CEU Cardenal Herrera University

Department of Animal Production, Animal and Public Health, Food Technology and Science

Characterization of oropharyngeal trichomonads in wild birds. Description of new variants, virulence assays and differential proteomic analysis using clonal cultures

DOCTORAL THESIS

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CERTIFICA:

Que la memoria titulada **"Characterization of oropharyngeal trichomonads in wild birds. Description of new variants, virulence assays and differential proteomic analysis using clonal cultures"**, que, para aspirar al grado de Doctor Internacional en Veterinaria presenta Dña. María del Carmen Martínez Herrero, realizada bajo mi dirección en la Universidad CEU Cardenal Herrera, cumple las condiciones adecuadas para su aceptación como Tesis Doctoral, por lo que,

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A la interesada a su presentación en la Facultad de Veterinaria de la Universidad CEU Cardenal Herrera.

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ABBREVIATION LIST

Abbreviation	Full name
3D	Three dimensions
ºC	Celsius degrees
Acc. n.	Accession number
ACN	Acetonitrile
amu	Atomic mass unit
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
BOE	Boletín Oficial del Estado - Official State Gazette (Spain)
BspA	*Bacteroides* sp. surface A-like protein
C	Clonal culture
cm	Centimeter
CNME	Centro Nacional de Microscopía Electrónica - National Center of Electron Microscopy
CPLM	Cysteine-Peptone-Liver-Maltose medium
CPE	Cytophatogenic effect
Da	Dalton
DH5α-T1	*Escherichia coli* strain resistant to lytic bacteriophage T1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate mix
DOI	Digital Object Identifier
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMEA	European Agency for the Evaluation of Medicinal Products
ER	Endoplasmic reticulum
FDR	False Discovery Rate
Fe	Iron
GO	Gene Ontology
GP63	Glycoprotein of 63 kDa
GTPase	Enzyme that binds guanosine triphosphate (GTP)
HF	Hollander fluid
HPI	Hours post-infection
i.e.	From latin “id est”, meaning that is
IM	Intramuscular
iTRAQ	Isobaric Tags for Relative and Absolute Quantification
ITS	Internal transcribed spacer
IU	International units
kDa	Kilodalton
kg	Kilogram
l	Liter
LC-MS/MS	Liquid chromatography with mass spectrometry
LPO	Ligue pour la Protection des Oiseaux
LTQ	Linear trap quadropole
M199	Medium 199
mA	Mili ampere
MANOVA	Multivariate analysis of variance
mg	Miligram
ml	Mililiter

Abbreviation	Full name
MS	Mass spectra
m/z	Mass-to-charge ratio units
NCBI	National Center for Biotechnology Information
P	Passage
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
ppm	Parts per million
PO	"Per os", oral administration
RAPD	Random Amplified Polimorphic DNA
Ras	Rat sarcome protein of small guanosine triphosphatases (GTPases)
rpb1	Ribonucleic acid polymerase II subunit
RP-LC-MS/MS	Reversed-phase liquid chromatography with mass spectrometry
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S	Svedberg
SAPLIP	Saposin-like pore-forming domain
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
SSU rRNA	Small subunit of ribosomal ribonucleic acid
TEM	Transmission Electron Microscopy
TPB	Tryptose-phosphate-broth
TV	*Trichomonas vaginalis* medium
TYM	Trypticase-Yeast-Maltose medium
UK	United Kingdom
USA	United States of America
UV	Ultraviolet light
V	Volt
vs	Versus
w/w	Weight/weight
µg	Microgram
µl	Microliter
µm	Micrometer

SUMMARY

Avian trichomonosis is a parasitic disease caused by the flagellated protozoan *Trichomonas gallinae* (Rivolta, 1878). Birds from a variety of orders, most frequently Accipitriformes, Columbiformes, Falconiformes, Passeriformes, Psittaciformes and Strigiformes, are susceptible to the infection and development of lesions. Columbiformes are the reservoir hosts of the parasite, presenting high levels of infection. The direct life cycle of the flagellate favours the transmission between gregarious species of birds. The sharing of food and water sources, courtship rituals and the feeding of squabs by adults are the situations in which infected individuals transmit the parasite. Birds of prey are primarily affected after consumption of infected preys or scavenging on their carcasses. Recent epidemics reported on wild finches had placed the status of emergent disease in several countries of Europe, Canada and North America. The presumed infection route were artificial feeders shared with infected columbiformes.

The pathogenicity varies according to several factors, such as the number of reinfections, the immune response of the host and the particular strain of the parasite. The upper alimentary tract of birds, from the oropharyngeal cavity to the oesophagus are the locations most commonly affected, although internal organs (liver, air sacs and lungs, pericardium, peritoneum, pancreas, bones) may also contain lesions. Death is a frequent outcome if treatment is not administered in birds with clinical signs.

Trophozoites initially start their multiplication by longitudinal binary fission at the surface of the mucosa and, as the infection progresses, deeper layers of the epithelium can be affected. The parasite induces necrosis of the cells and granulomatous reactions are generated as a result. The extent and dimensions of these lesions will impede the feeding of the bird and ultimately produce its death by starvation.

A notable genetic diversity of oropharyngeal trichomonads from wild birds has been described in several studies. Isolates with higher identity with *Trichomonas vaginalis*, *Trichomonas tenax* and *Trichomonas* sp. instead of *T. gallinae* have been identified in Europe and USA. Furthermore, new organisms have been detected in birds with

symptomatic infections or macroscopical lesions in the USA. *Trichomonas stableri* was described in outbreaks and mixed infections with *T. gallinae* in band-tailed pigeons (*Patagioenas fasciata monilis*) and a new trichomonad, more similar to tetratrichomonads, was reported in mockingbirds (*Mimus polyglottos*).

Considering this background, the main objective of this Doctoral Thesis is to study the diversity of oropharyngeal trichomonads present in different species of wild birds in Spain, as well as the relationship between isolates and pathogenicity. Samples of 1,688 bird admissions from wildlife recovery centres and field collaborators were screened for the protozoan by culture. Twenty three out of 53 avian species tested positive for *Trichomonas* spp. and protozoa isolates were sequenced for the first time in 16 hosts. Seven different genotypes of the ITS1/5.8S rRNA/ITS2 region were detected. Two were widely distributed in different host species (*T. gallinae*-1 and *T. gallinae*-2), but five were associated with particular ones: *Trichomonas* sp.-1, *T. canistomae*-like-1 and *T. tenax*-like-2 with European turtle doves (*Streptopelia turtur*), *T. canistomae*-like-2 with goshawks (*Accipiter gentilis*) and *T. vaginalis*-like-5 with Egyptian vultures (*Neophron percnopterus*). Gross lesions were present in infections with genotypes *T. gallinae*-1 and *T. gallinae*-2 and were not detected in genotypes associated to specific hosts. Infection with genotype *T. gallinae*-1 implied a higher likelihood of presenting lesions at the oropharyngeal cavity, in comparison with the rest of genotypes. Non-strict ornithophagous raptors had also a higher odds of presenting gross lesions, in contrast with strict ornithophagous ones. These data supports the hypothesis that the number of reinfections is a determinant factor for the development of lesions, having nestlings and non-strict ornithophagous birds of prey a higher risk.

Samples from Bonelli´s eagles (*Aquila fasciata*) and European turtle doves are discussed separately: in the first case, due to the special management of the species in different conservation programs including a European Life project, and as a result of the genetic diversity obtained with the ITS1/5.8S rRNA/ITS2 locus in the second case. Strains from Bonelli's eagle nestlings and European turtle doves were further characterized by the addition of sequences of the small subunit of rRNA and Fe-hydrogenase gene. Genotype *T. gallinae*-1 was detected in ten Bonelli's eagle nestlings with gross lesions indicative of avian trichomonosis and two mixed infections with

genotype *T. gallinae*-2. Also, six birds without lesions had genotype *T. gallinae*-2. The other genetic loci indicated that pathogenic strains, causative agents of the initial finch clonal epidemic in the UK, were present in birds with gross lesions. The MLST analysis of European turtle doves revealed that trichomonad organisms were different to *T. gallinae*, and more similar to *T. canistomae*, *T. tenax* and *Trichomonas* sp. Three novel genotypes, one from the small subunit of rRNA and two from the Fe-hydrogenase gene, and also mixed infections, were identified.

Additionally, a macroscopical evaluation of 77 cases with lesions at the oropharyngeal cavity compatible with avian trichomonosis where birds were classified into three grades was completed. The proposed methodology will aid in the patient evaluation, establishment of the prognosis, as well as in understanding and sharing information among wildlife related professionals.

Mixed infections and specific strains different from *T. gallinae* were selected for further characterization by MLST analysis of clonal cultures and morphometric comparisons. Two potentially new species of oropharyngeal trichomonads were detected; one of them was more similar to *T. tenax* and the other one to *T. canistomae*.

Moreover, in order to understand the pathogenicity of the parasite, four clones from genotype *T. gallinae*-1 and three of genotype *T. gallinae*-2 were tested in the cell culture line LMH. Differences were detected one and two days post-infection, with clones of genotype *T. gallinae*-1 showing higher virulence.

Finally, a comparative quantification of proteins was performed from the membrane and organelle membrane fraction of one clone with genotype *T. gallinae*-1, in addition to one with genotype *T. gallinae*-2, which were selected attending to their maximal and minimal degree of virulence demonstrated on the *in vitro* infections on LMH cells. Several proteins recognized as virulence factors in *T. vaginalis* and in a lower extent in *T. gallinae* (BspA-like, peptidases and cytoskeletal proteins involved in cytopathogenicity) were found differentially expressed in both clones. Besides, the *T. gallinae*-1 clone had a higher amount of immuno-dominant variable surface antigen-

like, GP63-like and a Ras protein, related with virulence or the chronicity of the infection in other protozoa, in comparison with the *T. gallinae*-2 clone.

Conclusively, this Doctoral Thesis has demonstrated the diversity of oropharyngeal trichomonads in wild birds, including the genetic characterization in 16 host species for the first time and the description of five novel genotypes in Spain associated to particular host species. Moreover, MLST and morphometric analysis indicated that potentially new species are present within the order Trichomonadida. Additionally, virulence factors were revealed from the two most widely distributed genotypes of the parasite, by the use of virulence assays on cell cultures and relative proteomic quantification. These findings have increased the current understanding of the epidemiology and pathogenicity of this emergent protozoal disease.

RESUMEN

La tricomonosis aviar es una enfermedad parasitaria causada por el protozoo flagelado *Trichomonas gallinae* (Rivolta, 1878). Las aves pertenecientes a los órdenes Accipitriformes, Columbiformes, Falconiformes, Passeriformes, Psittaciformes y Strigiformes, son susceptibles a la infección y desarrollo de lesiones con mayor frecuencia. Los columbiformes son el hospedador reservorio del parásito, con elevados niveles de prevalencia. El ciclo biológico directo de este flagelado favorece su transmisión entre especies de aves gregarias. Su transmisión se produce desde individuos infectados a través de fuentes de agua y alimento, en los rituales de cortejo y durante la alimentación de los pollos por parte de los adultos. Las aves rapaces se ven afectadas principalmente por el consumo de presas infectadas o mediante necrofagia. Debido a los recientes brotes descritos entre fringílidos silvestres, se considera una enfermedad emergente en diversos países europeos, Canadá y Norte América. La supuesta vía de transmisión fue la alimentación en comederos artificiales compartidos con columbiformes infectados.

La patogenicidad varía atendiendo a diversos factores, como el número de reinfecciones, la respuesta inmunitaria del hospedador y la cepa concreta del parásito. Las zonas más frecuentemente afectadas son el tracto digestivo superior, desde la cavidad orofaríngea hasta el esófago, si bien órganos internos tales como el hígado, sacos aéreos y pulmones, pericardio, peritoneo, páncreas o huesos pueden también contener lesiones. Si no se administra tratamiento, la muerte se produce de forma habitual en aves con signos clínicos.

Los trofozoítos inician su multiplicación por fisión binaria longitudinal en la superficie de la mucosa y, conforme el proceso avanza en el tiempo, las capas más profundas del epitelio pueden resultar infectadas. El parásito produce necrosis celular y, como resultado, aparecen reacciones granulomatosas. La extensión y dimensiones de estas lesiones dificultarán la alimentación del ave y finalmente producirán su muerte por inanición.

