. foetus* ATCC isolate from cattle. The similarity between porcine *T. foetus*, *T. mobilensis*, and feline *T. foetus* compared to the bovine *T. foetus* ATCC isolate were 99.9 % (T/C pos. 603), 99.5 % (A/G pos. 135; T/C pos. 300, C/T, pos. 651, A/G pos. 729) and 99.1 % (A/G pos. 135, T/C pos. 300, T/C pos. 331; T/C pos. 507, T/C pos. 579, C/T, pos. 651, A/G pos. 729), respectively (Fig. 1).

3.2. Internal transcribed region of the ribosomal DNA unit (ITS)

The ITS region was successfully amplified from all the bovine and feline *T. foetus* field isolates as well as the ATCC isolates included in the study. Sequence analysis showed that all cat *T. foetus* isolates were 100 % identical to each other, and bovine *T. foetus* isolates were also 100 % identical to each other. The alignment of the bovine and feline consensus sequences showed a single SNP (T to C substitution) at position 320 between feline and bovine isolates in the ITS2 region, which was previously described by Šlapeta et al. (2010) and subsequently confirmed by others (Reinmann et al., 2012). The porcine *T. foetus* sequence was 100 % identical to the bovine *T. foetus* sequences, whereas *T. mobilensis* showed a single SNP (C/T) at position 282 (Supplemental Fig. 1).

3.3. TR7/TR8 variable-length repeat region

The amplification of the TR7/TR8 variable-length DNA repeat showed complex and different patterns between feline and bovine *T. foetus* isolates, even within the same group (Fig. 2). These patterns, however, were not reproducible in our hands in different reactions, being only consistent the amplifications of the bands of approximately

Fig. 1. Sequences of *gap*2 gene, encoding for *Tritrichomonas foetus* glyceraldehyde-3-phosphate dehydrogenase 2 (GAPDH2). *T. foetus* bovine and feline genotypes aligned with the ATCC isolates: *T. foetus* MT TFC-5-1 (bovine *T. foetus*), *T. suis* C 19 F (= porcine *T. foetus*) and *T. mobilensis* USA:M776. No amplification was obtained for *Tetratrichomonas. gallinarum* or *Pentatrichomonas hominis*. Identical residues are shown as dots. TFGAPDH2F and TFGAPDH2R primers are indicated. Arrows depict the direction of amplification for each primer. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

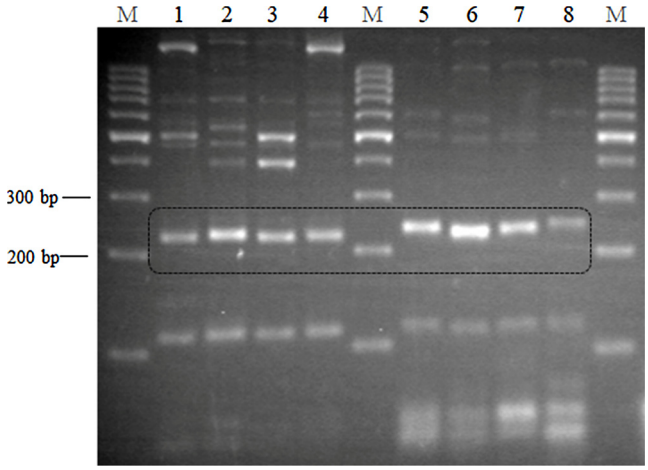

Fig. 2. Variable Length DNA repeats (VLR) amplification patterns in bovine and feline field isolates of *Tritrichomonas foetus*. Primers TR7 and TR8 were used. Lanes: M: molecular marker (each band stands for 100 bp), 1-4: *T. foetus* isolates from bulls, 5-8: *T. foetus* isolates from domestic cats. Delimited area indicates bands that were further assessed for RFLP.

210 and 110 bp. Sequence analysis of the larger band showed 100 % identity within the 9 feline isolates and 100 % identity within the 17 bovine isolates. It also revealed the existence of 3 SNPs (98.6 % homology), at positions 27 (G/A), 71 (T/C), and 197 (C/T) between cattle and cat sequences (Supplemental Fig. 2). We observed that the first SNP created a *BbvI* restriction site (5´-GCAGC-3´) in the feline sequences, which could be used for RFLP analysis (see below).

