Genetic and morphometric characterisation of clonal cultures from new avian oropharyngeal trichomonad variants

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ABSTRACT

Extensive diversity and even new species have been described within the avian oropharyngeal trichomonad complex in recent years. In this study we developed clonal cultures from four isolates selected by their different ITS1/5.8S/ITS2 (ITS) genotype and their association with gross lesions of avian trichomonosis. Isolates were obtained from an adult racing pigeon (*Columba livia*) with clinical signs of avian trichomonosis, a juvenile wood pigeon (*Columba palumbus*) and an European turtle dove (*Streptopelia turtur*) without clinical signs, and a nestling of Eurasian eagle owl (*Bubo bubo*) with gross lesions. Multi-locus sequence typing analysis of the ITS, small subunit of ribosomal rRNA (SSUrRNA) and Fe-hydrogenase (Fe-hyd) genes together with a morphological study by optical and scanning electron microscopy was performed. No differences in the structures were observed with scanning electron microscopy. However, the genetic characterisation revealed three novel sequence types: one for the SSUrRNA region and two for the Fe-hyd gene. Clones from the Eurasian eagle owl and the wood pigeon were identified as *Trichomonas gallinae* analysing each locus, with an ITS genotype of *T. gallinae*-1 and *T. gallinae*-2 groups, respectively, which are commonly reported in wild birds from Europe and America. Clones of trichomonads from the racing pigeon and European turtle dove showed higher similarity with *Trichomonas tenax* and *Tetratrichomonas canistomae* than with *T. gallinae* strains at their ITS region, respectively. SSUrRNA sequences grouped clones in a *T. gallinae, T. tenax* and *T. canistomae* clade. Further diversity of *T. gallinae* was detected within the Fe-hyd locus. Morphometric comparison by optical microscopy with clonal cultures of *T. gallinae* (*T. gallinae*-1 and *T. gallinae*-2 ITS genotypes), revealed significant statistical differences on axostyle projection in the clone from European turtle dove, which was 1.6 µm longer (mean 8.2 µm) than the mean values reported for *T. gallinae* (6.4 µm). Possible new species within the *Trichomonas* genus were detected in isolates obtained from the racing pigeon and the European turtle dove, but further reports will be needed to confirm their host distribution.

Introduction

Avian trichomonosis is one of the most important infectious diseases of wild birds with an emergent status. Latest outbreaks documented in passeriform species affected several countries with important consequences on the dynamics of avian populations (Forzán et al. 2010; Ganas et al. 2014; Lawson et al. 2011; Neimanis et al. 2010). *Trichomonas gallinae* (Rivolta 1878) is the causative agent of these mortality episodes, a flagellated protozoan unable to survive for long periods of time outside the host. Trophozoites from 6.2-20 µm in length are the parasitic stage of this parabasalid, with four anterior flagella and a fifth recurrent one that forms the undulating membrane (BonDurant and Honigberg 1994; Melhorn et al. 2009; Stabler 1941). Furthermore, pseudocysts formation has been described under suboptimal *in vitro* conditions, but it has not been detected in natural infections (Tasca and De Carli 2003). This stress-triggered morphotype lacks external flagella and undulating membrane, which seem to have been internalized through an invagination process (Tasca and De Carli 2003).

Direct transmission of trophozoites through contact with infected saliva or regurgitated food is the common route of infection, although contaminated water and carrion-feeding are also important (Erwin et al. 2000; Purple et al. 2015). Columbiform species are the reservoir host of the protozoan, with endemic levels of disease. Besides, they are the primary source of infection for other birds such as birds of prey or passeriforms that share feeding stations or water sources with them.

In recent years, new investigations of symptomatic cases have changed the etiology of avian trichomonosis as new agents were discovered. Several research groups reported other protozoans that were not genetically identified as *T. gallinae*. In 2009, a new *Trichomonas* sp. was isolated from mockingbirds (*Mimus polyglottos*) in USA, in 2012 a *Simplicomonas*-like organism was detected in green-winged saltators (*Saltator similis*) in Brasil and in 2014 the new species of *Trichomonas stableri* was described from white-winged pigeons (*Patagioenas fasciata monilis*) in USA (Anderson et al. 2009; Ecco et al. 2012; Girard et al. 2013 and 2014). Coinfections of *T. stableri* and *T. gallinae* were found in birds with gross lesions of avian trichomonosis (Girard et al. 2013). In addition, several authors reported strains with higher similarity to *Trichomonas tenax*, *Trichomonas vaginalis* or *Tetratrichomonas canistomae* organisms than with *T. gallinae* (Gerhold et al. 2008; Grabensteiner et al. 2010; Kelly-Clark et al. 2013; Martínez-Herrero et al. 2014). For instance, *T. tenax*-like organisms were found in symptomatic racing pigeons (*Columba livia*) from Austria, while *T. vaginalis*-like protozoans were isolated from a bearded vulture (*Gypaetus barbatus*) from the Czech Republic and an American bald eagle (*Haliaeetus leucocephalus*) from Canada without clinical signs (Grabensteiner et al. 2010; Zimre-Grabensteiner et al. 2011; Kelly-Clark et al. 2013). Later, the discovery of a novel organism, in this case associated with a particular host species in Spain, *Trichomonas gypaetinii* on scavenging birds of prey, determined that the previously described *T. vaginalis*-like organisms belonged to this new species (Martínez-Díaz et al. 2015). Additionally, *T. canistomae*-like isolates were found in European turtle doves (*Streptopelia turtur*) and Northern goshawks (*Accipiter gentilis*) without gross lesions of avian trichomonosis in Spain (Martínez-Herrero et al. 2014).

Despite this increase in the number of genetic variants and species of avian oropharyngeal trichomonads, there is still poor knowledge about the epizoological implications of these newly identified trichomonads. As a result, the diagnosis based on culture or cytology is turning difficult to interpret. Therefore, due to this increasing diversity and considering that both parasites and commensal organisms are found within the Trichomonadidae family, the genetic characterisation of avian trichomonosis outbreaks in the nature is strongly recommended. At least one genetic marker useful for their phylogenetic classification, such as the ribosomal region of the ITS1/5.8S/ITS2 (ITS) should be always included (Felleisen 1997; Kleina et al. 2004; Sansano-Maestre et al. 2016).

The objectives of this study were to perform a genetic and morphometric analysis of four clonal cultures of genetically different oropharyngeal trichomonads. The isolates were selected attending to their ITS genetic profile, that was previously described in other studies (Grabensteiner et al. 2010; Zimre-Grabensteiner et al. 2011; Martínez-Herrero et al. 2014). *Trichomonas gallinae*, *T. canistomae*-like and *T. tenax*-like strains from symptomatic and asymptomatic birds were considered. A multi-locus sequence typing (MLST) approach was used, adding the small subunit of ribosomal rRNA (SSUrRNA) and Fe-hydrogenase (Fe-hyd) genes. Finally, in order to fully characterise the protists, a morphometric study by optical microscopy as well as a structural evaluation by scanning electron microscopy (SEM) were performed.

Material and methods

Source of the isolates

Oropharyngeal trichomonads were recovered from four different host species, including raptors and columbiforms with or without lesions of avian trichomonosis (table 1). Birds with macroscopical lesions were a nestling of Eurasian eagle owl (*Bubo bubo*) sampled in the wild in collaboration with a bird ringing scientist in Murcia (Spain), and an adult of racing pigeon hospitalized at the Clinical Unit of Avian Medicine, University of Veterinary Medicine, Vienna (Austria). Birds without lesions were juvenile individuals of European turtle dove and a wood pigeon (*Columba palumbus*) from the wildlife recovery centre of "La Granja de El Saler" (Valencia, Spain).