Varios estudios han descrito la notable diversidad genética de tricomonádidos orofaríngeos de aves silvestres. En Europa y EEUU se han identificado aislados con mayor similitud con *Trichomonas vaginalis*, *Trichomonas tenax* y *Trichomonas* sp. que con *T. gallinae*. Es más, se han detectado nuevos organismos en aves con infecciones sintomáticas o lesiones macroscópicas en EEUU. *Trichomonas stableri* fue descrito en brotes e infecciones mixtas con *T. gallinae* en palomas de nuca blanca (*Patagioenas fasciata monilis*). Además, se ha encontrado un nuevo tricomonádido, más similar a los tetratricomonádidos, en sinsontes (*Mimus polyglottos*).

Considerando estos antecedentes, el objetivo principal de esta Tesis Doctoral es estudiar la diversidad de tricomonádidos orofaríngeos presentes en diferentes especies de aves silvestres en España, así como la relación entre los aislados y su capacidad patógena. Se tomaron muestras de 1.688 aves admitidas en centros de recuperación de fauna silvestre o directamente en la naturaleza a través de diversos colaboradores, y se analizaron para detectar la presencia del protozoo mediante cultivo. Se encontró infección por *Trichomonas* spp. en 23 de las 53 especies de aves estudiadas, siendo descritas por primera vez secuencias genéticas del flagelado en 16 hospedadores. En la región ITS1/5.8S rRNA/ITS2 fueron detectados un total de siete genotipos diferentes. Dos de ellos estaban ampliamente distribuidos en las diferentes especies de aves (*T. gallinae*-1 and *T. gallinae*-2), pero cinco se aislaron únicamente en ciertos hospedadores: *Trichomonas* sp.-1, *T. canistomae*-like-1 y *T. tenax*-like-2 en tórtolas europeas (*Streptopelia turtur*), *T. canistomae*-like-2 en azores (*Accipiter gentilis*) y *T. vaginalis*-like-5 en alimoches (*Neophron percnopterus*). Se detectaron lesiones macroscópicas en infecciones con los genotipos *T. gallinae*-1 y *T. gallinae*-2, pero no en los genotipos asociados a hospedadores específicos. La infección con el genotipo *T. gallinae*-1 supuso una mayor probabilidad de presentar lesiones en cavidad orofaríngea, en comparación con el resto de genotipos. Asimismo, las aves rapaces ornitófagas no estrictas también demostraron tener mayor probabilidad de presentar lesiones, en comparación con las ornitófagas estrictas. Estos datos apoyan la hipótesis de que el número de reinfecciones es un factor determinante para el desarrollo de lesiones, presentando un mayor riesgo los pollos y las aves rapaces ornitófagas no estrictas.

Las muestras de águila perdicera (*Aquila fasciata*) y tórtola europea se discuten separadamente. En el primer caso, debido al manejo especial de esta especie en diferentes programas de conservación incluyendo un proyecto europeo Life. En el segundo caso, como resultado de la diversidad genética obtenida en el fragmento ITS1/5.8S rRNA/ITS2. Las cepas de pollos de águila perdicera y tórtola europea fueron analizadas, además, incluyendo las secuencias de la subunidad menor ribosomal y el gen de la Fe-hidrogenasa. El genotipo *T. gallinae*-1 fue detectado en diez pollos de águila perdicera con lesiones características de tricomonosis aviar y en dos infecciones mixtas con el genotipo *T. gallinae*-2. Además, seis pollos sin lesión presentaron el genotipo *T. gallinae*-2. Los otros marcadores genéticos indicaron que cepas patógenas del parásito, productoras del brote clonal iniciado en fringílidos en Reino Unido, estaban presentes en las aves con lesiones. El análisis MLST de tórtola europea indicó que las aves presentaban tricomonádidos diferentes a *T. gallinae*, más similares a *T. canistomae*, *T. tenax* y *Trichomonas* sp. Se identificaron tres nuevos genotipos, uno de la subunidad menor ribosomal y dos del gen de la Fe-hidrogenasa, junto con infecciones mixtas.

Así mismo, se evaluaron 77 casos con lesiones macroscópicas en cavidad orofaríngea compatibles con tricomonosis aviar, siendo clasificados en tres grados. La metodología propuesta para dicha clasificación facilitará la evaluación del paciente, la asignación de un pronóstico y el entendimiento e intercambio de información entre profesionales.

Las infecciones mixtas y cepas específicas que habían resultado diferentes a *T. gallinae* fueron seleccionadas para realizar una caracterización más amplia mediante análisis MLST y morfometría comparada de cultivos clonales. Se detectaron dos potenciales nuevas especies de tricomonádidos orofaríngeos, una más similar a *T. tenax* y otra a *T. canistomae*.

Además, para entender la patogenicidad del parásito, cuatro clones de genotipo *T. gallinae*-1 y tres de genotipo *T. gallinae*-2 fueron evaluados en infecciones con cultivos celulares de línea LMH. Se detectaron diferencias en el primer y segundo día post-infección, siendo más virulentos los clones de genotipo *T. gallinae*-1.

Finalmente, se realizó un estudio comparativo de cuantificación proteica con la fracción de proteínas de membrana y membranas de orgánulos de un clon de genotipo *T. gallinae*-1 y otro de genotipo *T. gallinae*-2, seleccionados en función de su nivel máximo o mínimo de virulencia en las infecciones *in vitro* en células LMH.

Se detectaron, con expresión diferencial en ambos clones, proteínas reconocidas como factores de virulencia en *T. vaginalis,* y en menor medida en *T. gallinae* (BspA-like, peptidasas y proteínas del citoesqueleto relacionadas con la patogenicidad). Además, el clon *T. gallinae*-1 presentó una cantidad mayor de antígeno inmuno-dominante variable de superficie-like, de GP63-like y de una proteína Ras, relacionadas con la virulencia o la cronicidad de la infección en otros protozoos, en comparación con el clon *T. gallinae*-2.

En conclusión, esta Tesis Doctoral ha demostrado la diversidad presente en tricomonádidos orofaríngeos de aves silvestres, describiendo cinco nuevos genotipos asociados a hospedadores específicos y caracterizando genéticamente estos protozoos en 16 especies de aves por primera vez en España. Por otra parte, los análisis MLST y morfométricos indicaron la presencia de potenciales nuevas especies en el orden Trichomonadida. Finalmente, se identificaron factores de virulencia en los genotipos más ampliamente distribuidos del parásito, mediante el uso de infecciones en cultivos celulares y la cuantificación relativa de proteínas. Estos resultados han mejorado el conocimiento actual de la epidemiología y patogenicidad de esta enfermedad protozoaria emergente.

1. LITERATURE REVIEW

1.1. Historical background

Avian trichomonosis is an ancient disease that is known for centuries. The first documented descriptions come from falconry books, which explained the appearance of the lesions on birds of prey. Some of the oldest printed records that mentioned the disease in birds of prey are the Spanish falconry book "La caza de las aves" from Pedro López de Ayala (14th century) and the London's Turbervile book of 1575. Moreover, some authors have speculated about the presence of the organism even among the dinosaurs (Wolff *et al.*, 2009).

In 1878, Rivolta describes the disease in pigeons from Italy and the organism was named *Cercomonas gallinae*. Years later, in the USA, Dr. Robert M. Stabler, from the University of Colorado, would review its taxonomical classification and changed its name to *Trichomonas gallinae* in 1938. This American author, member of the Falconry Club of America, produced a huge number of research contributions on avian trichomonosis, being one of the world's leading scientist working to improve the knowledge and treatment of the disease.

During the 50's to the 70's decades a noteworthy number of investigations were carried out mainly in the USA. The morphology, pathogenicity, treatment, immunology and cultivation of the parasite were the objectives of these studies. Several american authors that must be cited for their contributions were: Dr. Richard M. Kocan, from the US Bureau of Sport Fisheries and Wildlife, Patuxent wildlife research center, Laurel, Maryland; Dr. Bronislaw M. Honigberg, from the University of Massachussets; Dr. Norman D. Levine from the University of Illinois and Dr. Lionel G. Warren, from Rice University, Houston, Texas, among others.

1.2. Etiological agent

1.2.1. Taxonomy

Trichomonas gallinae (Rivolta, 1878) belongs to the the family Trichomonadidae, order Trichomonadida, class Trichomonadea, phylum Parabasalia (Brugerolle and Lee, 2000; Cepicka *et al.*, 2010):

Empire Biota
Domain Eukaryotes (Chatton, 1925)
Phylum Parabasalia (Honigberg, 1973)
Class Trichomonadea (Cepicka, 2010)
Order Trichomonadida (Kirby, 1947)
Family Trichomonadidae (Chalmers and Pekkola, 1918)
Subfamily Trichomonadinae (Chalmers and Pekkola, 1918)
Genus *Trichomonas* (Donné, 1836)
Species *Trichomonas gallinae* (Rivolta, 1878)

Trichomonads are classified on the supergroup Excavata, which includes free-living, commensal and parasitic organisms. The phylum Parabasalia is divided in two orders that group around 550 species of flagellated protists (Sleigh, 1991).

The order Trichomonadida contains anaerobic protozoa with a complex cytoskeleton, characterized by the presence of three to five flagella at the apical pole, one recurrent flagellum, axostyle, costa and pelta (Mehlhorn *et al.*, 2009).

The family Trichomonadidae is divided in two subfamilies: Trichomonadinae and Tritrichomonadinae. *T. gallinae* is classified in the first one, where other parasites of human and veterinary importance are found, like *Trichomonas vaginalis* or *Tetratrichomonas gallinarum*. These eukaryotic parasites that lack mitochondria, have an alternative route for ATP synthesis that is fulfilled by hydrogenosomes, cytosolic organelles that do not require an oxygen influx.

The genus name, *Trichomonas*, derives from the Greek word "thrix-" meaning hair and "-monas" that indicates a single unit, regarding their one cell structure.

1.2.2. Morphology

The trophozoites of *T. gallinae*, the parasitic stage of this protozoan (from the Greek word "troph-" meaning feed and "–zoite", from "–zoon", animal) have an average cell size of 7 to 11 µm (Borror, 1988; Mehlhorn *et al.*, 2009; Martínez-Díaz *et al.*, 2015). Their shape is described as ovoid, pyriform or spherical with a great elasticity and frequent transition into these different forms (BonDurant and Honigberg, 1994; Tasca and De Carli, 2003; figure 1).

Four anterior flagella arise from the periflagellar canal at the apical pole of the cell, with mean length values reported from 11 to 18 µm (Mehlhorn *et al.*, 2009; Girard *et al.*, 2013; Martínez-Díaz *et al.*, 2015). Basal bodies, with a typical structure of a central centriole and external microtubule triplets, are at the origin of each flagellum. A fifth recurrent flagellum, which is attached to the cell membrane in all its extension, also emerges from the apical pole and is accompanying the undulating membrane. This membrane measures between 2/3 and 3/4 of the cell's length (Mehlhorn *et al.*, 2009).

Within the cell, the pelta, a curved microtubule complex, is located near the proximal pole, close to the nucleus. Another cytoskeleton structure of longer dimensions, the costa, is located below the undulating membrane and is described as B-type, with filaments arranged in a herringbone pattern or a v-shaped weaving pattern (Honigberg and Brugerolle, 1990; Cepicka *et al.*, 2010). The oval nucleus has mean measures of 2.5-3.4 µm length and 2.2 µm width and is found next to the basal bodies of the flagella (Mehlhorn *et al.*, 2009; Martínez-Díaz *et al.*, 2015).

Finally, a single row of microtubules extend throughout the central axis of the trophozoite, from the nucleus up to the end of the cell, the axostyle, a characteristic support structure with its final segment extending from the posterior pole of the cell (Stabler, 1941).

Figure 1. Schematic representation of a *T. gallinae* trophozoite. From Taylor *et al.*, 2016.

A first study by electron microscopy of *T. gallinae* revealed the complexity of its cytoskeletal structures (Mattern *et al.*, 1967). These authors described the existence of four types of kinetosome rootlets that were involved in the maintenance of trophozoite morphology, including the structures of the costa, pelta and parabasal filaments. Filamentous structures were also detected in cytoplasmic granules associated with the axostyle and costa.

More recently, Vieira *et al.* (2008) investigated the cytoskeleton of *T. gallinae* and *Tritrichomonas foetus*, a pathogenic urogenital trichomonad from cattle, by fluorescence microscopy. Their findings revealed that the microtubule organization of both parasite species did not differ and presented the same binding profile for the fluorescent reagent. Authors pointed out that an easy identification method for the diagnosis of these infections could be established with this technique.