3.4. TF1 region and RFLP analysis

Three *T. foetus* ESTs were retrieved and primers were designed to amplify the 503-bp TF1 region. This region was successfully amplified from all the bovine and feline *T. foetus* isolates included in the study, as well as the porcine *T. foetus* and *T. mobilensis* ATCC isolates. By contrast, no amplification was obtained for *T. gallinarum* or *P. hominis*. The sequences of all bovine *T. foetus* isolates were identical to porcine *T. foetus*, whereas the sequence obtained for *T. mobilensis* showed 2 SNPs (C/T) compared to the *T. foetus* bovine sequences (99.6 % similarity) at positions 60 (shared with the feline *T. foetus*) and 433. Five conserved SNPs (99.0 % similarity) at positions 38 (A/G), 60 (C/T), 113 (G/A), 157 (T/C) and 283 (C/T) were observed between the cat and the bovine *T. foetus* isolates (Fig. 3A). The analysis of these sequences revealed the existence of two *BbvI* restriction sites at positions 113 and 419 in the feline sequences and a single restriction site at position 419 in the bovine sequences. These generated different patterns that enabled the discrimination by RFLP: fragments of 406 and 97 bp in bovine *T. foetus* and 281, 125 and 91 bp in feline *T. foetus* (Fig. 3B).

3.5. Microsatellite sequences

A total of 5004 *T. foetus* sequences available in GenBank were screened for microsatellite sequences using the TROLL software (Castelo et al., 2002) and 357 with at least 5 di-nucleotide repeats, 4 tri-nucleotide repeats and 3 tetra- or penta-nucleotide repeats were selected. These sequences were then analysed with the Tandem Repeats Finder programme (Benson, 1999) and 77 potential micro- and mini-satellite sequences were identified. To avoid the analysis of redundant sequences and to identify possible homologous sequences in other organisms, a BLAST® search in GenBank® for each of the 77 sequences selected was performed. From these, a number of sequences were excluded on account of insufficient flanking sequences, or because they were unsuitable for primer design. A total of ten putative markers, named TFMS1-TFMS10, were finally selected, eight of them located in ESTs of unknown function and two in nucleotide sequences. Five of these markers, TFMS1, TFMS2, TFMS4, TFMS8 and TFMS9 either failed to amplify or produced complex patterns (data not shown) and were thus removed from the study.

For markers TFMS3, TFMS5, TFMS6, TFMS7 and TFMS10, no amplification was achieved for *T. gallinarum* or *P. hominis*. These markers were located in ESTs, except TFMS7 which was located within the sequence corresponding to *T. foetus* "putative fructofuranosidase genes, complete cds, and putative nucleoporin gene, partial cds" (Genbank accession number U66071). Sequence analysis of the five loci showed genetic homogeneity within the nine *T. foetus* cat isolates and within the 17 *T. foetus* bovine isolates. However, sequence differences were observed mostly between feline *T. foetus* and *T. mobilensis* compared to the rest of trichomonads analysed (described below). The most polymorphic marker was found to be TFMS10, followed by TFMS7.

In TFMS3, sequences from all bovine isolates of *T. foetus,* as well as the bovine and porcine *T. foetus* ATCC isolates were identical. *T. mobilensis* showed only a SNP at position 285 (G/A) with respect to the bovine *T. foetus* ATCC reference isolate (99.7 % similarity), which was shared with the *T. foetus* feline consensus sequence. The later also showed an insertion at position 235 of a 5´-CAACAACAAGTTCAG-3´ motif (94.8 % similarity) (Supplemental Fig. 3).

In TFMS5, sequences of all bovine isolates, as well as the porcine ATCC isolate of *T. foetus* were identical with the exception of a bovine isolate which showed a T/C substitution at position 70. This finding was confirmed in different DNA extractions and PCR amplifications from the original sample submitted and from different aliquots of the cultured isolate. The feline *T. foetus* sequence showed 5 SNPs (98.3 % similarity) at positions 30 (T/A), 52 (C/T), 127 (A/T), 130 (T/C), and 149 (A/C). Six SNPs, three of them shared with feline *T. foetus*, were observed in the *T. mobilensis* sequence (98.0 % similarity) at positions 28 (T/C), 30 (T/A), 52 (C/T), 107 (A/G), 127 (G/T), and 130 (T/C) (Supplemental Fig. 4).

In TFMS6, sequence differences were only found in feline *T. foetus* and *T. mobilensis*. Three SNPs at positions 146 (C/T), 265 (G/A) and 304 (T/C) and a deletion of an ATA motif were observed in the *T. foetus* feline consensus sequence (98.2 % similarity). These differences were shared with *T. mobilensis*, which showed two additional SNPs at positions 217 (T/A) and 220 (A/T) (Supplemental Fig. 5).

In TFMS7, differences were found in porcine and feline *T. foetus*, as well as in *T. mobilensis*, compared to the bovine *T. foetus*, with similarities of 97.7 %, 94.7 % and 92.5 %, respectively. Sequence diversity included the presence of SNPs and variation in two potential repeat regions (CTTTT and ATTCT repeats starting at positions 200 and 224, respectively) (Fig. 4A).