Culture of the parasite

Two types of culture medium were used for the primary isolation of the parasite. Five ml of Trypticase-Yeast-Maltose (TYM) medium, pH 6.5, in 10 ml sterile tubes were used for the isolates from Spain. For 1 liter of TYM medium, the composition was as follows: 20 g of trypticase, 10 g of D(+)-maltose, 10 g of yeast extract, 1 g of L-cysteine and 0.1 g of ascorbic acid (all Sigma-Aldrich, St. Louis, Missouri, USA). Filtration through 0.22 µm filters was used for sterilization (Millipore, Billerica, Massachusetts, USA). The culture medium was enriched with 10% of inactivated fetal bovine serum (FBS), and supplemented with the antibiotics ceftiofur, ticarcillin in combination with vancomycin (36 mg/L each) together with nystatin (24 ml/L, 10,000 IU/ml) as antimycotics (Sigma-Aldrich, St. Louis, Missouri, USA).

For the Austrian isolate, medium 199 (M199) with Earle's salts, L-glutamine, 25 mM of HEPES and L-amino acids (Gibco, Thermo Fisher Scientific, Vienna, Austria) was employed supplemented with 15% of inactivated FBS (Gibco, Thermo Fisher Scientific, Vienna, Austria) and 0.22% of rice starch sterilized by dry heat for 1 hour at 180ºC prior to use (Carl-Roth, Karlsruhe, Germany). In addition, a bacterial culture was added to improve the growth of the protozoan, using 0.5 ml of *Escherichia coli* DH5α-T1 strain previously incubated in agitation at 37ºC for 24 hours in 9 ml of M199. The cultures were routinely passaged every 48 hours in fresh growth medium.

Preparation of clonal cultures

For the establishment of single cell originated cultures, isolates were transferred to M199 with the bacterial strain until optimal growth was achieved. A passage using 1ml of culture in 9 ml of M199 without rice starch and bacteria was done 24 hours prior to the micromanipulation technique.. Single trichomonad cells were isolated and transferred to Eppendorf tubes with 1 ml of M199, using a micromanipulation method, following the protocol of Hess et al. 2006. For that, an inverted microscope (Diaphot 300, Nikon, Austria) was used with Narishige micromanipulators (Narishige, Japan). Growth was examined 48 hours post inoculation by optical microscopy and subcultivation was done in M199. Cryopreservation for long term storage was performed in a cryo freezing container (Mr. Frosty™, ThermoFisher Scientific, Vienna, Austria) at -180°C using 5% of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, Missouri, USA)

as cryoprotective. Clonal cultures were established from all the isolates with different success rates that ranged from 23% (isolate P196 from *S. turtur*) to 83% (isolate R17 from *B. bubo*).

For DNA extraction, 1 ml of the every clonal culture in exponential growth phase was used. Cells were centrifuged at 1,200 rpm for 3 minutes to remove the culture medium and washed with sterile PBS (pH 7.2). Silica-based purification of genomic DNA using columns of a commercial kit was employed following the manufacturer's instructions (DNeasy Blood and Tissue Extraction kit, QIAGEN, Valencia, California, USA).

Genetic characterisation

A MLST approach including the ITS, SSUrRNA and Fe-hyd regions was used to characterise the selected clonal cultures (Table 1). All reactions contained a final volume of 25 µL of: 12.5 µL HotStarTaq Master Mix Kit polymerase (QIAGEN, Hilden, Germany), 8 µL of PCR water (QIAGEN, Hilden, Germany), 1 µL of each primer and 2.5 µL of genomic DNA. Primers for the ITS region were: TFR1 (5'-TGCTTCAGTTCAGCGGGTCTTCC-3') and TFR2 (5'-CGGTAGGTGAACCTGCCGTTGG-3'), for the SSUrRNA: Hm-Long-f (5'-AGGAAGCACACTATGGTCATAG-3') and Hm-Long-r (5'-CGTTACCTTGTTACGACTTCTCCTT-3') and for the Fe-hyd gene: Fe-hyd-for (5'-GTTTGGGATGGCCTCAGAAT-3') and Fe-hyd-rev (5'-AGCCGAAGATGTTGTCGAAT-3').

The protocols of Ganas et al. (2014) were applied for the temperature cycles of polymerase chain reactions (PCR). For the ITS and SSUrRNA: 15 min at 95ºC for initial denaturation, 40 cycles of 30 s at 94ºC, 1 min at 55ºC, 2 min at 72ºC and a final extension of 10 min at 72ºC. For the Fe-hyd: 15 min at 95ºC for initial denaturation, 40 cycles of 30 s at 94ºC, 1 min at 58ºC, 2 min at 72ºC and finally, 10 min at 72ºC. A GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, California, USA) was used to carry out the PCRs. Products were charged on 1% agarose gels stained with ethidium bromide (Sigma-Aldrich, St. Louis, Missouri, USA). Electrophoresis was done at 100 mV, 400 mA for 30 minutes. Bands were visualized in a UV light transilluminator (SYNGENE, Cambridge, United Kingdom).

Sanger sequencing reactions were performed at the laboratory of Sistemas Genómicos, S. A. (Paterna, Valencia, Spain) in a 3730xl DNA Analyzer automated sequencer with the ABI PRISM® BigDye™ Terminator Ready Reaction Cycle Sequencing kit, v3.1 (Applied Biosystems, Foster City, California, USA). Chromatograms were manually examined and assembled with Lasergene SeqMan software version 7.0.0 (DNASTAR, Madison, Wisconsin, USA).

The Nucleotide Basic Local Alignment Search Tool (BLAST) version 2.3.1, from the National Centre of Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi/) was used for the genetic comparisons. Mega BLAST algorithm with optimization for highly similar sequences and low complexity region filter were chosen. Novel nucleotide sequences were submitted to the public database of Genbank (NCBI). Accession numbers are listed in Table 1. MEGA 6 version 6.0 was used for multiple alignments and phylogenetic trees (Figures 1-3, Tamura et al. 2013).

Optical microscopy

Prior to study the trophozoites by optical microscopy, clonal cultures were axenized in order to remove any possible source of bacterial or fungal contamination that could interfere with the visual examination of the cells. Initially, clones were thawed in M199 with the bacterial strain and transferred to TYM medium gradually. For this process, growth was closely monitored by optical microscopy. Passages in M199 without the bacterial strain and later with the addition of antibiotics, were done before the inoculation in TYM medium. Penicillin (200 IU/ml) and streptomycin (200 µg/ml) were used to remove the *E. coli* DH5α-T1 strain.

Once the clonal cultures grew properly in TYM medium, different aliquots of 100 µl were taken, mixing the culture content, to check for contamination. Aliquots were seeded in nutritive and Sabouraud agars (BioMérieux, Madrid, Spain). Nutritive agars were incubated at 37ºC for 48 hours and Sabouraud plates were kept at 28ºC for 10 days preserved from light. When the axenization process was completed, 1 mL of the clonal cultures was transferred to Eppendorf tubes. Trophozoites were sedimented by centrifugation (3 minutes, 1,500 rpm) and the culture medium was removed. The pellet was resuspended in 50 µl of fresh medium, smeared on glass slides and stained with Diff-Quick (Medion Diagnostics, AG, Düdingen, Switzerland).