Tasca and De Carli (2003) employed scanning electron microscopy for a further morphological analysis of *T. gallinae* trophozoites. Detailed descriptions of the flagella, undulating membrane and pelta were provided and an interesting new stage of the parasite was detected, the pseudocyst (figure 2). This new morphotype was discovered under *in vitro* conditions, probably induced by stressful conditions, such as low temperature. This stage was characterized by the lack of external flagella and undulating membrane, with an ovoid or spherical shape. Although the formation of a true cystic wall is not reported in trichomonads, the pseudocyst stage exists in intestinal

trichomonad species of amphibians, snakes, birds and rodents (Mattern *et al.*, 1973; Stachan *et al.*, 1984; Friedhoff *et al.*, 1991).

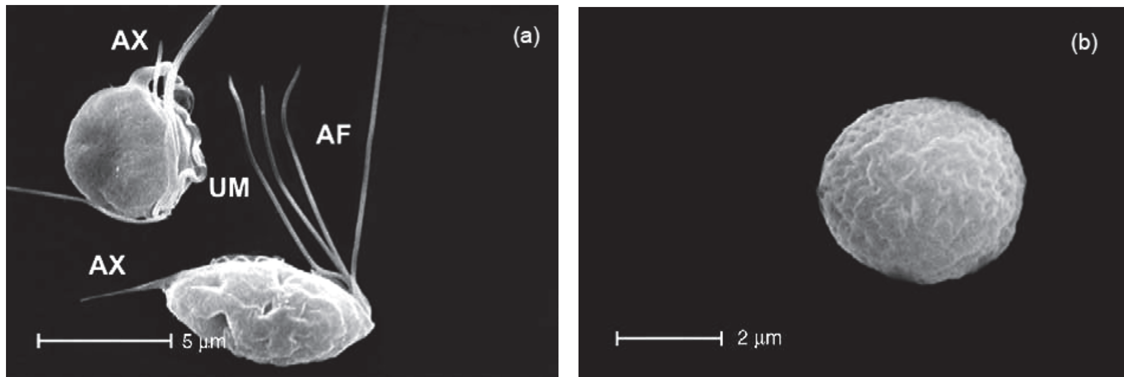


Figure 2. Trophozoites (a) and pseudocyst stage (b) of *T. gallinae*. AX: axostyle, AF: anterior flagella, UM: undulating membrane. From Tasca and De Carli, 2003.

Related trichomonad species, *T. foetus* and the human pathogen *Trichomonas vaginalis*, parasites from the urogenital tract, also develop pseudocysts as resistant forms that adhere to the host tissue at a higher rate than the trophozoite stage, indicating that they could have an important effect on the transmission and infective capacity of the parasite (Mariante *et al.*, 2004). In addition, their transition to trophozoite stage has been documented, being also competent to multiply (Granger *et al.*, 2000; Pereira-Neves *et al.*, 2003; Castro *et al.*, 2016).

A morphological comparison of the avian trichomonad parasites *T. gallinae* and *T. gallinarum* was performed by Mehlhorn *et al.* (2009) from clonal culture trophozoites by transmission electron microscopy. The typical ovoid or pyriform shape was observed in *T. gallinae* trophozoites, that measured an average of 7-11 µm. Their nucleus size displayed between 2.5 and 3 µm and was located closely to the flagella basal bodies. Pseudocyst stages were also characterized from *T. gallinae* and clear differences were seen in comparison with *T. gallinarum* trophozoites. For instance, a pear-shape morphology and wider cell size range (6-15 µm) were demonstrated in *T. gallinarum*. Furthermore, the fifth recurrent flagellum had a free posterior pole in constrast with the attachment present in the corresponding one of *T. gallinae* trophozoites.

1.2.3. Life cycle and transmission

T. gallinae has a direct life cycle, with trophozoites multiplying by longitudinal binary fission, one cell will divide to form two new ones (Stabler, 1941). Their preferred location is the mucosa of the upper digestive tract, from the oropharyngeal cavity and crop to the proximal oesophagus. Nevertheless, affection of internal organs such as liver, lungs, pericardium, air sacs and pancreas, has also been documented in certain strains of the parasite (Stabler and Engley, 1946; Jaquette, 1950; Kocan and Herman, 1971). Indeed, some strains target particular tissues, namely, Jones' Barn and Eiberg strains are hepatotrophic, while Mirza strain is cephalotrophic (head sinuses, orbital regions, brain and neck) (Pérez-Mesa *et al.*, 1961; Narcisi *et al.*, 1991). However, the Jones' Barn strain primary affected the lungs instead of the liver of mourning doves (*Zenaida macroura*) and rock pigeons (*Columba livia*) in experimental infections. Non pathogenic strains, like Lahore and Stabler-gallinae (SG), have also been described (Honigberg *et al.*, 1964; Kocan, 1969a; Nadler and Honigberg, 1988).

The primary route of transmission is by direct contact with the saliva of an infected bird, although contaminated food and water also act as sources of infection (Kocan, 1969b; Bunbury *et al.*, 2007). Indeed, experimental transmission of the parasite through the ingestion of contaminated water was demonstrated by Kietzmann (1990) in columbiformes. Recent studies have proved that bird baths may act as potential vehicles with viable trophozoites for at least 16 hours (Purple and Gerhold, 2015; Purple *et al.*, 2015).

Contaminated carcasses also remain infective for at least 48 hours after death, acting as a reservoir for ornitophagous and scavenging birds (Erwin *et al.*, 2000). Moist grains were also found to be able to maintain the parasite's viability for at least five hours (Kocan, 1969b).

Regarding the number of trophozoites needed to initiate the infection, Stabler and Kihara (1954) infected five rock pigeons with one trophozoite of the Jones' Barn strain. All birds died in less than 15 days post-infection.

1.2.4. Hosts spectrum

The main hosts are columbiformes, which are responsible of the worldwide distribution of the parasite (Forrester and Foster, 2008; Amin *et al*., 2014). Infections are constantly being produced due to their social behaviour, when birds produce crop milk to feed their young, by sharing food or water sources and through direct contact during courtship. Secondly, due to their feeding or drinking habits, accipitriformes and strigiformes are mostly affected. Birds of prey will mainly get the infection by predation on columbiformes or by their scavenging habits on their carcasses. Thirdly, psittaciformes and passeriformes are another major group of avian species affected by the disease (Baker, 1986; Anderson *et al.*, 2009; Forzán *et al.*, 2010; Grabensteiner *et al.*, 2010; Lawson *et al.*, 2012). Direct transmission occurs at feeding locations or ponds that are shared with columbiformes.

Other avian species are also capable of harbouring the infection but had a minor importance as sporadic cases are recorded in the literature, presumably because of their biological characteristics that determine a low contact with columbiformes. Examples of this type of birds are gulls (revised in Forrester and Foster, 2008) or secretary birds (*Sagittarius serpentarius*; Koyama *et al.*, 1971).

1.2.5. Diversity of avian oropharyngeal trichomonads

Recent findings have increased the number of possible etiological agents of avian trichomonosis. Thanks to the molecular identification of the parasite by polymerase chain reaction (PCR) and sequencing, the phylogenetic study of certain genes revealed new organisms isolated from symptomatic cases that were not genetically identified as *T. gallinae*. For instance, in 2009 a new *Trichomonas* species was isolated from mockingbirds (*Mimus polyglottos*) in the USA, in 2012 a *Simplicomonas*-like organism was detected in green-winged saltators (*Saltator similis*) in Brasil and in 2014 the new species *Trichomonas stableri* was described from white-winged pigeons (*Patagioenas fasciata monilis*) in the USA (Anderson *et al.*, 2009; Ecco *et al.*, 2012; Girard *et al.*, 2013 and 2014). Additionally, this latest species, *T. stableri*, was reported also in mixed infections with *T. gallinae* in birds where gross lesions of avian trichomonosis were detected (Girard *et al.*, 2013).

Also, several authors reported strains, not strictly related with disease, with higher similarity to *Trichomonas tenax*, *T. vaginalis* or *Tetratrichomonas canistomae* than to *T. gallinae* (Gerhold *et al.*, 2008; Grabensteiner *et al.*, 2010; Kelly-Clark *et al.*, 2013; Martínez-Herrero *et al.*, 2014). In this case, *T. tenax*-like organisms were found in symptomatic racing pigeons in Austria, while *T. vaginalis*-like protozoans were isolated from a captive bearded vulture (*Gypaetus barbatus*) in the Czech Republic and an American bald eagle (*Haliaeetus leucocephalus*) without clinical signs in Canada (Grabensteiner *et al.*, 2010 and 2011; Kelly-Clark *et al.*, 2013). Later, a description of a novel non pathogenic organism associated to scavenging birds of prey, *T. gypaetinii*, determined that these *T. vaginalis*-like organisms previously described belonged to this new species (Martínez-Díaz *et al.*, 2015).

Finally, *Tritrichomonas blagburni* n. sp.-like strain was found in two band-tailed pigeons in California, USA (Girard *et al.*, 2014). This trichomonad species was recently differentiated from the reproductive tract cattle pathogen *Tritrichomonas foetus*, as the causative agent of large bowel feline diarrhoea (Walden *et al.*, 2013). One of the birds had oral lesions from which only *T. gallinae* DNA was extracted. Due to this finding, authors suggested that *T. blagburni* could be present only at the oropharyngeal cavity but without a pathogenic character for band-tailed pigeons. The contamination with feline faeces may easily have occurred in urban areas with artificial bird feeders on gardens.

1.3. Description in different groups of birds

1.3.1. Order Columbiformes

Several species have been reported to be hosts of the parasite, with a distribution that includes every continent except the Antarctica. Infection is maintained with endemic levels among wild birds, which depends on the geographical location. For example, Schulz *et al.* (2005) reported 5.6% of prevalence in mourning doves from the USA. Lennon *et al.* (2013) found that 86% of European turtle doves and Eurasian collared doves were infected, while 47% of wood pigeons (*Columba palumbus*) and 40% of stock doves (*Columba oenas*) harboured the parasite in the UK.

Outbreaks of the disease are also known on columbiformes, with high mortality rates reported. As an example, latest epidemic studies estimated 20% mortality rate in Eurasian collared doves and African collared dove hybrids (*Streptopelia risoria*) in the Caribbean (Stimmelmayr *et al.*, 2012) and up to 2,600 dead wood pigeons in Spain (Höfle *et al.*, 2004). In California, USA, periodic outbreaks have affected the band-tailed pigeon population with negative consequences of up to 10,000 dead birds (Rogers *et al.*, 2016).

Avian trichomonosis has also notable economical impacts for domestic pigeon breeders. Pigeon farms are valuable industries for certain areas in Asia where the consumption of birds is an important protein food resource. In China, nearly 60 million pigeons are produced each year and several studies detected a high prevalence of the infection. Huang *et al.* (2008) found 75.1% of birds infected in pigeon farms and markets of China. Qiu *et al.* (2012) reported an overall prevalence of 33.9%, that ranged from 23.7-45.1% on seven Chinese pigeon farms. Latest studies detected similar results, with a global prevalence of 33.8% in five farms and one slaughterhouse in China (Jiang *et al.*, 2016).

Finally, the industry of pigeon fanciers has special importance in some countries of Europe and USA. *T. gallinae* infection is one of their major concerns due to the consequences in the breeding and racing performance of the birds, the difficult eradication of the infection from large flocks and treatment failures (Lumeij and Zwijnenberg, 1990; Charlton *et al.*, 1991; Franssen and Lumeij, 1992; Stoute *et al.*, 2009; Rouffaer *et al.*, 2014). In 2007, Stenzel and Koncicki found 100% infection rate in fancy pigeon lofts in Poland.

1.3.2. Order Accipitriformes

Accipitriformes and columbiformes are probably the orders presenting the highest prevalence rates of the parasite. Especially for those with an ornitophagous diet, the predation on columbiformes preys makes diurnal raptors one of the easiest hosts where the flagellate will replicate (Lierz *et al.*, 2002). In this sense, multiple researches have studied the prevalence on several species. Retrospective studies showed that *T. gallinae*

is the most frequent infectious disease as cause of admission for wild birds in wildlife recovery centers (Molina-López *et al.*, 2011; Stenkat *et al.*, 2013).

Considering major ornitophagous species, like goshawks (*Accipiter gentilis*), Eurasian sparrowhawks (*Accipiter nisus*) or peregrine falcons (*Falco peregrinus*), different studies have demonstrated that the proximity to urban areas is one of the most important risk factors for the infection. The abundance of infected columbiformes on these areas results in an increase on the chick raptors prevalence. In the USA, Boal *et al.* (1998) observed that 85% of the urban nests from Cooper's hawks (*Accipiter cooperii*) were infected, in contrast with only 9% of the non-urban ones. Wieliczko *et al.* (2003) monitored the presence of the parasite in goshawk chicks of Wroclaw, Poland. Interestingly, a maximum increase in prevalence, from 35% in 14 day-old chicks to 100% at 40-day-old, was observed. In 2005, Krone *et al.*, found 65% of goshawk nests infected in the urban surroundings of Berlin, Germany.