Analysis of the TFMS10 marker showed greater genetic heterogeneity in the trichomonad sequences analysed. In addition to SNPs, highly polymorphic regions were observed in the alignment (Fig. 4B), which displayed differences in repeat patterns AGACTC / TGACTC at positions 61–84; and GAAGAACCAAAGAAA / GAAGAGCCAAAGAAA / GAAGAACCGAAGAAAA / ATAGAACCAAAGAA in the region spanning from positions 128-277. Percentages of similarity with respect to the bovine *T. foetus* ATCC isolate were 94.5 % for porcine *T. foetus*, 94.5 % for feline *T. foetus*, and 85.9 % for *T. mobilensis*. In addition, differences in the first region (AGACTC at positions 67–72,) and a SNP (A/G at position 313) were found between the sequence obtained from the bovine *T. foetus* ATCC isolate and the consensus sequence of the *T. foetus* from the bovine isolates included in this study, showing a similarity of 92.6 % (Fig. 4B).

Phylogenetic analysis of these markers confirmed the results. The neighbour-joining analyses of the multiple alignments performed with the sequences obtained in this study showed that porcine *T. foetus* clusters together with the bovine isolates of *T. foetus*, whereas *T. mobilensis* and the feline isolates of *T. foetus* form a separate cluster (Fig. 5). These results were confirmed when removing the *T. mobilensis* sequence from the analysis (Supplemental Fig. 6).

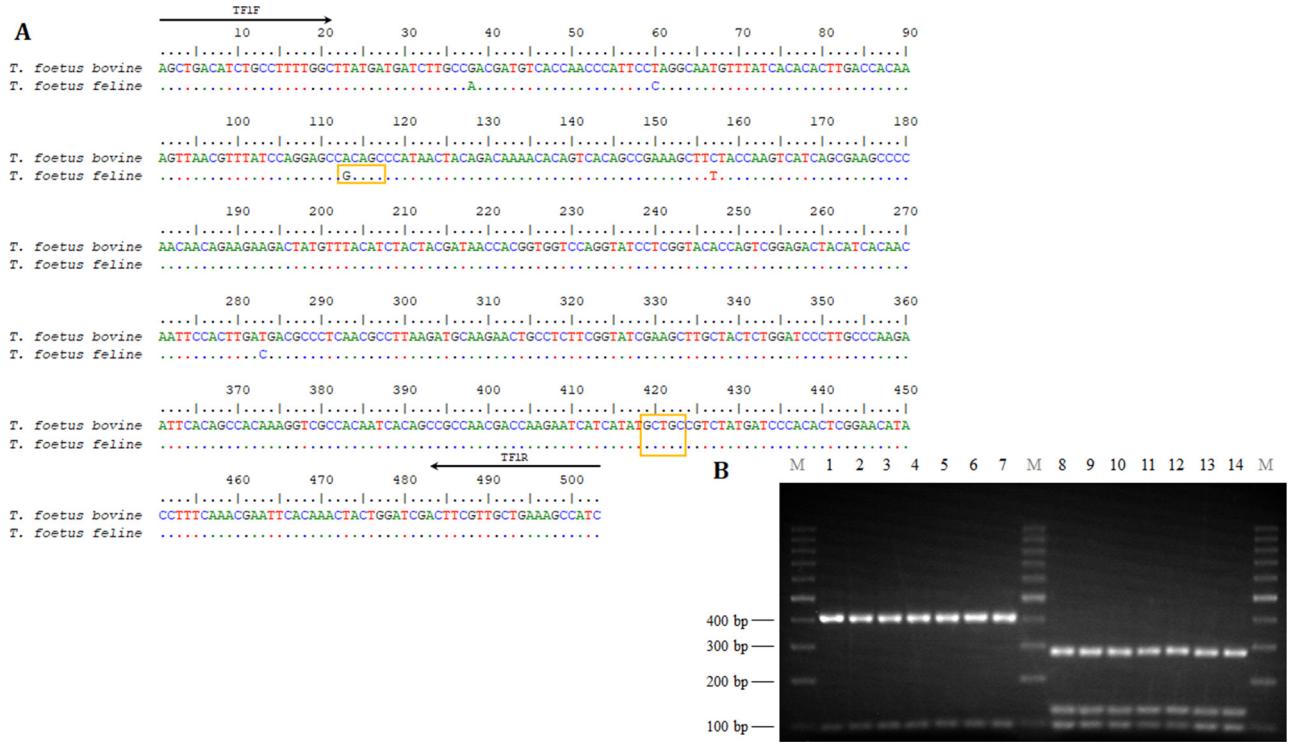

Fig. 3. Restriction fragment length polymorphism (RFLP) analysis of region TF1 in bovine and feline field isolates of *Tritrichomonas foetus*. (A) Alignment of the 503 bp region named TF1 showing consensus sequences of both bovine and feline *T. foetus* field isolates. Position and direction of primers TF1F and TF1R are indicated by black arrows. *BvbI* recognition sites are delimited by yellow square boxes. (B) TF1 RFLP patterns after cutting the restriction enzyme BvbI. Lanes: M, molecular marker (each band stands for 100 bp); 1–7, *T. foetus* isolates from bulls; 8-14, *T. foetus* isolates from domestic cats. Note the presence of 2 bands of 97 and 406 bp in the bovine isolates and 97, 125 and 281 bp in the feline isolates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