For the morphometric study, 30 trophozoites of each preparation were randomly selected using an optical microscope (Zeiss Axioskop 20, Carl Zeiss, Germany) with a CMEX 10.0 digital camera (Euromex, Holland). Different criteria related to cell body, nucleus, four anterior flagella, undulating membrane and axostyle were measured using the software ImageFocus 4, 2.6 version (Euromex, Holland). The parameters considered were length, width, perimeter and surface of the cell; length of four anterior flagella; length of the axostyle projection and total length of the axostyle.

Scanning electron microscopy

For electron microscopy analysis, trophozoites were washed twice with sterile PBS (pH 7.2) for 15 minutes and centrifuged at 1,500 rpm for three minutes. Samples were fixed with a PBS solution containing 2.5% glutaraldehyde and 4% paraformaldehyde for at least 12 hours. Cells were washed during 10 minutes in sterile PBS (pH 7.2) and centrifuged at 700 rpm for ten minutes, this process was repeated three times to remove the fixing solution. Trophozoites were transferred to 0.22 µm cellulose filters and dehydrated in acetones of increasing concentration (30%, 50%, 70%, 80%, 90% and 100%). Critical point drying and metalization with gold were performed at the National Center of Electron Microscopy (Madrid, Spain) before examination using a JEOL JSM 6400 scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Statistical analysis

The morphometric data from the clones was compared by a multivariate analysis of variance (MANOVA) to assess if there were differences between the clones on a linear combination of the measured variables. To avoid problems of collinearity, highly correlated variables ($r \geq 0.700$, $p < 0.01$) were identified by calculating the Pearson's correlation coeficients and discarded for the analysis. Variables showing statistical differences among the clones were detected by univariate ANOVA tests and Scheffé post hoc tests were employed to identify their possible grouping. All statistical comparisons were performed using the IBM SPSS Statistics software version 21 (IBM Corp., Armonk, New York, USA).

Results and discussion

Genetic characterisation

Nucleotide sequences were submitted to the GenBank public database (Table 1). Overall, the MLST and phylogenetic analysis revealed three new genotypes, one of the SSUrRNA region and two novel of the Fe-hyd gene in two clones, 7895-C2 and P196-C20 (Table 1, Figures 1-3). Clones P178-C7 and R17-C1 were identified as *T. gallinae* organisms by all three genetic loci, with ITS genotype *T. gallinae*-1 and *T. gallinae*-2, SSUrRNA genotype VI and KM246609 and Fe-hyd subtypes A1 and C4, correspondingly (genotype nomenclature according to Grabensteiner et al. 2010; Chi et al. 2013; Martínez-Herrero et al. 2014). Additionally, one single nucleotide polymorphism (SNP) was detected in the ITS sequence of clone P178-C7. Interestingly, the ITS region of clones 7895-C2 and P196-C20 demonstrated a higher similarity with *T. tenax* and *T. canistomae,* respectively, than with *T. gallinae* strains,.

Clone 7895-C2

Sequences of the ITS and SSUrRNA were published in a previous study with Genbank accession numbers FN433474 and FN433479, respectively. The organism was designated as *Trichomonas sp.* with genotype II for the ITS region and genotype I for the SSUrRNA (Grabensteiner et al. 2010).

BLAST analysis indicated that the ITS region had the highest similarity (99% identity with one gap) with a *Trichomonas* sp. sequence from Australasian columbiformes (Genbank acc. n. JQ755287). Interestingly, Lennon et al. (2013) found the same genetic variant in columbiformes from the UK (sequence type 2 showing 100% identity with JQ030996). Considering only the sequences deposited in Genbank from organisms identified up to the species level (that is, excluding the *genus* sp. registers), the next match (99% identity, 294/297 nucleotide differences with one gap) corresponds to a *T. tenax* sequence isolated from a man (Genbank acc. n. KF164607).

The other ribosomal marker, the SSUrRNA region, obtained maximum similarity with a *Trichomonas* sp. from a grey-capped Emerald dove (*Chalcophaps indica*) (Genbank acc. n. JQ030997). However, the first match with an organism that was identified to the species level corresponded to a *T. gallinae* strain

from an Eurasian collared-dove (Genbank acc. n. FN433481, 99% identity, 6 different nucleotides). In addition, another clone was obtained from this isolate (7895-C1, not included in this study) with identical nucleotide sequences for the ITS and SSUrRNA genes (Grabensteiner et al. 2010).

Finally, for the Fe-hyd, a new sequence was obtained. This novel subtype had only 97% identity, 819/842 nucleotides, with a *T. gallinae* strain isolated from a Zebra dove (*Geopelia striata*) from Seychelles islands (Genbank acc. n. JF681142).

These results indicate that this organism, found in a racing pigeon with clinical signs of avian trichomonosis in Austria, seems to be more genetically related to *T. tenax* than to *T. gallinae*, as the ITS region demonstrated only three different nucleotides (294/297 with one gap) with the *T. tenax* strain, while the double number of base pairs (1122/1128) was found different for the SSUrRNA region that matched with a *T. gallinae* strain. For the Fe-hyd gene, a lower identity with *T. gallinae* was found (97%, 819/842 nucleotides). Ribosomal genetic markers (ITS and SSUrRNA) had more conserved sequences than the Fe-hyd single-copy gene, but also the recent inclusion of this gene for fine molecular typing of avian trichomonads implies that a lower number of nucleotide sequences are available on public databases which could be another explanation for this difference.

Clone P178-C7

This clone was also completely identified as *T. gallinae*, as the ITS region indicated genotype *T. gallinae*-2 (Genbank acc. n. EU881912), although one SNP was detected (transition of A to G). The SSUrRNA fragment showed 100% identity with the *T. gallinae* Genbank sequence KM246609 and the Fe-hyd belonged to C4 subtype (Genbank acc. n. KJ184172; genotype names according to Chi et al. 2013; Martínez-Herrero et al. 2014).

Clone P196-C20

The ITS fragment from this clone revealed that maximum identity (99%, 254/256 nucleotides) was found with a *Trichomonas* sp. strain obtained from a Northern goshawk (Genbank acc. n. KF993706). The clone sequence was identical to the ITS region of the original isolate P196-13, previously published as genotype *T. canistomae*-like-1 (Genbank acc. n. KF993705, Martínez-Herrero et al. 2014). However, the next highest similarity matched with an organism defined at least to the species level (97%, 252/259 with three gaps) was found with a *T. canistomae* strain isolated from the oral cavity of a dog (Genbank acc. n. AY244652).

Our results are in contrast with the ITS genotypes found by Lennon et al. (2013) on eight European turtle doves from the UK. In the last mentioned work, three distinct genotypes were found, two of them typed as *T. gallinae* in previous studies (Sansano-Maestre et al. 2009; Reimann et al. 2012), which corresponded with the ITS sequence group *T. gallinae*-1 and *T. gallinae*-2. One nestling that showed clinical signs harboured genotype *T. gallinae*-1 that was associated

with the presence of gross lesions on previous studies (Sansano-Maestre et al. 2009; Martínez-Herrero et al. 2014). Interestingly, the third genotype was from an undetermined *Trichomonas* sp. organism phylogenetically related to *T. tenax* (Peters and Raidal, unpublished data). The phylogenetic analysis of the ITS region of clone 7895-C2 indicated 99% similarity (one gap) with this third genotype described by Lennon et al. (2013). Besides, Stockdale et al. (2014) studied 25 European turtle doves from the same country, with clinical signs of avian trichomonosis on three adult birds. Their results indicated that four genotypes of the ITS region were present, two were *T. gallinae-1* and *T. gallinae-2* groups (Martínez-Herrero et al. 2014) and two others were *Trichomonas* sp. Once more, genotype *T. gallinae-1* was isolated from birds with clinical signs. The other *Trichomonas* sp. genotypes were similar to *T. tenax* and *T. canistomae* strains. However, our results from clone 7895-C2 and P196-C20 did not have complete identity with these genetic variants.