Endangered species, like the Mediterranean population of Bonelli's eagle (*Aquila fasciata*), are also affected by the parasite. Höfle *et al.* (2000) revealed that up to 87.5% of the nestlings had oropharyngeal lesions compatible with avian trichomonosis in the South-west of Portugal, and Real *et al.* (2000) reported that 41% of the broods were infected in Catalonia, Spain. In addition, those nests with columbiformes carcasses had higher risk to develop disease than others (Real *et al.*, 2000). Sansano-Maestre *et al.*, in 2009, referred to 52% prevalence in the Valencian Community, Spain.

1.3.3. Order Falconiformes

There is scarce number of studies that have focussed on detecting the prevalence of the disease among wild falconiformes. Sansano-Maestre *et al.*, (2009) found 12.5% prevalence in peregrine falcons and 10.3% in common kestrels (*Falco tinnunculus*) in eastern Spain.

1.3.4. Order Strigiformes

Nocturnal raptors are also susceptible hosts for oropharyngeal trichomonads. The diet of particular owl species will be the determinant factor to establish the likelihood of the disease. Large-sized species, like tawny owls (*Strix aluco*) or Eurasian eagle-owls

(*Bubo bubo*) will be more predisposed to prey on columbiformes. In 1980, Jessup reported severe infections in great horned owls (*Bubo virginianus*). Sansano-Maestre *et al.* (2009) found 14% prevalence in barn owls (*Tyto alba*) of Spain, while Pokras *et al.* (1993) described severe cases with gross lesions in this species in the USA. First documented reports date from 1925, when Tanabe cultured the parasite from a nocturnal raptor. Schulz (1986), surveyed more than 1,600 barn owls from wildlife recovery centers of California, USA, and found that trichomonosis was the most common infectious disease. Ecco *et al.* (2012) reviewed several cases with gross lesions including Brazilian owls. However, symptomatic cases in small-sized species like Eurasian scops-owls (*Otus scops*) have been also documented (Martínez-Herrero *et al.*, 2014).

1.3.5. Order Passeriformes

In recent years, passeriformes have become a suitable and effective vector for the infection. In 2005 and 2006, different outbreaks of disease were reported on greenfinches (*Chloris chloris*) and chaffinches (*Fringilla coelebs*) in the UK (Cousquer, 2005; Pennycott *et al.*, 2005; Lawson *et al.*, 2006; Duff *et al.*, 2007; Robinson *et al.*, 2010). In addition, similar outbreaks were detected in North America affecting American goldfinches (*Carduelis tristis*) and purple finches (*Carpodacus purpureus*) in Canada and house finches (*Carpodacus mexicanus*) in California, USA (Anderson *et al.*, 2010; Forzán *et al.*, 2010). For this reason, the parasite was catalogued as an emergent disease for wild finches, and other wild bird species, such as raptors or columbiformes, could be potentially at risk (Forzán *et al.*, 2010; Robinson *et al.*, 2010; Chi *et al.*, 2013).

In subsequent years, the disease spread from the UK to other north European countries. In 2008 and 2009, avian trichomonosis was observed in Sweden, Norway and Finland (Neimanis *et al.*, 2010). In 2010, France was also included, while first cases in Austria and Slovenia were seen in 2012 (Gourlay *et al.*, 2011; Ganas *et al.*, 2014).

In the USA, Anderson *et al.* (2009) reported some cases in house finches (*Carpodacus mexicanus*), corvids and mockingbirds. Joseph (2003) examined captive passeriformes in Australia, finding low prevalence rates.

The infection route for passeriformes is, mainly, through the ingestion of contaminated food or water. These feeding resources must have been infected by other birds, most frequently columbiformes. Besides, the gregarious social behaviour of some species and similar feeding habits that columbiformes, increase the risk of contact with reservoir hosts and favors the transmission of the parasite. Indeed, the emergence and spread of finch trichomonosis in Europe is thought to be initiated by sharing feeding stations with columbiformes (Robinson *et al.*, 2010).

1.3.6. Order Psittaciformes

Budgerigars (*Melopsittacus undulatus*) are a popular cage bird and avian trichomonosis is a frequently reported disease affecting them. McKeon *et al.* (1997) examined Australian budgerigars kept in captivity finding low infection rates (11%) and Park (2011) reviewed 143 symptomatic cases from veterinary clinics. Other species of psittaciformes, such as cockatiels (*Nymphicus hollandicus*), purple-crowned lorikeets (*Glossopsitta porphyrocephala*) and coconut lorikeets (*Trichoglossus haematodus*) also hosted the protozoan (Park, 2011). Amazon parakeets (*Amazona* spp.) and conure species such as Aratinga parakeets (*Aratinga* spp.) may also suffer the disease (Lumeij, 1994).

1.3.7. Order Gruiformes

Death and gross lesions of avian trichomonosis have been reported in captive rufous-crested bustards (*Eupodotis ruficrista*). Birds were probably infected by contaminated food or water in facilities that do not regulate the entrance of columbiformes (Bailey *et al.*, 1996).

Silvanose *et al.* (1998) found positive houbara bustards (*Chlamydotis undulata*) and Kori bustards (*Ardeotis kori*) with different signs of infection including caseous lesions and inflammation at the oropharynx. Birds were housed in aviaries that were easily accessed by columbiformes; therefore, the most likely route of transmission was by infected food or water.

1.3.8. Order Galliformes

This avian order is present in the scientific name of the parasite, *T. gallinae*, although the parasite was first described from rock pigeons (Stabler, 1941). Domestic poultry (*Gallus gallus domesticus*) and turkeys (*Meleagris gallopavo*) may become infected (Davidson *et al.*, 1985; Willoughby *et al.*, 1995). Latest studied outbreaks in turkeys were seen in animals reared with pigeons (Mirzaei *et al.*, 2016).

1.3.9. Order Anseriformes

Tsai *et al.* (1997) described an outbreak of trichomonosis in farmed ducks. The authors described a respiratory and intestinal presentation and the organism was identified by morphological criteria, as *Tetratrichomonas anatis.* However, the clinical signs observed in the respiratory form of the disease resembled some of *T. gallinae* infections. The upper respiratory tract, infraorbital sinuses and nasal cavity were affected with tracheitis, mucofibrino-purulent sinusitis and catarrhal rhinitis. The involvement of infraorbital sinusitis and rhinitis are also described in *T. gallinae* infections (Samour *et al.*, 1995; Samour, 2000). For the intestinal presentation, catarrhal enteritis, diarrhoea and low mortality were seen in adult ducks, with lesions at the lower small intestine.

1.3.10. Others

Hees, in 1938, informed about one species of gull of the Shetland Islands that harboured the parasite (revised in Forrester and Foster, 2008) and secretary birds (Koyama *et al.*, 1971).

1.4. Pathogenicity

Differences in the pathogenic behaviour are known to exist, with non pathogenic, milder and highly pathogenic strains described in the literature.

The evolution of the disease originated by pathogenic strains has been studied by some authors in experimental infections with columbiformes.

Stabler and Engley (1946) employed a virulent strain from a peregrine falcon that was inoculated in pigeon squabs. Initially, small, yellowish single or multiple gross lesions were detected in the oropharyngeal mucosa. The most common finding was a unique

lesion at the mid-line between the pair of soft palatal flaps. Lesions appeared from day three post-infection to day 14, with an average of 7.4 days. Gross lesions rapidly increased their size and extended through the oropharyngeal roof, their large dimensions being a risk for digestive or tracheal obstruction. The bones of the floor of the skull were also affected. Conjunctivitis and parasite detection in the orbital inflammatory exudate were as well described. The oesophagus was found with necrotic lesions in some cases, although much less frequently than the oropharyngeal cavity. In this region, lesions were referred as small mounds, from the centers of which extend short spurs. Crop lesions were seen at the base, if present, and a greenish fluid accumulation was frequent. The liver was affected in certain animals, with up to 90% of the organ presenting caseous necrosis. Hepatic lesions were described from small and numerous to few and large foci. Furthermore, a nodule was found on the small intestine serosa in one case, just next to a large liver caseation. The pancreas was involved also in one case with extensive hepatic and small intestinal lesions. Lungs appeared also affected in birds with extremely caseous liver and small intestine. Finally, the myocardium was another target organ for the parasite with lesions found on the ventricles, apparently due to its close location with an affected liver. Moreover, the authors were able to establish a *T. gallinae* culture from this cardiac blood. Serous exudates were present in the coelomic cavity of the birds and contained numerous trophozoites. Lesions on internal organs generated adhesions between viscera, peritoneum and posterior air sacs.

Later, Stabler (1948) observed a wide range of pathogenic effects after experimental infections in pigeons. Highly pathogenic parasites, isolated from birds with gross lesions that died of trichomonosis, induced fatal caseonecrotic lesions on pigeons. However, strains from healthy carriers of the parasite induced lesions that lasted only for two to four days. Finally, one strain from a healthy pigeon recovered from a clinical infection with gross lesions produced two types of presentation, lesions that disappeared after ten days, but also fatal infections.

Pérez-Mesa *et al.* (1961) performed experimental infections in domestic pigeons with a virulent hepatotrophic strain (Jones' barn). These authors observed that trophozoites extended in a layer similar to columnar epithelium in the mucosa of the pharynx and inside the lumen of the pharyngeal mucous glands at 48 hours post-infection. White

spots were detected in the mucosa of the oropharyngeal cavity, mainly at the pharyngeal papillae with mild inflammation of mononuclear cells initially circumscribed to the gland openings. On day three post-infection, a mucous film was formed at the oropharynx and superficial pharyngeal ulcers appeared with parasites present that seemed to cause the separation of squamous epithelial cells. A severe leucocyte infiltration was present at the submucosa surrounding the oropharyngeal and laryngeal glands. Internal organs were affected at this stage. The liver had multiple small whitish-yellow, solid nodules up to 0.2 cm in all organ lobes. Parasites were present at the Disse's spaces and sinusoidal capillaries, and originated necrotic foci with mononuclear cells and heterophils. Several abscesses up to 0.4 cm appeared in the lungs, close to secondary and tertiary bronchi, with lymphocytes, mononuclear cells and giant cells surrounding the lesions. Trophozoites were detected at the periphery. Besides, tracheal ulceration and trophozoites were seen in the epithelium of this organ in one bird. At 96 hours post-infection, extensive inflammation appeared on the pharynx, with a yellow pseudomembrane, mucosal ulcers and a massive inflammatory reaction with hyperplastic epithelium. Liver abscesses were larger (up to 0.4 cm), starting to coalesce, with heterophils and mononuclear cells. On day five after infection, congestion was detected at the mucosal surface, with deeper pharyngeal ulcers and liver lesions up to 1.0 cm. One week post-infection, trophozoites had reached the dermis from the pharyngeal epithelium. Congestion was present at the submucosa. Liver abscesses reached 2.0 cm, with a yellow colouration and thickening of the organ capsule. Animals died between five and ten days post-infection due to the hepatic necrosis.

Kietzmann (1993) infected ringed doves (*Streptopelia risoria*) with a pathogenic strain and observed trophozoites attached to the cells of the epithelium at 6 hours post-infection. Potentially secreted parasite factors initiated the damage to the host's cells, causing cell border separation and removal. Necrotic foci appeared after 19 to 240 hours post-inoculation in the palatal-oesophageal junction. The parasite penetrated deeper in the epithelium, producing necrotic foci with inflammatory cell infiltration on day ten post-infection. Oropharyngeal lesions started as small, superficial ulcerations that developed spur-like projections and coalesced to form larger ones. Caseonecrotic areas, with a yellow coloration, were ultimately formed.

Fatal infections have been reported to occur at only four days post-infection in pigeon squabs experimentally infected (Stabler, 1977). However, birds will frequently die between four and 18 days post-infection, according to the pathogenicity of the strain and host susceptibility (Taylor *et al.*, 2016).