Previous studies on the morphological, physiological and molecular levels have questioned the phylogenetic relationship among some *Trichomonas* species of veterinary importance, particularly for bovine, feline and porcine genotypes of *T. foetus* and *T. mobilensis* (Šlapeta et al., 2012; Frey and Müller, 2012). Although in the last years it has become clear that these species are closely related, the study of their diversity has been hampered by the lack of genetic information. In the present study, we provide further insight into the genetic characterization of *T. foetus* by employing different markers and a novel set of microsatellites that shed new light on the molecular characterisation of trichomonads.

When this study was designed, it was focused on some markers that have been already used in previous studies in *T. foetus* (Frey and Müller, 2012; Yao and Köster, 2015). We confirmed that the ITS2 presents no differences between bovine and porcine genotypes of *T. foetus* and shows 1 SNP between these two and *T. mobilensis*, as well as between the feline genotype of *T. foetus* (Šlapeta et al., 2010; Reinmann et al., 2012). Furthermore, the analysis of variable-length DNA repeats showed complicated patterns which were not reproducible, as previously described (Tachezy et al., 2002; Frey and Müller, 2012). Nevertheless, the analysis of a constant band of 217 bp revealed novel differences between feline and bovine *T. foetus* sequences, which gave rise to unique restriction sites that allowed us to use RFLP analysis. The readily available RFLP technique can be easily used to differentiate isolates, drastically reducing the cost of genotyping. Moreover, a new region of 503 bp named TF1 was amplified and revealed differences between *T. foetus* and *T. mobilensis*, as well as between the feline and bovine isolates of *T. foetus*.

A new gene encoding for GAPDH2 in *T. foetus* (*gap2*) was used. The sequences of *gap2* gene show differences for all amplified isolates when compared to bovine *T. foetus*, including porcine *T. foetus*. This gene, together with others encoding for proteins such as malate dehydrogenase, enolase and α- and β-tubulin have also been used in the past to investigate the evolutionary relationships between different trichomonads (Viscogliosi and Müller, 1998; Wu et al., 1999; Gerbod et al., 2004). However, these genetic markers have been found to be insufficient to reliably resolve inter- and intraspecific relationships, because they undergo recombination, horizontal gene transfer or duplication (Viscogliosi and Müller, 1998; Wu et al., 1999; Gerbod et al., 2004; Stechmann et al., 2006; Rogers et al., 2007; Oyhenart and Breccia, 2014). Therefore, other genetic markers are needed to study the diversity of trichomonads., Consequently, a set of five novel MS were analysed for its use as potential markers to distinguish trichomonads species. MS are generally considered to be neutral alleles that have not evolved under selective pressure. Indeed, MS markers have provided an excellent resource for studying population genetics of several eukaryotic parasites, including *T. vaginalis* (Conrad et al., 2011; Prokopi et al., 2011). To our knowledge, the use of MS for the study of *T. foetus* is described here for the first time. Among the MS markers analysed, TFMS10 and TFMS7 proved to be highly polymorphic, revealing marked differences in repeat patterns between *T. foetus* bovine, feline and porcine isolates and *T. mobilensis*.

As a matter of fact, the phylogenetic analysis of these markers confirmed that *T. foetus* porcine genotype clusters together with the bovine genotype, whereas *T. mobilensis* and the feline genotype of *T. foetus* form a separate cluster, which is in accordance to previous reports (Frey and Müller, 2012). In this regard, several studies of genomic DNA have shown a high degree of genetic similarity between *T. foetus* bovine and porcine genotypes using multilocus analysis (*i.e.* RFLP, RAPD, and PCR based analysis of variable-length DNA repeats), as well as direct comparison of sequences in highly conserved regions, such as ITS1, 5.8S-rRNA and ITS2 (*e.g.* Felleisen, 1997; 1998; Šlapeta et al., 2010, 2012); and some metabolic genes such as Elongation Factor 1