For the other two loci, SSUrRNA and Fe-hyd, new genotypes were obtained. The SSUrRNA fragment had 99% identity (1398/1407 nucleotides) with a *T. gallinae* sequence (Genbank acc. n. KM246609) and the Fe-hyd had only 95% (814/853 nucleotides) with a *T. gallinae* strain (Genbank acc. n. KJ184172).

In the present work, the ITS region indicated that this organism is genetically related with *T. canistomae*, a trichomonad species from the oral cavity of canines. This result was also obtained in other two isolates from European turtle doves that were described in a previous study (Martínez-Herrero et al. 2014). The addition of the SSUrRNA and Fe-hyd regions revealed that these isolates (n=3) also shared the same nucleotide sequence for thegenetic markers. These findings suggest that new oropharyngeal trichomonads are present, at least, in this host species, with novel sequences for the SSUrRNA and Fe-hyd genes. Their association with pathology is still unknown, as cultures were recovered from birds without macroscopical lesions, consequently further studies will be required to investigate their pathogenicity.

Clone R17-C1

This clone had 100% identity with other *T. gallinae* Genbank sequences for the three different loci. Genotype group *T. gallinae*-1 was found for the ITS region (Genbank acc. n. EU881913), genotype VI for the SSUrRNA (Genbank acc. n. KM095107) and A1 subtype for the Fe-hyd (Genbank acc. n. KC244201; genotype nomenclature according to Grabensteiner et al. 2010; Chi et al. 2013; Martínez-Herrero et al. 2014).

Optical microscopy

The criteria of cell perimeter, cell surface and individual anterior flagellar length were not considered for statistical comparisons between the clones due to their high correlation.

All variants showed mean values for cell body size ranged from 8-11 µm length and 4.3-7.2 µm width (Table 2, Figure 4). Clones P196-C20 and R17-C1 had the smallest cell size values with statistical significance, with mean values of 8.3 x 4.4 µm and 8 x 4.3 µm, respectively. In contrast, clone P178-C7 obtained the highest values (11 x 6.5 µm), while clone 7895-C2 had intermediate results for these criteria (9.2 x 7.2 µm, Tables 2 and 3). The results from the clone with the highest trophozoite dimensions (11 x 6.5 µm, clone P178-C7), are in accordance with the isolates of identical ITS genotype (*T. gallinae*-2) of Martínez-Díaz et al. (2015) (10.9 x 6.2 µm).

Mehlhorn et al. (2009), reported average cell size dimensions of 11 x 7 µm from a *T. gallinae* clone of genotype *T. gallinae*-1 for the ITS region and VI for the SSUrRNA. Similar values were measured by Martínez-Díaz et al. (2015), from isolates of ITS genotype *T. gallinae*-1 with mean values of 10.6 x 6.7 µm. However, mean values for clone R17-C1, that had the same genetic profile, were comparatively smaller, of 8 x 4.3 µm. Recently described species within the oropharyngeal avian trichomonads, like *T. gypaetinii* and *T. stableri* had also smaller dimensions in comparison with *T. gallinae*. *T. gypaetinii* was described with mean values of 8.1 x 5.8 µm for cell size (Martínez-Díaz et al. 2015), while two morphotypes were found for *T. stableri*: a slender (13 x 6 µm) and rounded (8.1 x 5.9 µm) form (Girard et al. 2013). Therefore, regarding trophozoite dimensions, clone R17-C1 is more similar to *T. gypaetinii* and the rounded form of *T. stableri,* than with the average size reported for *T. gallinae* by other authors (Mehlhorn et al. 2009; Martínez-Díaz et al. 2015). The use of clonal cultures, instead of isolates, implies that a more homogeneous sample was obtained. Indeed, our standard deviation was considerably smaller (1 µm for cell length and 0.7 µm for cell width) than observed by Martínez-Díaz et al. (2015; 1.9 µm for cell length and 1.8 µm for cell width).

In relation to individual anterior flagellar length, mean values varied from 10.0-17.9 µm, a wider range in comparison with previous reports of oropharyngeal avian trichomonads in the literature. Hence, the clone studied by Mehlhorn et al. (2009) measured 11-13 µm, while the isolates of Martínez-Díaz et al. (2015) had a lenght of 13.9-17.8 µm, *T. gypaetinii* of 12.1-14.4 µm (Martínez-Díaz et al. 2015) and a *T. gallinae*- together with a *T. stableri* isolate of 13.87 µm (Girard et al. 2013). Furthermore, significant statistical differences were calculated for these criteria, with the longest flagella of clones P178-C7 and P196-C20, intermediate lengths of clone 7895-C2 and the shortest ones of clone R17-C1. Clone P178-C7, of ITS genotype *T. gallinae*-2, had an anterior flagella with mean values between 12.8-17.1 µm in length. These results include the measurement determined by Girard et al. (2013) in a *T. gallinae* isolate with the same genotype, however, Martínez-Díaz et al. (2015) reported different values from the same type of isolates (14.1-18.2 µm). For clone R17-C1, anterior flagellar length ranged from 10-14 µm, similar results in relation to the study of Mehlhorn et al. 2009 (11-13 µm), that included one *T. gallinae* clone with the same genetic profile.

Axostyle projection mean values ranged from 5.9-8.2 µm length, with the largest measurements (range 6-11 µm) of clone P196-C20 showing a significance statistical difference. *T. gallinae* isolates from Martínez-Díaz et al. (2015) had an average length of 6.6 µm, while *T. gypaetinii* had 7.6 µm. Our results show a broader range of lenght for this criterion..

On the whole, the results revealedd significant morphological differences present among the clones (MANOVA Pillai's Trace test, $p<0.000$). As it is shown in Table 3, clone P196-C20 is clearly distinguishable by its smaller cell size and its larger axostyle projection.The latter characteristic allowed the differentiation from the also small-sized clone R17-C1.

The MLST analysis of clones 7895-C2 and P196-C20 revealed that the ITS region, validated for the phylogenetic classification of trichomonads (Felleisen, 1997; Kleina et al. 2004), indicated higher similarity (99% and 97%) with *T. tenax* and *T. canistomae* than with *T. gallinae* strains, respectively (Table 1, Figure 1). These results agree with morphometric observations, as both clones had very similar trophozoite dimensions. In fact, *T. tenax* had mean values of 7 µm length, with a 5-12 µm range (Gutierrez, 2000), and mean values of clone 7895-C2 were 9.2 µm x 7.2 µm. *T. canistomae* was described to be 7-12 µm in length and 3-4 µm width (Taylor et al. 2016), and clone P196-C20 had an average size of 8.3 x 4.4 µm. In consequence, MLST and morphometric evidences indicate that both clones are different organisms from *T. gallinae*. The intermediate position demonstrated by the ribosomal markers (ITS and SSUrRNA) could reflect an early stage of speciation that will lead to the establishment of novel species within the avian oropharyngeal trichomonad complex.