1.5. Clinical signs

Avian trichomonosis, also known as "canker" by pigeon fanciers or "frounce" for birds of prey, is described with a wide variety of clinical signs. Fundamentally due to the primary location of the trophozoites at the oropharyngeal cavity and crop, sialorrhea, dyspnoea, regurgitation and weight loss are frequent findings (figure 3). Animals may have ruffled feathers, listlessness and a foul smell. Involvement of the head tissues and sinuses is sometimes detected, with swollen eye orbits that protrude externally and nasal-ocular discharge, usually reported from passeriformes and falconiformes (St. Leger *et al.*, 1998; Samour, 2000; Anderson *et al.*, 2010).

In some cases, caseonecrotic nodules could be externally seen if they are located next to the beak commisures or crop. This necrotic material could block the alimentary tract, obstructing the passage of food or air, if the lesion occludes the trachea. Therefore, the bird will become cachectic and die of starvation, as the oral lesions do not allow a proper feeding. Death could also be produced because the lesions detach and block the trachea, or by vital organ failure. Nevertheless, infections without clinical signs also occur. Indeed, columbiformes are frequently infected without showing symptoms and act as carriers.

Figure 3. External appearance of birds with trichomonosis, note the matted feathers surrounding the beak indicative of sialorrhea. From left to right: tawny owl (*Strix aluco*, case R18-12) and common kestrel (*Falco tinnunculus*, case R186-13). See section 4.4., for further information.

Subclinical infections are also important in the epidemiology of the disease as no clinical signs could be detected in infected birds. Therefore, the transmission of the parasite to other individuals will easily occur if a proper diagnostic method is not employed. Bunbury *et al.* (2007) studied the infection in columbiformes of Mauritius, finding high prevalence values (19-59% according to the species) and low pathogenicity, highlighting the risk of transmission to an endangered endemic bird, the pink pigeon (*Neosoenas* [*Columba*] *mayeri*). Lennon *et al.* (2013) also reported a high prevalence level in columbiformes of the UK.

Recent studies have detected infected passeriformes without clinical signs in the UK (Zu Ermgassen *et al.*, 2016). Greenfinches, chaffinches and bullfinches (*Pyrrhula pyrrhula*) were carriers of the protozoan. These birds will be efficient vectors as a result of their gregarious behaviour, contributing to the spread of the parasite.

1.6. Lesions

The upper alimentary tract, from the oropharyngeal cavity to the crop and oesophagus are the primary tissues where lesions appear (Amin *et al.*, 2014). Initial lesions start as ulcerations with a white colouration of the surface or pseudomembranes that will later evolve to necrotic foci with the formation of yellowish granulomas (Pérez-Mesa *et al.*, 1961; Kietzmann, 1993; figures 3 and 4). However, initial lesions could be produced by other etiological agents. Therefore, a proper differential diagnosis is essential. Other processes that have a similar appearance are: tuberculosis, mycoplasmosis, salmonellosis, coligranuloma, aspergillosis, candidiosis, poxvirus, capillariosis, vitamin A deficiency or squamous cell carcinoma (Kocan and Herman, 1971; Cole, 1999; Nordio *et al.*, 2016).

Internal organs such as lungs, air sacs, liver, pericardium and pancreas as well as head sinuses could also be involved, especially in fatal infections by the most pathogenic strains (Stabler and Engley, 1946; Jaquette, 1950; Kocan and Herman, 1971). In addition, affection of the muscles from the neck and mandible are sometimes reported (Stabler and Engley, 1946; Kocan and Herman, 1971; Ecco *et al.*, 2012). Birds that recover from a symptomatic infection have been often observed without pharyngeal folds, according to Kocan and Herman (1971).

1.6.1. Lesions in columbiformes

In the order Columbiformes, the reservoir hosts of the parasite, clinical infections with severe symptomatology are described. Caseonecrotic granulomas are the type of lesion that is mainly reported at the superior digestive tract: oropharyngeal cavity, crop and/or oesophagus (Harmon, 1987; Höfle *et al.*, 2004; Bunbury *et al.*, 2007; Hegemann *et al.*, 2007; Sansano *et al.*., 2009; Stimmelmayr *et al.*, 2012; Lennon *et al.*, 2013; Amin *et al.*, 2014; Girard *et al.*, 2014). Their description is as diphtheritic membranes (Narcisi *et al.*, 1991; Lumeij, 1994) or solid nodules with a yellow colouration, focally or multifocally distributed that may coalesce and reach several centimeters of diameter in late stage infections (Stabler and Engley, 1946; Kietzmann, 1993). Due to this condition, the blockage of the digestive tract or tracheal opening may occur. Nevertheless, the initial stages of the infection are characterized by small areas of superficial ulcerations that will become visible during gross examination as small, whitish lesions (Pérez-Mesa *et al.*, 1961; Bunbury *et al.*, 2007).

Harmon (1987) detected possible healed areas of caseous lesions of avian trichomonosis in endemic Galapagos doves (*Zenaida galapagoensis*). The author described these findings as scarred palatal flaps. Also, the yellow caseous lesions were found in clinical cases at the oropharyngeal cavity and upper crop.

Histopathological studies had revealed extensive and severe tissue inflammation with congestion and cell infiltration with mononuclear cells, heterophils and hyperplastic epithelium in the oropharyngeal cavity (Pérez-Mesa *et al.*, 1961; Stimmelmayr *et al.*, 2012). Ingluvitis, cellulitis, necrotizing stomatitis and pharyngitis were the main reported findings (Stimmelmayr *et al.*, 2012; Girard *et al.*, 2013). Deep tissue invasion of the lesions, involving the mucosa, submucosa and muscularis mucosae have been also reported (Girard *et al.*, 2014).

Structures resembling trophozoites are found within affected areas, particularly at the margins (Höfle *et al.*, 2004; Girard *et al.*, 2013 and 2014). However, the detection of the parasite in the affected areas is difficult, and other techniques such as immunohistochemical stains or chromogenic in situ hybridization could be employed (Mostegl *et al.*, 2010; Girard *et al.*, 2013 and 2014).

Internal organ involvement has been described, with lesions in the brain (Pérez-Mesa *et al.*, 1961), conjunctive (Stabler and Engley, 1946; Zimre-Grabensteiner *et al.*, 2011; Stimmelmayr *et al.*, 2012; Stockdale *et al.*, 2015), myocardium (Stabler and Engley, 1946; Pérez-Mesa *et al.*, 1961; Höfle *et al.*, 2004), pancreas (Stabler and Engley, 1946; Pérez-Mesa *et al.*, 1961), liver (Stabler and Engley, 1946; Pérez-Mesa *et al.*, 1961; Stimmelmayr *et al.*, 2012; Girard *et al.*, 2014), kidneys (Pérez-Mesa *et al.*, 1961), trachea (Höfle *et al.*, 2004), lungs and air sacs (Pérez-Mesa *et al.*, 1961; Höfle *et al.*, 2004; Girard *et al.*, 2013).

Regarding the myocardium, Stabler and Engley (1946) described caseonecrotic areas in the ventricles that seemed to be consequence of the extense liver lesions and the close location of both organs. Fibrinous pericarditis and fibrinonecrotic lesions on the right atrium were reported by Höfle *et al.* (2004).

Lesions on the liver are described as caseonecrotic foci with a focal or multifocal distribution with inflammation on the organ capsule in the affected area/s. Mononuclear cell infiltration with heterophils are found surrounding the lesions (Pérez-Mesa *et al.*, 1961).

Bone destruction has been referred by some authors in the skull and oral cavity, due to the extreme severity of the lesions. In 2007, Hegemann *et al.*, described one case with mandibular affection (figure 4). Also, cartilage and mandibular bones with alterations were reported by Stimmelmayr *et al.* (2012). The invasion of the encephalic cavity has been detected in cases where lesions were located on the hard palate (Höfle *et al.*, 2004; Stimmelmayr *et al.* (2012).

Figure 4. Lesions of avian trichomonosis infection in a stock dove (*Columba oenas*). From Hegemann *et al.* (2007).

Considering the respiratory tract, caseonecrotic foci could be present in the lungs with extensive necrotizing pneumonia (Stabler and Engley, 1946; Girard *et al.*, 2013). Tracheal obstruction is also frequent when large granulomatous lesions are present at the oropharyngeal cavity.

Skeletal muscles are also involved in some cases due to the spread of caseonecrotic lesions through deeper anatomical layers and the subsequent inflammatory response (Stimmelmayr *et al.*, 2012; Girard *et al.*, 2014).

Finally, in reference to the distribution of the lesions, Stimmelmayr *et al.* (2012) detected that the majority of the birds had multiple lesions (94%) during an outbreak of the disease. In this group of birds with multiple lesions, 53% of them coalesced and may extend up to the 90% of the oral mucosa. Fifty percent of the lesions were located only in the oropharyngeal cavity, while 28% were also present in the crop, and 22% in three locations, oropharyngeal cavity, crop and oesophagus.

1.6.2. Lesions in accipitriformes

Oropharyngeal caseonecrotic granulomas at the upper digestive tract are predominantly found in diurnal birds of prey, although invasion of the ocular and encephalic cavities are also frequent presentations.

The study of Real *et al.* (2000) on Bonelli's eagle nestlings identified nodular gross lesions, some larger than 3 cm, in the oropharyngeal cavity, located at the lower jaw, upper jaw, posterior part, glottis or at the lateral areas. These lesions may have caused the death of one nestling by obstruction of the pharynx, as the animal had good physical condition with recently ingested contents at the crop and stomach. The necropsy revealed a large nodule on the inferior surface of the tongue that occluded the glottis. Höfle *et al.* (2000) found fibrous lesions at the oesophagus in nestlings of this bird species, with trophozoites being identified on impression smears from the borders of the lesions.

Large oropharyngeal caseous lesions of more than one cm in diameter were described by Krone *et al.*, (2005) at the oropharynx of goshawk nestlings. Deformations of the cranio-mandibular apparatus and death of the bird were also reported.

Labrut *et al.*, (2012) described one fatal case of avian trichomonosis from a sparrowhawk with multiple, transmural lesions of oval shape at the oesophagus, ranging from two to seven mm in diameter. One of these ulcers had perforated the oesophageal wall and severe inflammation extended to the subcutaneous tissue. Necrotic and suppurative oesophagitis with vasculitis were the main histopathological findings. The inflammatory cell infiltrates were composed of lymphocytes, plasma cells, macrophages and heterophils.

1.6.3. Lesions in falconiformes

An extensive revision of clinical cases in falconiformes was done by Samour and Naldo (2003). Lesions were found at the oropharynx in 63% of the birds, 31% at the crop, 10% on the oesophagus, 4% at the nasal cavity, 5% on infraorbital sinus and 2% on the syrinx. More than one location was affected in some birds.

At the oropharynx, caseous nodular proliferation was commonly found at the base of the tongue, within the choana and in the caudal area of the oropharynx. At the crop two clinical presentations were reported: a nodular and a membranous type. In the nodular one, small to large, nodular, caseous lesions measured up to 7 x 5 cm. A single nodule was most commonly observed, but multiple were also found. In the membranous type, a

plaque-like growth affected a limited area of all the crop. Caseous nodules in the crop were easily identified by palpation, but membranous growths were more difficult to detect, unless the lesion was well developed.

The distal portion of the thoracic oesophagus was the area reported with lesions. Half of the affected birds had severe oesophageal stenosis as a consequence. The two types of presentations, nodular and membranous, were also detected at the oesophagus.

In the nasal cavity, partial or complete obstruction of one or both nares was found, with thick mucoid discharge from the choana. In some cases the nodule was large enough to protrude through the hard palate or create a fistula. Infraorbital sinuses were unilaterally affected, with enophthalmos and abundant discharge. Finally, the syrinx could be unilateral or bilaterally affected, with membranous caseous growths that caused partial obstruction.

1.6.4. Lesions in strigiformes

Caseonecrotic granulomas tend to be located at the hard palate with involvement of the choanal slit and extension to the skull and cephalic sinuses in this group of birds (Pokras *et al.*, 1993; Ecco *et al.*, 2012). However, the floor of the pharynx could also be affected, as Pokras *et al.* (1993) detected in one barn owl. Sansano-Maestre (2009) also found severe, large caseonecrotic lesions in one barn owl and one tawny owl that extended to the skull tissues.

Furthermore, Ecco *et al.*, (2012) described that lesions may also be present at the surrounding mandibular musculature. In this case, striped owl (*Asio clamator*) was the species involved.

1.6.5. Lesions in passeriformes

For passeriformes, lesions tend to be circumscribed to the proximal oesophagus (figure 5), with necrotic foci of small size that extend through the mucosal and submucosal layers (Forzán *et al.*, 2010; Neimanis *et al.*, 2010; Robinson *et al.*, 2010; Ganas *et al.*, 2014).