Fig. 4. Sequences of the microsatellite region TFMS7 and TFMS10. Amplification of **(A)** TFMS7 and **(B)** TFMS10 was achieved by using primers TFMS7F-TFMS7R and TFMS10F-TFMS10R, respectively (direction of amplification indicated by arrows). *T. foetus* isolates from bulls and domestic cats were aligned with the ATCC bovine isolate of *T. foetus* MT TFC-5-1, *T. suis* C 19 F (= porcine *T. foetus*) and *T. mobilensis* USA:M776. No amplification was obtained for *Tetratrichomonas gallinarum* or *Pentatrichomonas hominis*. Identical residues are shown as dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

alpha (EF-1α) (Reinmann et al., 2012), several cysteine proteins (CPs) (Šlapeta et al., 2012; Sun et al., 2012; Casteriano et al., 2016; Suzuki et al., 2016) or malate dehydrogenase 1 (MDH-1) (Šlapeta et al., 2012; Casteriano et al., 2016; Suzuki et al., 2016). Remarkably, identical sequences have been shown for all of them when comparing *T. foetus* bovine and porcine isolates, with the exception of EF-1α and CP8, accounting for less than 1 % (Šlapeta et al., 2012; Yao and Köster, 2015; Suzuki et al., 2016). However, an interesting finding arose when we compared the tandem repetitions in the microsatellite markers designed in the present study. Although TFMS3, TFMS5 and TFMS6 showed 100 % similarity between bovine and porcine isolates, TFMS7, and specially TFMS10, showed certain degree of difference (2.3 % and 5.5 %, respectively). These results show that, despite the high degree of similarity between these genotypes, there are nevertheless differences.

On the other hand, recent studies have demonstrated genetic differences between bovine and feline genotypes of *T. foetus* by sequencing some loci (Šlapeta et al., 2010, 2012; Reinmann et al., 2012; Sun et al., 2012), as well as by a transcriptomic approach (Morin-Adeline et al., 2014; Stroud et al., 2017)., Several SNPs have been found when sequencing the conserved region ITS2 and other genes such as EF-1α, CP2, 5 and 8 (among others) and MDH1 (Reviewed in Frey and Müller, 2012). All the nine feline and the 17 bovine analysed isolates proved to be identical within themselves and different from each other for all the markers used in this study. As stated by others, this strongly suggests that these two lineages have a distinct, non-interbreeding evolutionary trajectory and may be considered as two *T. foetus* genotypes displaying intra-specific variation (Šlapeta et al., 2012; Morin-Adeline et al., 2014).Bull isolates showed also identical sequences compared to the the cattle ATCC isolate (MT TFC-5-1). In one of the isolates, however, a single nucleotide substitution was detected consistently in the TFMS5 marker. Considering that from all the markers this was the only SNP detected among all the *T. foetus* bovine isolates, we hypothesize that this SNP is due to a punctual mutation in that particular isolate. Nevertheless, further studies using a broader collection of isolates with this and other markers would help to elucidate this matter.

Finally, with the exception of ITS2, we were not able to amplify any of the novel markers used in this study with either *T. gallinarum* or *P. hominis* isolates, suggesting that the differences with these species are too big to be suitably studied with these markers. To our knowledge, there is only one study in which a comparison of these two isolates and other trichomonads was performed (Reinmann et al., 2012). In that study, the presence of a high number of SNPs was revealed by sequencing the EF-1α and ITS2 loci, accounting for less than 80 % of similarity. On the other hand, analysis of *T. mobilensis* using the same markers showed a closer relationship with the feline *T. foetus* than bovine or porcine genotypes (Šlapeta et al., 2012; Suzuki et al., 2016).