Scanning electron microscopy

Digital images were inspected to assess superficial structures and external trophozoite shape. The external membrane appeared rumpled in all samples. This phenomenon has been also reported by other authors and could be probably originated by the sample preparation protocol needed for the SEM (Tasca and De Carli 2003). Dehydration and other physicochemical reactions of this previous procedure would damage the integrity of the plasmatic membrane of the parasite.

The outer shape of the trophozoites was pyriform or rounded which is in consistence with the plasticity previously reported (Martínez-Díaz et al. 2015; Melhorn et al. 2009; Tasca and De Carli, 2003). Differences were found with clone P196-C20 in comparison with the other clones, as it showed a rounded appearance, a comparative smaller cell size and a longer axostyle projection. These differences were easily appreciated by visual examination of the images at the same scale (Figure 5).

Conclusion

The comparative study of clonal cultures from oropharyngeal trichomonads with different genetic profile revealed a wide diversity of organisms. Two of the clonal cultures analysed, clones P178-C7 and R17-C1, were genetically identified with complete homology for all loci as *T. gallinae*. Also, these genotypes belonged to the two most commonly distributed among wild birds populations, as it has been demonstrated in epidemiological studies carried out in Europe and America (Gerhold et al. 2008; Anderson et al. 2009; Sansano-Maestre et al. 2009; Chi et al. 2013; Ganas et al. 2014; Martínez-Herrero et al. 2014; McBurney et al. 2015).

However, clones 7895-C2 and P196-C20 were not identified as *T. gallinae* strains. Their ITS region showed maximum identity with a *T. tenax* and *T. canistomae* isolates (99% with 3 different nucleotides and 97% with 7 different nucleotides), respectively. Thus, they were initially named as *Trichomonas* sp.

organisms on previous studies. We have fully characterised these clones by MLST, adding the SSUrRNA and Fe-hyd genes, and by morphometric analysis. The SSUrRNA fragment also revealed that they were newly identified organisms, as full identity was not obtained with *T. gallinae* strains. A 99% identity with different *T. gallinae* strains was demonstrated, with 1122/1128 and 1396/1407 modified nucleotides for clone 7895-C2 and P196-C20, respectively. In addition, further diversity was revealed by the analysis of Fe-hyd gene, as novel sequences were revealed for both clones, with low conformity values with *T. gallinae*. Clone 7895-C2 had 97% identity with 23 distinct nucleotides and clone P196-C20 only 95% with 39 dissimilar nucleotides, a high degree of divergence.

Finally, the morphometric criteria indicated that both clones, 7895-C2 and P196-C20, were within the range of other trichomonad species, although the characteristic plasticity of trophozoites makes difficult their final classification in one or other species attending only to cell size measurements. Therefore, a proper evaluation with genotyping information should be made, in order to avoid misidentifications and to confirm the obtained data. The evidences presented in this study, including the genetic characterization of the parasites, suggest that novel species are present within the avian oropharyngeal trichomonad complex. However, more isolates and clonal cultures would be desirable to confirm their host distribution.

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Table 1. Information of the clonal cultures that were analysed in this study. BLAST sequence identity shows first match and further relevant identifications for comparison. Genotype nomenclature according to Grabensteiner et al. 2010; Chi et al. 2013; Martínez-Herrero et al. 2014. ITS: ITS1/5.8S/ITS2, SSU: small rRNA subunit and Fe: Fe-hydrogenase gene. REF: reference of the sequence, 1: Peters and Raidal, unpublished, 2: López-Escamilla et al. 2013, 3: Girard et al. 2014, 4: Grabensteiner et al. 2010, 5: Lawson et al. 2011, 6: Sansano-Maestre et al. 2009, 7: Martínez-Díaz et al. 2015, 8: McBurney et al. 2015, 9: Martínez-Herrero et al. 2014, 10: Kutisova et al. 2005, 11: Kunca et al. 2015.

							BLAST			
CLONE	**HOST**	**YEAR**	**LESION**	**ACC. N. / GENOTYPE**	**PCR**	**SEQUENCE LENGTH**	**IDENTITY (%)**	**ACC. N.[REF]**	**ORGANISM**	**HOST**
7895-C2	*Columba livia*	2006	Yes	FN433474 / II / *T. tenax*-like-1	ITS	296	99	JQ755287[1]	*Trichomonas* sp.	Australasian columbid
							99	KF164607[2]	*Trichomonas tenax*	Man (*Homo sapiens*)
							95	KC215388[3]	*Trichomonas gallinae*	Band-tailed pigeon (*Patagioenas fasciata monilis*)
				FN433479 / I	SSU	1128	100	JQ030997[1]	*Trichomonas* sp.	Grey-capped Emerald dove *(Chalcophaps indica)*
							99	FN433481[4]	*Trichomonas gallinae*	Eurasian collared-dove *(Streptopelia decaocto)*
				/ Novel	Fe	842	97	JF681142[5]	*Trichomonas gallinae*	Zebra dove (*Geopelia striata*)
P178-C7	*Columba palumbus*	2013	No	/ *T. gallinae*-2, 1 SNP	ITS	244	99	EU881912[6]	*Trichomonas gallinae*	Rock pigeon *(Columba livia)*
					SSU	1407	100	KM246609[7]	*Trichomonas gallinae*	Eurasian buzzard (*Buteo buteo*)
				/ C4	Fe	820	100	KJ184172[8]	*Trichomonas gallinae*	Rock pigeon *(Columba livia)*
P196-C20	*Streptopelia turtur*			/ *T. canistomae*-like-1	ITS	256	100	KF993705[9]	*Trichomonas* sp.	European turtle dove *(Streptopelia turtur)*
							99	KF993706[9]	*Trichomonas* sp.	Northern goshawk *(Accipiter gentilis)*
							97	AY244652[10]	*Trichomonas canistomae*	Dog *(Canis familiaris)*
							97	KC215388[3]	*Trichomonas gallinae*	Band-tailed pigeon (*Patagioenas fasciata monilis*)
				/ Novel	SSU	1407	99	KM246609[7]	*Trichomonas gallinae*	Eurasian buzzard (*Buteo buteo*)
				/ Novel	Fe	853	95	KJ184172[8]	*Trichomonas gallinae*	Rock pigeon *(Columba livia)*
R17-C1	*Bubo bubo*	2012	Yes	/ *T. gallinae*-1	ITS	260	100	EU881913[6]	*Trichomonas gallinae*	Common barn-owl *(Tyto alba)*
					SSU	1396	100	KM095107[11]	*Trichomonas gallinae*	Eurasian sparrowhawk (*Accipiter nisus*)
				/ A1	Fe	909	100	KC244201[3]	*Trichomonas gallinae*	Band-tailed pigeon (*Patagioenas fasciata monilis*)

Table 2. Morphometric results of *Trichomonas* sp. trophozoites from clonal cultures. Values are expressed in micrometers. The four free flagella are ordered from longest to shortest.