The first evidence of lesions of avian trichomonosis in passeriformes was reported by Pennycot *et al.* (2005) in chaffinches and greenfinches from the UK. Gross lesions on the mucosa of the oesophagus and crop were identified as small, focal, yellow nodules, from 2-4 mm in diameter. However, diffuse lesions and larger masses were also found in some birds, with a yellow-orange colouration, a deep ulceration of the mucosa and obstruction of the upper digestive tract. Furthermore, these alterations were detected in some occasions through the skin of the birds. The histopathological data indicated severe ulcerative oesophagitis and ingluvitis, with trophozoite-like structures near the borders of the lesions.

Anderson *et al.* (2009) described clinical cases from wild passeriformes of North America. Conjunctivitis, sinusitis and neurologic disease were detected in mockingbirds. Conjunctivitis, oronasal discharge and caseous stomatitis were seen in house finches (*Carpodacus mexicanus*). Stellar's jays (*Cyanocitta stelleri*), scrub jays (*Aphelocoma californica*), crows (*Corvus brachyrhynchos*) and ravens (*Corvus corax*) also had ocular or oral cavity alterations. Sinusitis was found in wood thrushes (*Hylocichla mustelina*), robins (*Turdus migratorius*), cat birds (*Dumetella carolinensis*), brown thrashers (*Toxostoma rufum*) and blue jays (*Cyanocitta cristata*). In house finches, the histopathological study identified granulomatous inflammation with lymphoplasmacytic cells and heterophils (Anderson *et al.*, 2010). Inflammation was seen at the oesophagus, oropharyngeal cavity, eyelids and crop. Caseous sinusitis or conjunctivitis was found in 10% of the cases, caseous stomatitis in 20% and oronasal discharge in 30%.

In Canada, Forzán *et al.* (2010) described slight to marked thickened crop, and rarely, white to yellow masses in the oropharyngeal cavity in purple finches (*Carpodacus purpureus*) and American goldfinches (*Carduelis tristis*). Hyperplasic epithelium and a necrotic pseudomembrane were detected at the mucosa of the oropharynx, crop and/or oesophagus. Multifocal areas of erosive to ulcerative necrosis and inflammation with heterophils, macrophages and lymphocytes were present. Trophozoite-like forms were detected between the keratinized epithelial cells. Hepatic trichomonosis was found in one American goldfinch with multifocal necrotic foci containing degenerated

macrophages, plasma cells and lymphocytes. Trophozoites were also identified next to the necrotic foci and hepatic sinusoids.

These type of lesions described for the oropharyngeal cavity of finches were later reported from subsequent outbreaks of the disease in the UK (Robinson *et al.*, 2010), Fennoscandia (Neimanis *et al.*, 2010), central Europe (Gourlay *et al.*, 2011; Ganas *et al.*, 2014). Furthermore, Neimanis *et al.* (2010) reported that some birds may have the mandibular bone affected, periesophageal fasciitis and myositis.

Madani *et al.* (2015) described fatal infections in captive zebra finches (*Taeniopygia guttata*) with fibrinonecrotic lesions in the oesophagus and crop and severe inflammation. The histopathological analysis revealed mucosal necrosis, ingluvial thickening and heterophil infiltration of the submucosa. Trophozoites were identified at the necrotic mucosal foci and tracheal epithelium.

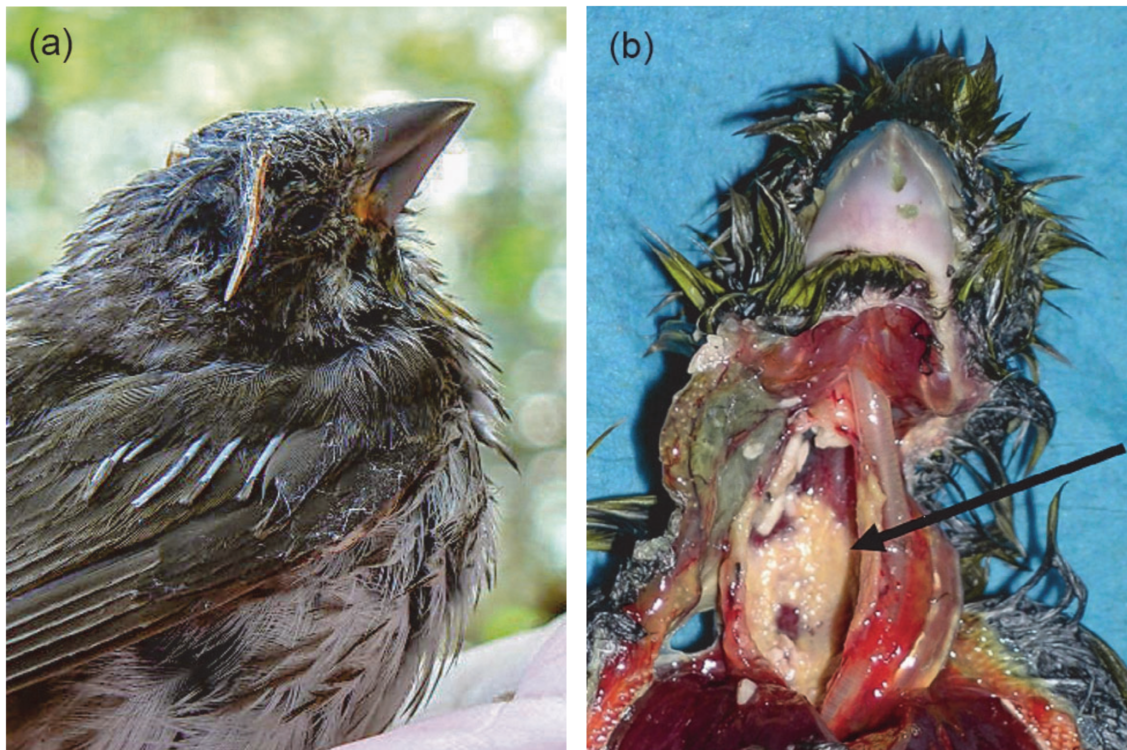


Figure 5. (a): clinical signs of trichomonosis in an immature purple finch (*Carpodacus purpureus*), note the matted feathers around the beak indicative of sialorrhea and lethargic appearance (from Forzán *et al.*, 2010). (b): avian trichomonosis infection in a greenfinch (*Chloris chloris*), the black arrow indicates the lesion of necrotic ingluvitis (from Robinson *et al.*, 2010).

1.6.6. Lesions in psittaciformes

Park (2011) reported that lesions in budgerigars were located in the oropharyngeal cavity, crop or thoracic/distal oesophagus. The type of lesions were described as abscesses and were focally distributed in one of the previous mentioned regions. A maximum size of 5 mm and occlusion of the digestive tract were also detected in some cases.

A case of a cockatiel (*Nymphicus hollandicus*) with a 5 mm abscess was also reported by this author. Its location was at the left pharynx and oesophagus and partially blocked the access to the digestive tract. A thick layer of purulent material in the oropharyngeal cavity was recorded also in a fatal infection from a purple-crowned lorikeet (*Glossopsitta porphyrocephala*).

Zimre-Grabensteiner *et al.* (2011) also found thickened crop mucosa in budgerigars that presented symptomatic infections.

1.6.7. Lesions in galliformes

Stockdale *et al.* (2015) described the case of a positive red-legged partridge (*Alectoris rufa*) with a caseous lesion at the proventriculus that penetrated through the organ wall and affected the liver. This organ was pale, with necrotic areas and adjacent connective tissue was also necrotic.

1.6.8. Lesions in piciformes

Ecco *et al.* (2012) examined a toco toucan (*Ramphastos toco*) that died of trichomonosis. The bird presented two caseous masses at the oropharyngeal cavity and caseous necrosis in the proximal oesophagus musculature. Trichomonad compatible organisms were present in the necrotic foci, oval or rounded structures with a pale eosinophilic colouration. Coagulative necrosis with heterophils and macrophages surrounded the caseous necrotic foci. Fibroblasts were covering these areas in moderate numbers. Mineralization and fibrinoid necrosis of vessel endotheliums were also identified.

1.7. Immune response

Regarding the immunological reaction originated by avian trichomonosis, cellular and humoral types of response are involved. For the cellular response, leukocytes phagocyte trophozoites in mild and avirulent infections, with an appropriate control of the parasite number, which does not multiply (Kocan and Herman, 1971). Inflammatory cells are always seen surrounding the necrotic foci in severe infections. For instance, heterophils, macrophages, lymphocytes and plasma cells were observed at the mucosa and submucosa of oropharyngeal lesions (Robinson *et al.*, 2010; Ecco *et al.*, 2012). Plasma cells, heterophils, macrophages and lymphocytes were detected in the liver and giant cells; lymphocytes and other mononuclear cells were observed in lung lesions (Pérez-Mesa *et al.*, 1961; Forzán *et al.*, 2010).

The humoral response has been proved to protect against natural infections. First approaches demonstrated that the subcutaneous inoculation of lyophilized antigens from *T. gallinae* secreted products in protein-free medium produced 50% of animals resistant to the development of lesions in challenge infections using a mouse model (Warren *et al.*, 1961). Later, Honigberg (1978), reported immunization of mice after intraperitoneal injections of living organisms from a strain of *T. gallinae* of mild pathogenicity. Other experiments demonstrated that plasma and serum will transfer this type of immunity to non immune columbiformes (Kocan, 1970; Kocan and Herman, 1970). Secretory IgA produced at the oropharyngeal mucosa could be another important response against the disease, but no further investigations have been carried out (Forrester and Foster, 2008).

Premunition generated from successive reinfections of the animals, could play a major role in the maintenance of an immune response (Forrester and Foster, 2008). Immunity will be lost with time, although specific studies have not been performed to determine this hypothesis.

Urban and Mannan (2014) speculated that the pH of the oropharyngeal cavity of the birds may influence the establishment of the infection. These authors reported more acidic pH values in older birds (fledglings and breeding Cooper's hawks) in comparison with nestlings, which had a higher prevalence of infection.

Other trichomonad parasites, such as *T. foetus* and *T. vaginalis* may provide useful information to design future experiments for oropharyngeal avian trichomonads. IgA and IgG produced at the mucosal surface and serum IgG have been proved to provide protection against *T. foetus* infection (Corbeil *et al.*, 2003; Cobo *et al.*, 2009). For *T. vaginalis*, humoral and cellular responses are both important, being the loss of humoral antibodies a marker for chronic infection or therapeutic failure (Schwebke and Burgess, 2004; Ton Nu *et al.*, 2015).

1.8. Diagnosis

1.8.1. Cytology

Two types of cytology have been used for detection of oropharyngeal trichomonads: wet-mount microscopy or stained cytologies. For the first type, a recently taken swab from the oropharyngeal cavity and crop is used, previously moistened in a sterile solution such as physiologic saline, which is extended on a microscopic glass slide. Then, a drop of the sterile solution is added to the sample, in order to observe the parasite, if present, in a liquid medium. Trophozoites will be directly visualized through optical microscopy and their characteristic movement would facilitate their detection. The main inconvenience of this method is that a direct examination should be performed, immediately after obtaining the sample. Moreover, a low specificity, more than twice times lower, has been reported in comparison with the culture (Bunbury *et al.*, 2005). However, it is still used as an inexpensive and simple method for screening and some authors have obtained good sensitivity results in certain host species, such as house finches (Anderson *et al.*, 2009).

In case of stained cytologies, the sample would be taken by the same procedure, but it would be left to air dry and subsequently stained with a metachromatic stain, such as Diff-Quick or Giemsa. Obviously, a more time-consuming type of diagnosis is required, especially early at the infection when low trophozoite numbers may be present, and the risk of false negative diagnosis is higher.

1.8.2. Culture

Cultivation of oropharyngeal trichomonads is regarded as the most sensitive method for the diagnosis of the infection (Cooper and Petty, 1988; Cover *et al.*, 1994; Bunbury *et*

al., 2005). A proper culture medium is required for the growth and isolation of the parasite, and numerous types have been tested for this purpose.

First investigations determined that certain ingredients are essential for the metabolism of the parasite. Hence, the addition of serum is vital for its content of diverse nutrients: amino acids, lipids, fatty acids and trace metals. Inactivated fetal bovine serum has been proved to obtain best results than chicken serum (Amin *et al.*, 2010). Maltose as carbohydrate source is efficiently used by *T. gallinae* trophozoites, providing the highest cell yields in comparison with glucose (Daly, 1970; Matthews, 1986).