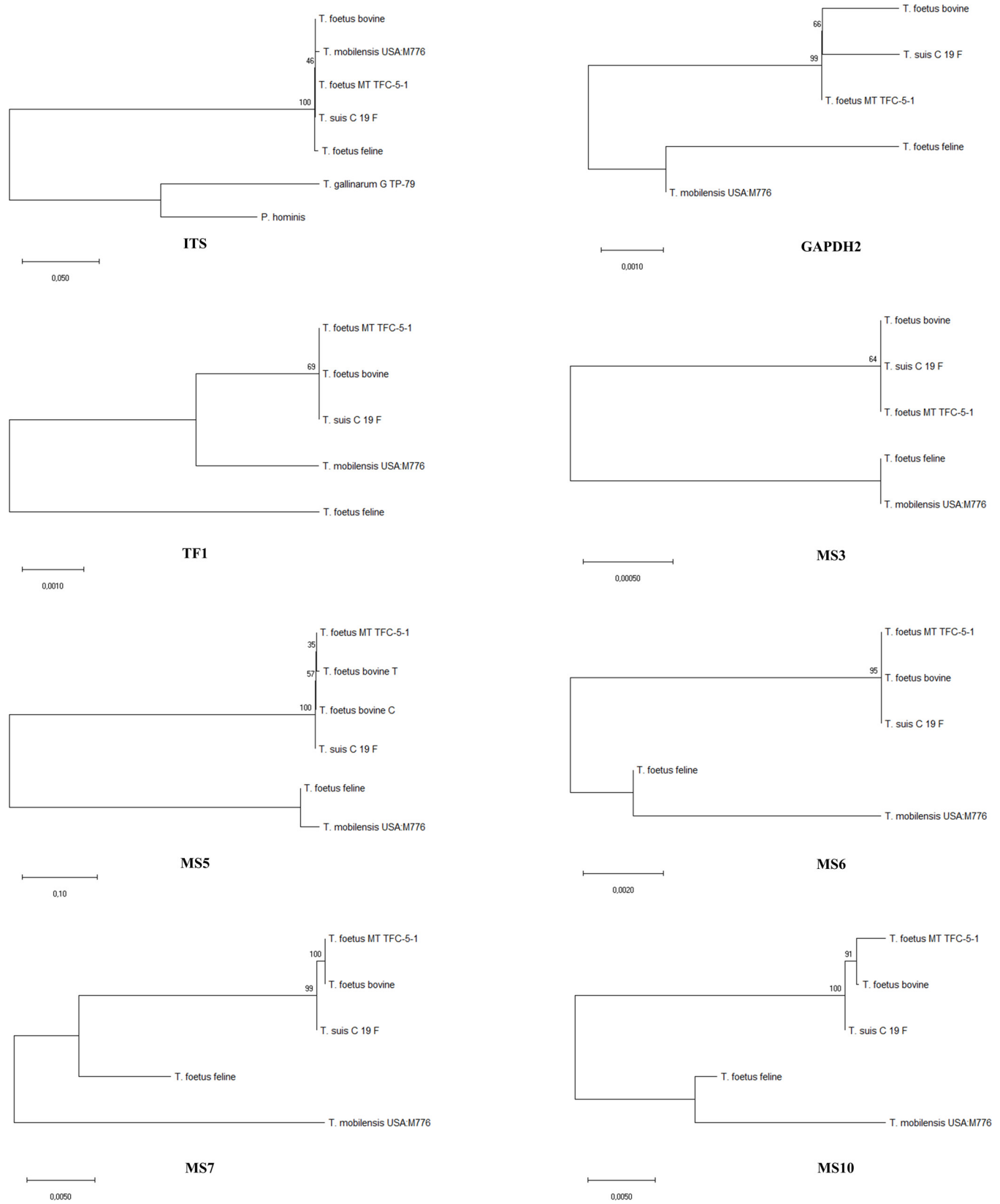

Fig. 5. Neighbour-joining tree showing the phylogenetic relationship of the multiple alignments performed with the sequences obtained in this study. The phylogenetic trees of the porcine (indicated as *Tritrichomonas suis* C19 F according to the name of the isolate in the ATCC), feline and bovine *T. foetus* genotypes, *T. mobilensis*, *Tetratrichomonas gallinarum* and *Pentatrichomonas hominis* were drawn using the neighbour-joining analysis (MEGA software v. 5.05). Evolutionary distances were computed using the Kimura-2 parameter method. Bootstrap values greater than 50 % from 1000 replicates are shown. Branch lengths are proportional to sequence divergence and relate to the scale bar shown (bottom left of each picture).

Similarly, all markers analysed in the present study showed at least 1 SNP between bovine *T. foetus* and *T. mobilensis*, sharing some of these SNPs with *T. foetus* feline genotype.

5. Conclusion

The results obtained in the present study offer further insight into the genetic differences between the bovine, feline and porcine genotypes of *T. foetus*. The analysis of the TF1 region, *gap*2 gene, as well as TFMS3, TFMS5, TFMS6, TFMS7 and TFMS10 showed several SNPs that accounted for more than 5 % of dissimilarities in some cases. These findings, together with previous descriptions at molecular, pathogenic and species-specificity levels, among others, confirm the clear separation of feline from bovine *T. foetus* isolates and reveal further genetic differences between bovine and porcine genotypes. Specifically, microsatellite marker can be adopted as molecular tools for understanding the diversity and population structure of *T. foetus*. Future studies should examine *T. foetus* isolates using multiple gene regions, or full genome sequencing, in order to provide more detailed information.

CRediT authorship contribution statement

Susana Pedraza-Díaz: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization, Supervision. **David Arranz-Solís:** Validation, Investigation, Writing - original draft. **Hipólito Gómez-Couso:** Investigation. **Lumila Fuschs:** Investigation. **Marcelo Fort:** Investigation, Funding acquisition. **Claudia Rengifo-Herrera:** Investigation. **Vanesa Navarro-Lozano:** Investigation. **Luis M. Ortega-Mora:** Conceptualization, Writing - review & editing, Funding acquisition. **Esther Collantes-Fernández:** Conceptualization, Writing - original draft, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.108965.