Parameter	Mean ± standard deviation (range)			
	Clone 7895-C2	Clone P178-C7	Clone P196-C20	Clone R17-C1
Cell length	9.2 ± 0.9 (8-11)	11 ± 1.5 (14-8)	8.3 ± 1.2 (6-11)	8 ± 1 (6-10)
Cell width	7.2 ± 1.3 (4-9)	6.5 ± 1.5 (4-10)	4.4 ± 0.8 (3-6)	4.3 ± 0.7 (3-6)
Cell perimeter	26.7 ± 3 (21-32)	29.2 ± 3.9 (21-38)	21.2 ± 2.9 (15-29)	21.3 ± 2.1 (18-28)
Cell surface	53.8 ± 13 (32-81)	55.5 ± 16.3 (31-89)	29.1 ± 8.2 (15-53)	29.7 ± 5.9 (18-42)
Length of flagellum 1	16.2 ± 1.3 (14-19)	17.1 ± 1.2 (15-20)	17.9 ± 2.2 (14-23)	14 ± 1.3 (12-18)
Length of flagellum 2	14.5 ± 1.3 (12-18)	16 ± 1.2 (13-18)	15.8 ± 1.6 (13-18)	12.7 ± 1.2 (11-16)
Length of flagellum 3	12.3 ± 1 (10-14)	13.8 ± 1.5 (9-17)	14.2 ± 1.7 (11-18)	11.5 ± 1 (10-14)
Length of flagellum 4	11.5 ± 1.2 (9-14)	12.8 ± 1.7 (7-16)	13.1 ± 1.7 (10-16)	10 ± 1.1 (7-12)
Length of axostyle	16 ± 1.5 (13-20)	18.4 ± 2 (16-23)	17.2 ± 1.7 (13-22)	15.2 ± 1.9 (12-20)
Length of axostyle projection	5.9 ± 1 (4-8)	6.4 ± 2.1 (2-12)	8.2 ± 1.1 (6-11)	6.5 ±1.2 (5-10)

Table 3. Statistical results from the different morphometric criteria considered in this study. Scheffé post hoc tests are indicated with their statistical significance. Mean values correspond with original measurements (non transformed data) in µm.

Transformed variable	Homogeneous group/s	Significance	Clones	Mean
Cell length	1	1.000	7895-C2	9.2
	2	1.000	P178-C7	11
	3	0.911	P196-C20	8.3
			R17-C1	8
Cell width	1	0.996	P196-C20	4.4
			R17-C1	4.3
	2	0.156	7895-C2	7.2
			P178-C7	6.5
Total flagellar length	1	1.000	7895-C2	54.5
	2	1.000	R17-C1	48.2
	3	0.916	P178-C7	59.7
			P196-C20	61
Axostyle projection	1	0.451	7895-C2	5.9
			P178-C7	6.4
			R17-C1	6.5
	2	1.000	P196-C20	8.2

Figure 1. Neighbor-Joining phylogenetic tree based on the ITS1/5.8S/ITS2 region of clonal cultures from this study and other trichomonad species. Evolutionary distances were computed by the Tamura 3-parameter method and are in the units of the number of base substitutions per site. The final alignment contained 217 nucleotides. Codon positions included were 1st+2nd+3rd+Noncoding. Numerical values represent bootstrap percentages from 2000 replicates.

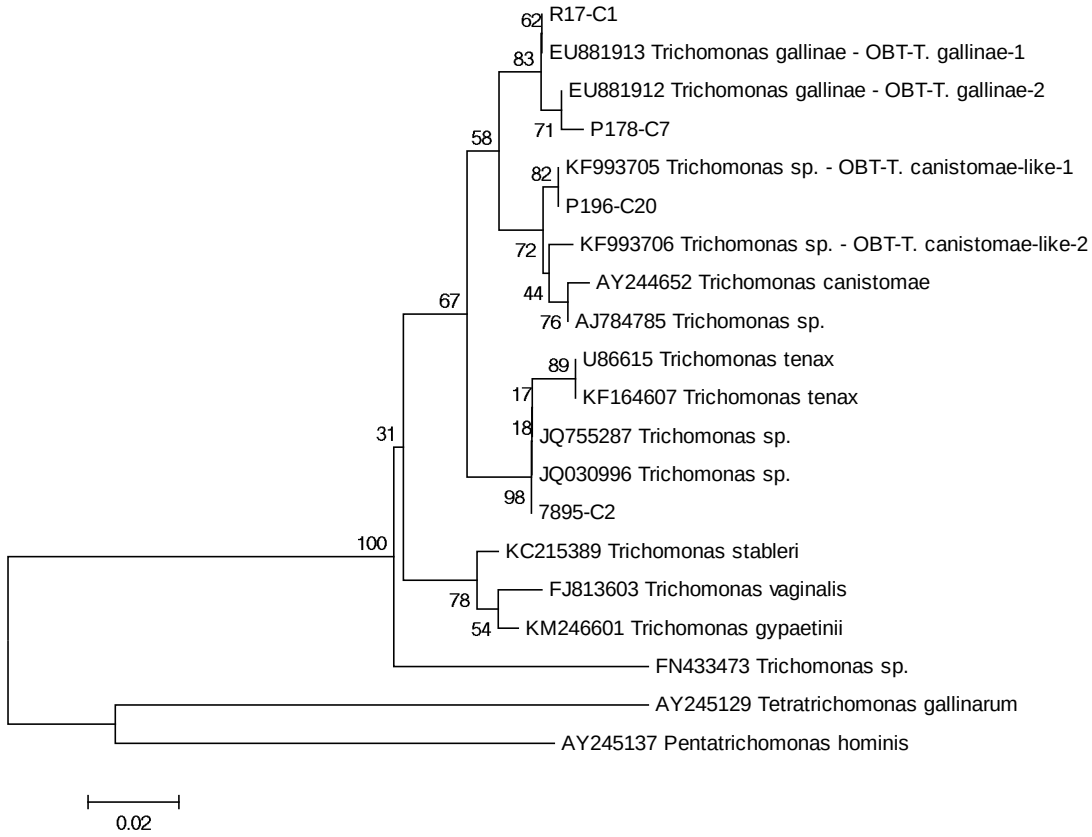


Figure 2. Neighbor-Joining phylogenetic tree based on the sequences from the small subunit of ribosomal rRNA of clonal cultures from this study and other trichomonad species. Evolutionary distances were computed by the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The final alignment contained 1,106 nucleotides. Codon positions included were 1st+2nd+3rd+Noncoding. Numerical values represent bootstrap percentages from 2000 replicates.

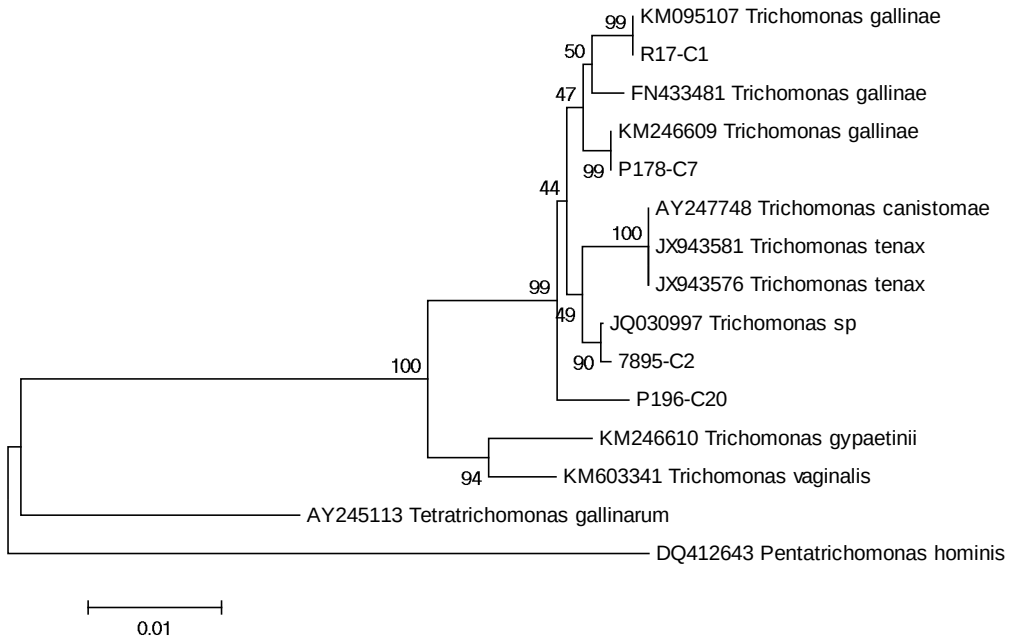


Figure 3. Neighbor-Joining phylogenetic tree based on Fe-hydrogenase sequences of clonal cultures from this study and other trichomonad species. Evolutionary distances are in units of the number of base substitutions per site and were computed by the Kimura 2-parameter method with a discrete Gamma distribution with five rate categories. The final alignment contained 591 nucleotides. Codon positions included were 1st+2nd+3rd+Noncoding. Numerical values represent bootstrap percentages from 2000 replicates.