The iron content is another important molecule, as studies carried out with *T. vaginalis* determined that the adherence to epithelial cells and multiplication rate of the strain could be increased (Lehker *et al.*, 1991; Ryu *et al.*, 2001; De Jesus *et al.*, 2007; Torres-Romero and Arroyo, 2009). In *T. gallinae*, optimal growth is also achieved in culture media with iron (Diamond, 1957; Amin *et al.*, 2010). Ascorbic acid was also reported as decisive ingredient (Forrester and Foster, 2008).

Considering liquid media, Diamond's medium or a modified recipe of it have been used in several studies (Diamond, 1954; Kocan and Amend, 1972; BonDurant and Honigberg, 1994; revised in Forrester and Foster, 2008). The media employed for *T. vaginalis* gave good results, as examples: *Trichomonas vaginalis* medium (TV), modified *Trichomonas vaginalis* medium (modified TV), Trypticase-Yeast extract-Maltose medium (TYM), Trypticase-Yeast-Iron serum 33 medium (TYS-S-33), Cysteine-Peptone-Liver infusion-Maltose broth (CPLM) or Simplified-Trypticase-Serum medium (STS) (Kupferberg *et al.*, 1948; Smith, 1983; Garber *et al.*, 1987; Höfle *et al.*, 2004; Amin *et al.*, 2010; Grabensteiner *et al.*, 2010).

CPLM broth supplemented with 5% of inactivated rabbit serum also enables trophozoite growth (Muñoz *et al.*, 1998; Höfle *et al.*, 2000). Loeffler's dried blood serum at 0.2% in Ringer's solution or 2% of pigeon serum in an isotonic salt solution are also other media that could be used for trophozoite propagation (Gerhold, 2014).

Medium 199 (M199), a general media for maintenance of eukaryotic cells, supplemented with rice starch and inactivated fetal bovine serum is another standard procedure for the isolation of these protozoa (Grabensteiner *et al.*, 2010).

Commercial diagnostic tests for the isolation of bovine, feline or human trichomonads, InPouch™ TF and TV Kits (BioMed Diagnostics, White City, Oregon, USA) have proved to be useful for sampling of avian oropharyngeal trichomonads in field conditions (Cover *et al.*, 1994; Boal *et al.*, 1998; Glass *et al.*, 2001; Schulz *et al.*, 2005; Bunbury *et al.*, 2007; Chi *et al.*, 2013; Girard *et al.*, 2013 and 2014; Martínez-Herrero *et al.*, 2014). Results indicated that this medium had the same sensitivity as Diamond's medium (Cover *et al.*, 1994).

Semisolid media, like Hollander fluid (HF) containing microbiology agar-agar are another option for cultivation. In 2010, Amin *et al.* developed a study for the optimization of growth and axenization process of clonal cultures from *T. gallinae* and *Tetratrichomonas gallinarum*. Their findings indicated that a modification of HF medium with inactivated fetal bovine serum or chicken serum instead of pooled human serum, obtained the best results for cell yield and survival, probably due to its ferric and maltose content.

T. gallinae is capable of multiplication in a range of pH reported between 6.5 – 7.5 (Urban and Mannan, 2014). Although the InPouch™ TV and TF Kits (BioMed Diagnostics, White City, Oregon, USA) employed in several studies have a pH of 6.1 ± 0.5, lower sensitivity has not been reported (Cover *et al.*, 1994; Boal *et al.*, 1998; Bunbury *et al.*, 2005 and 2007; Gerhold *et al.*, 2008; King *et al.*, 2010; Chi *et al.*, 2013; Girard *et al.*, 2013 and 2014; McBurney *et al.*, 2015). In fact, InPouch™ kits had the same sensitivity in comparison with Diamond's medium and offer advantages for field sampling conditions since the pouch does not need to be opened to check for trophozoite growth, reducing the risk of external contamination, and storage prior to use could be done at room temperature (Cover *et al.*, 1994).

Optimal incubation temperatures have been investigated as well. Average cloacal body temperature registered in chickens ranges from 40.6 to 43.0 ºC (Robertshaw, 2004).

Diamond (1957) recommended 35.5 ºC for incubation, but later, Amin *et al.* (2010) compared the growth of clonal cultures of *T. gallinae* at 37 ºC and 40 ºC and reported a higher number of live trophozoites at 37 ºC. Consequently, the majority of studies use this incubation temperature (Wieliczko *et al.*, 2003; Krone *et al.*, 2005; Gerhold *et al.*, 2008; Sansano-Maestre *et al.*, 2009; Grabensteiner *et al.*, 2010; Zimre-Grabensteiner *et al.*, 2011; Chi *et al.*, 2013; Girard *et al.*, 2013 and 2014; Martínez-Herrero *et al.*, 2014; Kunca *et al.*, 2015; Martínez-Díaz *et al.*, 2015; McBurney *et al.*, 2015). However, some authors used lower temperatures (30 ºC) in order to obtain a controlled parasite growth without a lower diagnostic sensitivity (Robinson *et al.*, 2010).

1.8.3. PCR

Polymerase chain reactions (PCR) of different loci have been used for confirmation of parasite DNA. Initially, a nested PCR of the α-tubulin gene, in addition to the ITS1/5.8S rRNA/ITS2 and small rRNA subunit amplifications were used for detection and phylogenetic classification (Felleisen, 1997; Gerhold *et al.*, 2008; Höfle *et al.*, 2004; Villanúa *et al.*, 2006; Gaspar da Silva *et al.*, 2007; Grabensteiner *et al.*, 2010; Chi *et al.*, 2013, Ganas *et al.*, 2014; Martínez-Herrero *et al.*, 2014; Martínez-Díaz *et al.*, 2015). Nowadays, a single-copy gene, called Fe-hydrogenase has been added for molecular fine-typing characterization in outbreaks (Lawson *et al.*, 2011; Ganas *et al.*, 2014; Girard *et al.*, 2013; McBurney *et al.*, 2015; Sansano-Maestre *et al.*, 2016; Zu Ermgassen *et al.*, 2016). However, up to date there are no studies that compared PCR results from oropharyngeal swabs with culture. This comparison would be interesting as maybe some strains are not capable to multiply in the selected medium and false negative results could appear. For instance, Sansano-Maestre (2009) reported that strains of genotype *T. gallinae*-1 had a slower *in vitro* growth in comparison with strains of genotype *T. gallinae*-2.

1.8.4. Others

Commercially available diagnostic tests for specific detection of oropharyngeal trichomonads without the requirement of cultures are not available yet. However, for *T. vaginalis* there are two main systems on demand.

The APTIMA® *Trichomonas vaginalis* system (APTIMA® TV, Gen-Probe Inc.) has been officially recognized by the USA Food and Drug Administration (FDA) for screening of urogenital swabs or urine samples (Chapin and Andrea, 2011). The sensitivity and specificity is of 95% and 98%, respectively. This device is based on the qualitative detection of ribosomal RNA. This technology processes the sample, produces a transcription-mediated amplification and hybridization using a chemiluminiscent probe.

In addition, a rapid diagnostic immunochromatographic test exists for *T. vaginalis*, the OSOM® test (Sekisui Diagnostics, L. L. C., San Diego, California, USA). The potential trichomonad antigens would be solubilised in a cellulose matrix and antibody-antigen complexes will be formed. Antibodies are conjugated with coloured particles that will be visualized by the examiner. Interestingly, this test has been proved for detection of *T. gallinae* infections in columbiformes (Valek *et al.*, 2013). The results revealed that a 93% and 90% specificity and sensitivity were obtained, respectively, in comparison with culture, the gold standard method. The test could be another alternative in those situations where cultures could not be performed.

For post-mortem diagnosis, a reliable method from formalin-fixed or paraffin-embedded tissue is essential. In 2010, Mostegl *et al.* developed a technique for *in situ* hybridization of different protozoan species from the order Trichomonadida based on the ribosomal 18S RNA gene, including *T. gallinae*.

1.9. Treatment

Nitroimidazole drugs are the first choice for the treatment of avian trichomonosis. Their mechanism of action is initiated by susceptible organisms that are capable of inducing the activation of the drug that will ultimately reach the interior of the cell by passive diffusion. They are considered to be inert substances, also named prodrugs, which need the reduction of their nitro group to allow their absorption by the trophozoite (Nagel and Aronoff, 2015). The reduced compound will produce DNA damage and the death of the cell (Edwards, 1980). Anaerobic and microaerophilic bacteria and protists are susceptible to their action; some of the more important species are, as an example, *Helicobacter pylori*, *Giardia duodenalis*, *Entamoeba histolytica* and *T. vaginalis*.

For the treatment of avian trichomonosis on birds of prey, metronidazole or carnidazole are the recommended medications (Redig, 2003; Samour and Naldo, 2003). For columbiformes, dimetridazole, carnidazole or ronidazole are employed in drinking water, food or by direct administration to the bird (Inghelbrecht *et al.*, 1996; Hawkins *et al.*, 2013).

Metronidazole could be used for most avian species (Hawkins *et al.*, 2013). A dose of 10-30 mg/kg is prescribed PO or IM every 12 hours for 10 days in psittacines, with 25 mg/kg PO every 12 hours during 2-10 days for their chicks or nestlings (Joyner, 1991; Tully, 2000; Bailey and Apo, 2008). For birds of prey, 30 mg/kg PO every 12 hours for 5-10 days or 50 mg/kg once every 24 hours for 5-7 days have been employed (Redig, 1992; Huckabee, 2000; Cooper, 2002; Bailey and Apo, 2008; Forbes, 2008). In some cases, doses up to 100 mg/kg PO every 24 hours for three days have been proved to be useful (Samour and Naldo, 2003). Columbiformes could be treated with 50 mg/kg PO every 12 hours for 5 days or with 1,057 mg/ml in drinking water (Rupiper and Ehrenberg, 1994; Harlin, 2006; Chitty and Lierz, 2008). In passeriformes, drinking water is the suitable vehicle to administer the drug, with 400 mg/l for 5-15 days as appropiate dosage when sinusitis is detected (Stadler and Carpenter, 1996; St. Leger *et al.*, 1998).

Carnidazole has been also extensively used in columbiformes, with different protocols. For example, 5 mg/kg PO in squabs or up to 30 mg/kg PO for two days in adults (Rupiper and Ehrenberg, 1994; Bailey and Apo, 2008; Chitty and Lierz, 2008; Taylor *et al.*, 2016). In birds of prey, a two-day treatment of 20 mg/kg PO every 24 hours, 30 mg/kg PO every 12 hours for three days or 50 mg/kg PO once have been used (Joseph, 1995; Heidenreich, 1997; Huckabee, 2000; Jones, 2006). In particular species, such as American kestrels (*Falco sparverius*) and Eastern screech owls (*Megascops asio*), but also for infections resistant to treatment with lower dosages, 120 mg/kg PO once or this dose divided during 2-5 days has been recommended (Ueblacker, 2000). The general recommendation for most species is to treat with 20-30 mg/kg PO for one or two days (Bailey and Apo, 2008; Chitty and Lierz, 2008). As a result, this drug is very common in any wildlife recovery center or conservation project that needs to treat wild birds,

because of the convenience of a single dose treatment, in contrast with the longer administration schedule required for metronidazole.

Dimetridazole is also an active compound against the protozoan, although some hepatotoxicity has been reported due to their low therapeutic index in lories, passeriformes, anseriformes and columbiformes (Stadler and Carpenter, 1996; Bailey and Apo, 2008). In columbiformes, central nervous system signs of intoxication, such as ataxia and, consequently, inability to fly and feed, have been reported as well with high doses (Reece *et al.*, 1985; Harlin, 2000). Besides, it is not available for food-producing animals in the USA and the European Union since 1995 due to the latest scientific evidence of its potential genotoxic carcinogenicity for humans (European Commission, 2015). However, it is reported as the drug of choice in bustards with a dosage of 900 mg/l in drinking water for five days, followed by 700 mg/l for ten days (Bailey and Apo, 2008).

Finally, ronidazole, the latest commercial active ingredient of the nitroimidazole group against avian trichomonosis, is used at 6-10 mg/kg PO every 24 hours for six to ten days in most species (Marshall, 1993). An increased dosage of up to 20 mg/kg PO every 24 hours for seven days could be administered in columbiformes, while 200 mg/l in drinking water during seven days could be needed if resistant infections are present in columbiformes and cockatiels (Marshall, 1993; Chitty and Lierz, 2008). Doses of 400 mg/l in drinking water for five to seven days are also recommended as preventive medications or treatments for canaries and columbiformes (Bailey and Apo, 2008). For canaries, 400 mg/kg in soft feed is another treatment option (Dorrestein, 1995; Hawkins *et al*., 2013).