References

Arranz-Solís, D., Pedraza-Díaz, S., Miró, G., Rojo-Montejo, S., Hernández, L., Ortega-Mora, L.M., Collantes-Fernández, E., 2016. *Tritrichomonas foetus* infection in cats with diarrhea from densely housed origins. Vet. Parasitol. 221, 118–122.

Benson, G., 1999. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res. 27 (2), 573–580.

BonDurant, R.H., 1997. Pathogenesis, diagnosis and management of trichomoniasis in cattle. Vet. Clin. North Am. Food Anim. Pract. 23, 345–361.

Castelo, A.T., Martins, W., Gao, G.R., 2002. TROLL–tandem repeat occurrence locator. Bioinformatics 18 (4), 634–636.

Casteriano, A., Molini, U., Kandjumbwa, K., Khaiseb, S., Frey, C.F., Šlapeta, J., 2016. Novel genotype of *Tritrichomonas foetus* from cattle in Southern Africa. Parasitology 143 (14), 1954–1959.

Collantes-Fernández, E., Moreno-Gonzalo, J., Sánchez-Sánchez, R., García-Bocanegra, I., Horcajo, P., Ortega-Mora, L.M., 2019. Prevalence of bovine trichomonosis and associated risk factors in bulls from Spanish beef herds. Theriogenology. 2 (128), 116–121.

Conrad, M., Zubacova, Z., Dunn, L.A., Upcroft, J., Sullivan, S.A., Tachezy, J., Carlton, J.M., 2011. Microsatellite polymorphism in the sexually transmitted human pathogen *Trichomonas vaginalis* indicates a genetically diverse parasite. Mol. Biochem. Parasitol. 175 (1), 30–38.

Felleisen, R.S., 1997. Comparative sequence analysis of 5.8S rRNA genes and internal transcribed spacer (ITS) regions of trichomonadid protozoa. Parasitology 115 (Pt 2), 111–119.

Felleisen, R.S., Lambelet, N., Bachmann, P., Nicolet, J., Muller, N., Gottstein, B., 1998. Detection of *Tritrichomonas foetus* by PCR and DNA enzyme immunoassay based on rRNA gene unit sequences. J. Clin. Microbiol. 36 (2), 513–519.

Frey, C.F., Müller, N., 2012. *Tritrichomonas*–systematics of an enigmatic genus. Mol. Cell. Probes 26 (3), 132–136.

Gerbod, D., Sanders, E., Moriya, S., Noel, C., Takasu, H., Fast, N.M., Delgado-Viscogliosi, P., Ohkuma, M., Kudo, T., Capron, M., Palmer, J.D., Keeling, P.J., Viscogliosi, E., 2004. Molecular phylogenies of Parabasalia inferred from four protein genes and comparison with rRNA trees. Mol. Phylogenet. Evol. 31 (2), 572–580.

Gookin, J.L., Breitschwerdt, E.B., Levy, M.G., Gager, R.B., Benrud, J.G., 1999. Diarrhea associated with trichomonosis in cats. J. Am. Vet. Med. Assoc. 215 (10), 1450–1454.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35 (6).

Levy, M.G., Gookin, J.L., Poore, M., Birkenheuer, A.J., Dykstra, M.J., Litaker, R.W., 2003. *Tritrichomonas foetus* and not *Pentatrichomonas hominis* is the etiologic agent of feline trichomonal diarrhea. J. Parasitol. 89 (1), 99–104.

Lun, Z.R., Chen, X.G., Zhu, X.Q., Li, X.R., Xie, M.Q., 2005. Are *Tritrichomonas foetus* and *Tritrichomonas suis* synonyms? Trends Parasitol. 21 (3), 122–125.

Mendoza-Ibarra, J.A., Pedraza-Díaz, S., García-Peña, F.J., Rojo-Montejo, S., Ruiz-Santa-Quiteria, J.A., San Miguel-Ibáñez, E., Navarro-Lozano, V., Ortega-Mora, L.M., Osoro, K., Collantes-Fernández, E., 2012. High prevalence of *Tritrichomonas foetus* infection in Asturiana de la Montaña beef cattle kept in extensive conditions in Northern Spain. Vet. J. 193, 146–151.

Morin-Adeline, V., Lomas, R., O'Meally, D., Stack, C., Conesa, A., Šlapeta, J., 2014. Comparative transcriptomics reveals striking similarities between the bovine and feline isolates of *Tritrichomonas foetus*: consequences for in silico drug-target identification. BMC Genomics 15 955-2164-15-955.