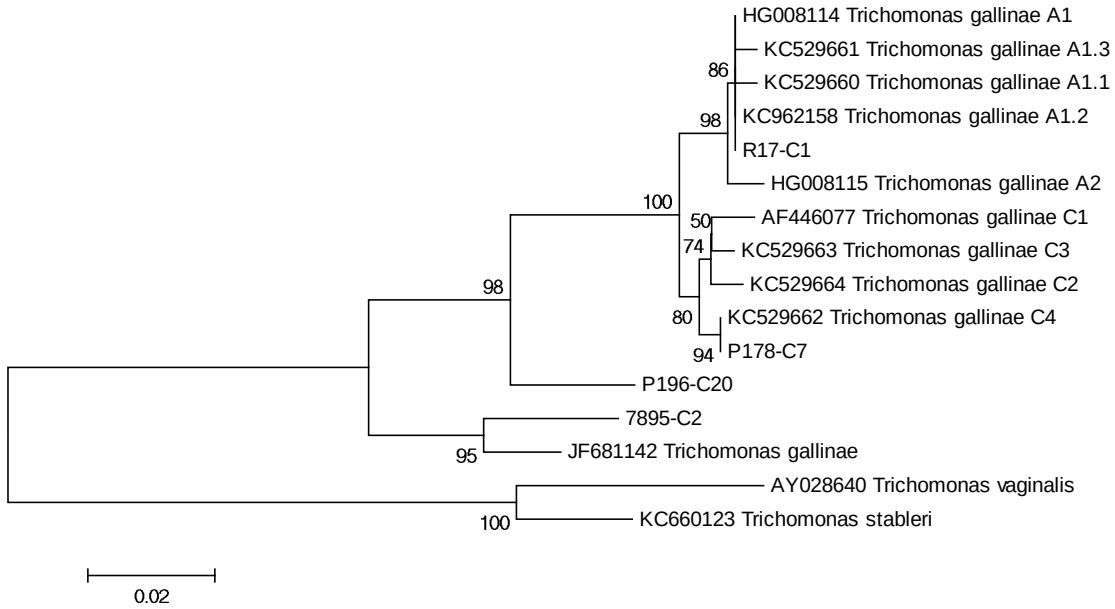
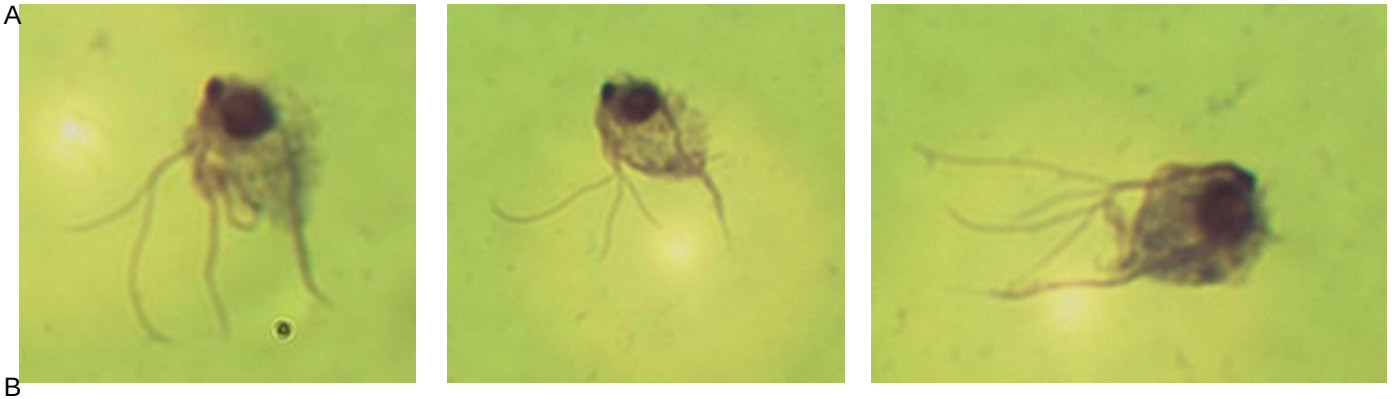
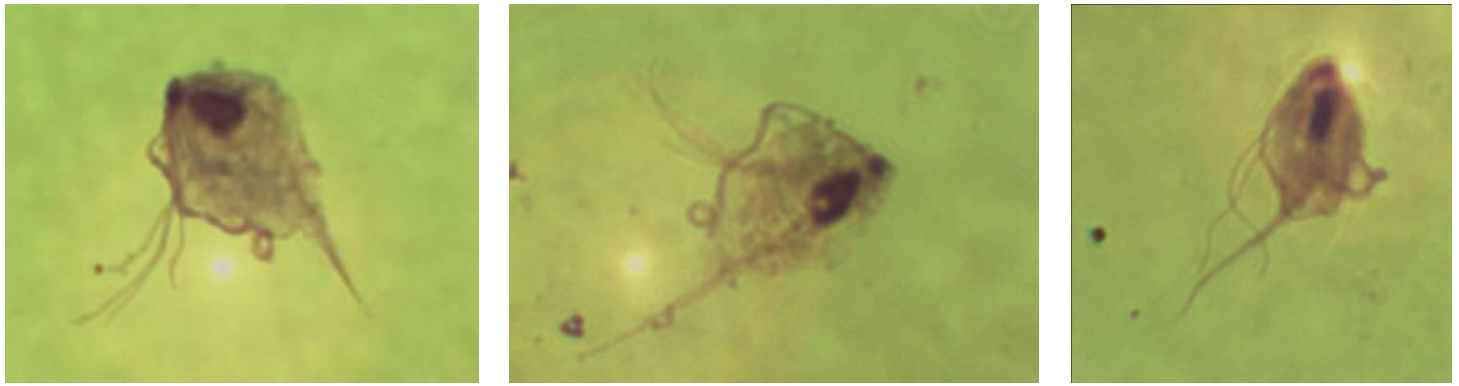


Figure 4. Images of trophozoites from clonal cultures of this study. Diff-Quick staining. A: clone 7895-C2. B: clone P178-C7. C: clone P196-C20. D: clone R17-C1.