Oyhenart, J., Breccia, J.D., 2014. Evidence for repeated gene duplications in *Tritrichomonas foetus* supported by EST analysis and comparison with the *Trichomonas vaginalis* genome. Vet. Parasitol. 206 (3-4), 267–276.

Prokopi, M., Chatzitheodorou, T., Ackers, J.P., Clark, C.G., 2011. A preliminary investigation of microsatellite-based genotyping in Trichomonas vaginalis. Trans. R. Soc. Trop. Med. Hyg. 105 (8), 479–481.

Reinmann, K., Muller, N., Kuhnert, P., Campero, C.M., Leitsch, D., Hess, M., Henning, K., Fort, M., Muller, J., Gottstein, B., Frey, C.F., 2012. *Tritrichomonas foetus* isolates from cats and cattle show minor genetic differences in unrelated loci ITS-2 and EF-1alpha. Vet. Parasitol. 185 (2-4), 138–144.

Rogers, M.B., Watkins, R.F., Harper, J.T., Durnford, D.G., Gray, M.W., Keeling, P.J., 2007. A complex and punctate distribution of three eukaryotic genes derived by lateral gene transfer. BMC Evol. Biol. 7 89-2148-7-89.

Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132, 365–386.

Sager, H., Ferre, I., Henning, K., Ortega-Mora, L.M., 2007. Tritrichomonosis. In: Ortega-Mora, L.M., Gottstein, B., Conraths, F.J., Buxton, D. (Eds.), Protozoal Abortion in Farm Ruminants: Guidelines for Diagnosis and Control. CAB International, Oxfordshire, U.K, pp. 232–263.

Šlapeta, J., Craig, S., McDonell, D., Emery, D., 2010. *Tritrichomonas foetus* from domestic cats and cattle are genetically distinct. Exp. Parasitol. 126 (2), 209–213.

Šlapeta, J., Muller, N., Stack, C.M., Walker, G., Lew-Tabor, A., Tachezy, J., Frey, C.F., 2012. Comparative analysis of *Tritrichomonas foetus*(Riedmuller, 1928) cat genotype,*T. foetus* (Riedmuller, 1928) cattle genotype and *Tritrichomonas suis* (Davaine, 1875) at 10 DNA loci. Int. J. Parasitol. 42 (13–14), 1143–1149.

Stechmann, A., Baumgartner, M., Silberman, J.D., Roger, A.J., 2006. The glycolytic pathway of *Trimastix pyriformis* is an evolutionary mosaic. BMC Evol. Biol. 6 101-2148-6-101.

Stroud, L.J., Šlapeta, J., Padula, M.P., Druery, D., Tsiotsioras, G., Coorssen, J.R., Stack, C.M., 2017. Comparative proteomic analysis of two pathogenic *Tritrichomonas foetus* genotypes: there is more to the proteome than meets the eye. Int. J. Parasitol. 47 (4), 203–213.

Sun, Z., Stack, C., Šlapeta, J., 2012. Sequence differences in the diagnostic region of the cysteine protease 8 gene of *Tritrichomonas foetus* parasites of cats and cattle. Vet. Parasitol. 186 (3–4), 445–449.

Suzuki, J., Kobayashi, S., Osuka, H., Kawahata, D., Oishi, T., Sekiguchi, K., Hamada, A., Iwata, S., 2016. Characterization of a human isolate of *Tritrichomonas foetus* (cattle/swine genotype) infected by a zoonotic opportunistic infection. J. Vet. Med. Sci. 78 (4), 633–640.

Tachezy, J., Tachezy, R., Hampl, V., Sedinova, M., Vanacova, S., Vrlik, M., Van Ranst, M., Flegr, J., Kuldaa, J., 2002. Cattle pathogen *Tritrichomonas foetus* (Riedmuller, 1928) and pig commensal *Tritrichomonas suis* (Gruby & Delafond, 1843) belong to the same species. J. Eukaryot. Microbiol. 49 (2), 154–163.

Viscogliosi, E., Müller, M., 1998. Phylogenetic relationships of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase, from parabasalid flagellates. J. Mol. Evol. 47 (2), 190–199.

Wu, G., Fiser, A., ter Kuile, B., Sali, A., Müller, M., 1999. Convergent evolution of *Trichomonas vaginalis* lactate dehydrogenase from malate dehydrogenase. Proc. Natl. Acad. Sci. U. S. A. 96 (11), 6285–6290.

Yao, C., Ketzis, J.K., 2018. Aberrant and accidental trichomonad flagellate infections: rare or underdiagnosed? Trans. R. Soc. Trop. Med. Hyg. 112 (2), 64–72.

Yao, C., Köster, L.S., 2015. *Tritrichomonas foetus* infection, a cause of chronic diarrhea in the domestic cat. Vet. Res. 46 35-015-0169-0.