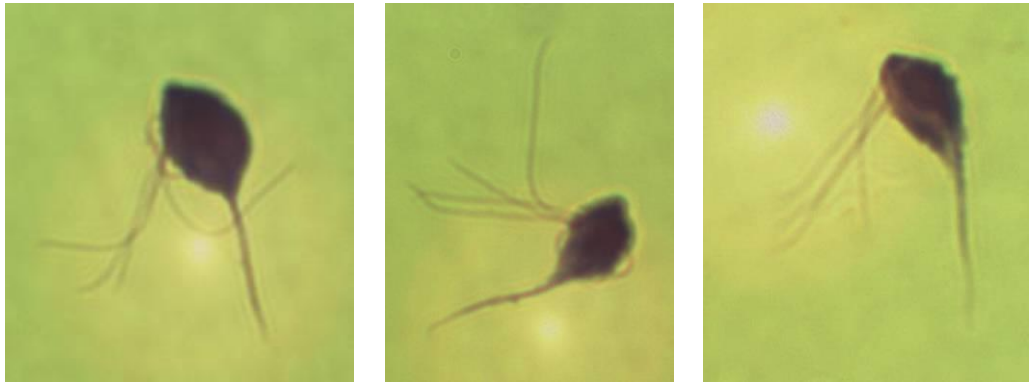
A



B



C



D

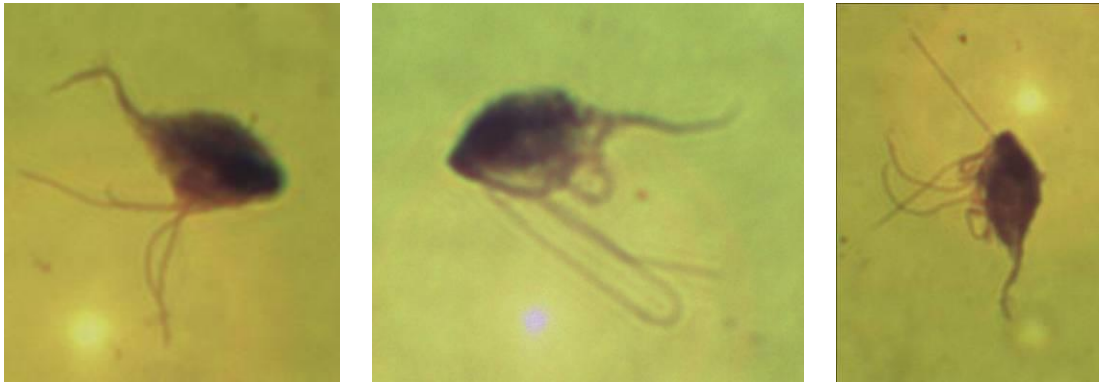
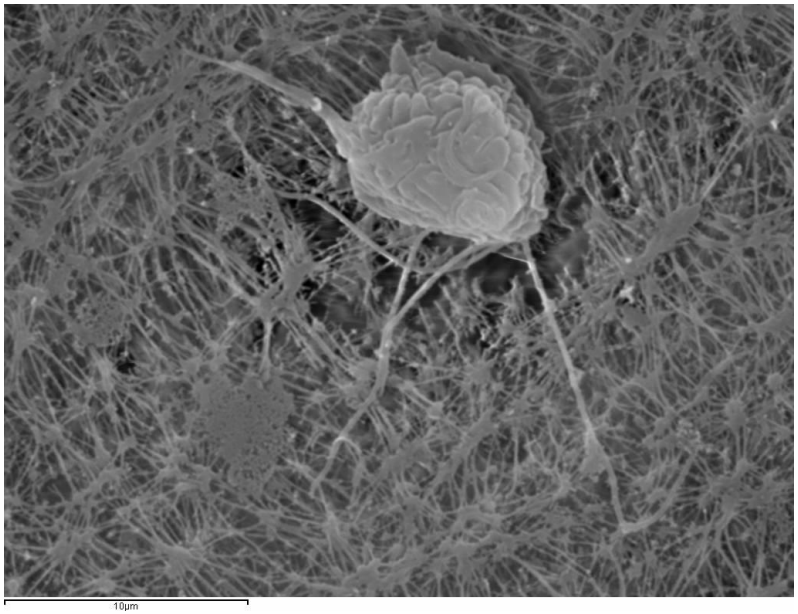
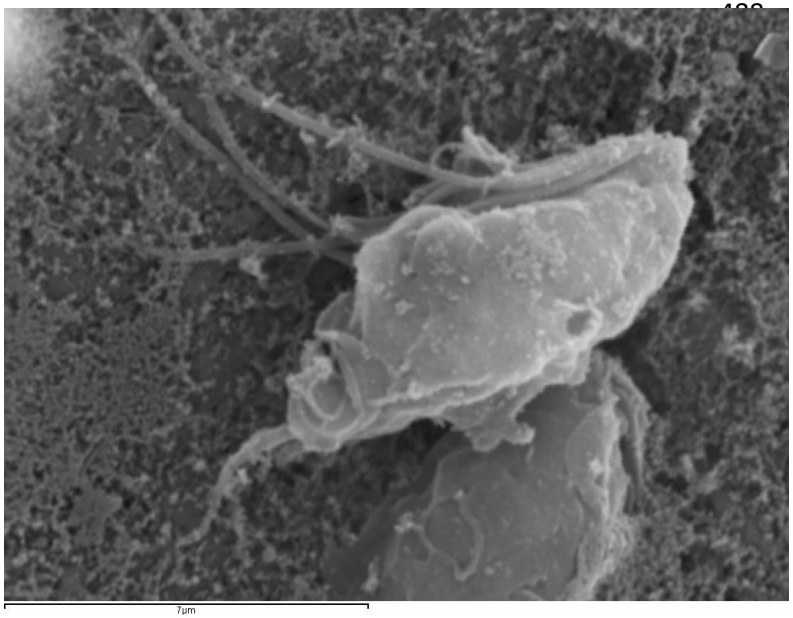


Figure 5. Images of trophozoites from clonal cultures obtained by scanning electron microscopy. Scale bar is denoted at the bottom of each photograph. A: clone 7895-C2. B: clone P178-C7. C: clone P196-C20. D: clone R17-C1.

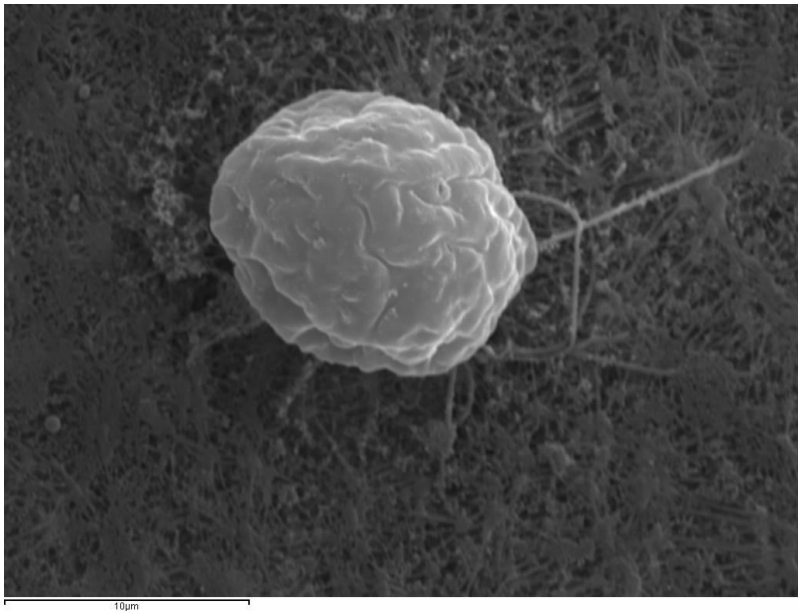
A



B



C



D