Nevertheless, for food-producing animals, ronidazole was banned in 1993 in the European Union due to its carcinogenic potential risk for human health (European Commission, 2015). Furthermore, metronidazole and dimetridazole are not approved for use in birds in the USA, but could be prescribed by veterinarians for non-food-producing birds (Gerhold, 2014).

Nitroimidazoles are potential carcinogenic agents, with genotoxic and mutagenic effects known in humans after oral administration (European Agency for the Evaluation of Medicinal Products-EMEA, 1997). Mutagenicity was also observed after *in vitro* experiments with mammalian and human cells and carcinogenic effects were seen in mice and rats (Voogd *et al.*, 1974; Legator *et al.*, 1975; Lindmark and Muller, 1976; Rosenkranz and Speck, 1976; Speck *et al.*, 1976; Connor *et al.*, 1977; EMEA, 1997). Actually, metronidazole is classified as "possibly carcinogenic to humans" on group 2B by the International Agency for Research on Cancer (IARC, 2016) due to the sufficient evidence of carcinogenicity in experimental animals and limited or inadequate evidence in humans. Despite this information, it is currently used in human and veterinary medicine.

Some authors have reported the use of dimetridazole as treatment and preventive medication for outbreaks in wild birds. Höfle *et al.* (2004) investigated an outbreak in wood pigeons in Spain and Portugal and its control by the use of this compound added to the grain on the game feeders for red legged partridges (*Alectoris rufa*) that wood pigeons also used. Mortality rate was controlled but non desired effects were recognized on red legged partridges as a lower number of adults and chicks were registered during the next hunting season.

In Mauritius, carnidazole, ronidazole and dimetridazole were used to increase the survival of an endangered wild population of pink pigeons (Swinnerton *et al.*, 2005). Treatment was administered individually or through drinking water with good results.

Resistant strains to different nitroimidazoles have been demonstrated in *T. gallinae.* Treatment failures were reported by Franssen and Lumeij (1992) and Muñoz *et al.* (1998) in isolates obtained from clinical infections of pigeons. Muñoz *et al.* (1998) found different drug sensitivities, from highest to lowest: ronidazole, ornidazole, carnidazole, dimetridazole and metronidazole. In addition, ronidazole and carnidazole resistant strains were detected.

Zimre-Grabensteiner *et al.* (2011) tested clonal cultures from symptomatic budgerigars and pigeons for nitroimidazole resistance, with positive results to metronidazole, ornidazole, dimetridazole and ronidazole in the clones isolated from pigeons.

The widespread use of nitroimidazoles by pigeon fanciers explains the abundance of resistant strains in pigeons, being an issue of concern as these resistant organisms could be easily introduced in wild bird populations during their competitions (Rouffaer *et al.*, 2014). In fact, predation by raptors is one the most claimed damages by this sector.

Recent investigations have reviewed nitroimidazole compounds to improve its efficacy. Nitroimidazole carboxamides have demonstrated activity against *T. vaginalis* trophozoites, with lower *in vitro* toxicity to kidney and liver mammalian cells (Jarrad *et al.*, 2016).

Alternative compounds from African plants have been evaluated *in vitro* against *T. gallinae* trophozoites isolated from pigeons. Hence, Adebajo *et al.* (2006 and 2009) found anti-trichomonad activity on carbazole alkaloids extracted from curry tree (*Murraya koenigii*), leaves and wampee (*Clausena lansium*) stem bark, also known as "fool's curry leaf". Iwalewa *et al.* (2008) tested haronga (*Harungana madagascariensis*) stem bark extract against *T. gallinae* trophozoites isolated from pigeons with good results. Finally, Ibikunle *et al.* (2011) investigated the properties of Surinam cherry (*Eugenia uniflora*) leaves, a plant used in tropical countries against bacterial infections, malaria and trypanosomiasis, with promising results tested *in vitro.*

Synthetic chalcones are alternative compounds that have also shown activity against *T. gallinae* and low host toxicity (Oyedapo *et al.*, 2004). These chemicals are anti-inflammatory and potentially chemotherapeutic agents as well (Won *et al.*, 2005; Wu *et al.*, 2011).

Chitosan, obtained from the exoskeleton of shellfish, was presented as a potential new drug, with good *in vitro* results (Tavassoli *et al.*, 2012). Recently, Seddiek *et al.* (2014) evaluated the properties of garlic extracts for the treatment of avian trichomonosis in pigeons, with promising *in vivo* results.

1.10. Genetic characterization

1.10.1. ITS1/5.8S rRNA/ITS2 sequence

The ITS1/5.8S rRNA/ITS2 region has been used as a genetic marker for the phylogenetic classification of trichomonads. This fragment is non-coding DNA, present in tandem repetitions at the ribosomes of the cell, with internal transcribed spacers (ITS1 and ITS2) that are eliminated during pre-rRNA maturation. The ribosome of *T. gallinae* has two subunits, the large and the small. The large subunit has two sequences, 5.8S and 28S, while the small has the 16S. Genetic diversity has been detected at the ITS1 and ITS2 regions, that are less evolutionary conserved.

Felleisen (1997) studied the diversity of the genus *Tritrichomonas* and *Trichomonas* by the comparison of ITS1/5.8S rRNA/ITS2 sequences from *T. foetus*, *Tritrichomonas suis*, *Tritrichomonas mobilensis* and *T. vaginalis*, *T. tenax*, *T. gallinae* and *Pentatrichomonas hominis*. A higher diversity was detected on the genus *Trichomonas.* Later, Kleina *et al.* (2004) reported that this region was a solid marker for the taxonomical differentiation of the order Trichomonadida, from species to at least the family level.

The study of the ITS1/5.8S rRNA/ITS2 region has been used for the genetic characterization of avian trichomonosis outbreaks around the world (Höfle *et al.*, 2004; Gaspar da Silva *et al.*, 2007; Robinson *et al.*, 2010; Stimmelmayr *et al.*, 2012; Chi *et al.*, 2013; Ganas *et al.*, 2014; Girard *et al.*, 2013 and 2014; McBurney *et al.*, 2015).

Höfle *et al.* (2004) reported a severe outbreak of avian trichomonosis in wood pigeons from Spain and Portugal, with more than 2,600 dead birds. The ITS1/5.8S rRNA/ITS2 region was employed for the diagnosis of the protozoan, in addition to post-mortem examination and culture of the parasite.

The Mauritian columbiformes of Madagascar turtle doves (*Streptopelia picturata*) and pink pigeons, an endangered endemic species on the verge of extinction, were studied by Gaspar da Silva *et al.* (2007). No strain variation at the ITS1/5.8S rRNA/ITS2 sequences was detected on *T. gallinae* organisms and this genetic locus was determined as an appropriate species marker.

Gerhold *et al.* (2008) characterized the parasite in the USA, with five *T. gallinae* genotypes and seven *T. vaginalis*-like genotypes being described for this genomic region (A-L sequence groups). In Europe, Granbesteiner *et al.* (2010) reported further diversity in birds from Austria and the Czech Republic, with three types of organisms: *T. gallinae*, *T. vaginalis*-like and *T. tenax*-like. Six ITS1/5.8S rRNA/ITS2 sequence types were distinguished (I-VI). In addition, these authors detected mixed infections by the analysis of clonal cultures obtained from two clinical isolates of pigeons.

In the UK, Robinson *et al.* (2010) used this locus for the diagnostic confirmation of *T. gallinae* as the causative agent of the epidemic finch mortality. In Canada, Forzán *et al.* (2010) also employed this sequence for the molecular confirmation of finch trichomonosis. Later, Chi *et al.* (2013) detected three genotypes of this region in birds of prey, columbiformes, passeriformes and psittaciformes from the UK (A and C genotypes from Gerhold *et al.*, 2008 and genotype II from Grabensteiner *et al.*, 2010). Ganas *et al.* (2014) studied finch trichomonosis in Austria and Slovenia and identified the same strain involved in the finch epidemic (genotype A from Gerhold *et al.*, 2008). In Canada, McBurney *et al.* (2015) found two ITS1/5.8SrRNA/ITS2 genotypes. One of them belonged to the same genotype found in the UK (type A corresponding with A and B genotypes from Gerhold *et al.*, 2008) and was observed also in healthy birds; the other was seen in columbiformes without clinical signs (type B corresponding with C, D and E genotypes from Gerhold *et al.*, 2008).

In North America, Anderson *et al.* (2009) discovered a new oropharyngeal trichomonad from mockingbirds and reported a *T. gallinae* strain from house finches and corvids, clade III from Kleina *et al.* (2004), which corresponds with genotypes A and B from Gerhold *et al.* (2008). In the Caribbean, Stimmelmayr *et al.* (2012) determined *T. gallinae* and a *Trichomonas*-like parabasalid in an outbreak of Eurasian collared doves and African collared dove hybrids. Girard *et al.* (2013 and 2014) described a new species isolated from band-tailed pigeons with and without clinical signs, *T. stableri*, which was also found in mixed infections with *T. gallinae* in California, USA. Besides, a *T. blagburni* n. sp-like strain was also detected in two birds.

Epidemiological studies carried out in Spain revealed the presence of two main genotypes for the ITS1/5.8S rRNA/ITS2 region (A and B) and reported that one of them was associated with the presence of gross lesions on the birds (genotype B from Sansano-Maestre *et al.*, 2009; ITS-OBT-*T. gallinae*-1 from Martínez-Herrero *et al.*, 2014).

As it has been mentioned in this section, several genotypes have been established from the study of this sequence, although a lack of nomenclature consensus among the different authors still exists.

1.10.2. Small subunit of rRNA

The small subunit of rRNA has been analyzed as a preserved locus for the genetic characterization of avian oropharyngeal trichomonads. This ribosomal fragment is useful for phylogenetic analysis due to its ubiquitous presence in tandem repetitions on the cells and its highly conserved regions. As a result, this multicopy coding gene has been studied by several authors (Gerhold *et al.*, 2008; Grabensteiner *et al.*, 2010; Ganas *et al.*, 2014; Kunca *et al.*, 2015).

In the USA, Gerhold *et al.* (2008) reported five different genotypes, while Grabensteiner *et al.* (2010) described eight genotypes (I-VIII), with also mixed infections, in Europe.

Ganas *et al.* (2014) performed a multi-locus sequence typing (MLST) study of finch trichomonosis isolates from Austria, Slovenia and the UK including this genetic marker. Finally, Kunca *et al.* (2015) used this gene and the ITS1/5.8SrRNA/ITS2 region for the confirmation of infection in sparrowhawk nestlings from the Czech Republic.

1.10.3. Fe-hydrogenase gene

This single-copy gene is one of the latest genetic markers that have been added for the molecular typing of avian trichomonosis outbreaks. In fact, Lawson *et al.* (2011) employed this sequence for the fine-scale molecular typing of avian trichomonosis outbreaks in the UK. Previously, it was validated for the evolutionary analysis of protists, including *T. vaginalis* (Voncken *et al.*, 2002).

Several authors have declared their utility for deeper genetic characterization, useful to investigate the host and/or geographical origin of the outbreaks, with further resolution in comparison with the ribosomal markers ITS1/5.8S rRNA/ITS2 and small subunit of rRNA (Chi *et al.*, 2013; Ganas *et al.*, 2014; McBurney *et al.*, 2015; Sansano-Maestre *et al.*, 2016). This greater sequence diversity has provided new genotypes or sequence variants (single nucleotide polymorphisms, SNPs) within isolates belonging to the same ITS1/5.8SrRNA/ITS2 genotype (Chi *et al.*, 2013).

1.10.4. Others

Other loci that have been studied for avian trichomonosis are the α-tubulin and rpb1 genes. Firstly, α-tubulin was used with a nested PCR protocol described by Gerhold *et al.* (2008). This gene is a conserved genetic marker that codes for the α-subunit of microtubules, an important cytoskeleton structure vital for the cells. Seven genotypes were distinguished by Gerhold *et al.* (2008) for this locus. Its analysis was abandoned on posterior studies due to the appearance of new genetic markers, for instance, the Fe-hydrogenase gene, that showed superior capacity for the molecular fine-scale typing of isolates (Lawson *et al.*, 2011).

The study of the rpb1 gene, a single-copy gene that codes for the largest RNA polymerase II subunit, resolved phylogenetic relationships up to the species and isolate levels within ten parabasalid genera, including *T. vaginalis* and *T. gallinae* isolates (Malik *et al.*, 2011). Girard *et al.* (2013) found that *T. stableri* isolates from band-tailed pigeons of California, USA, were more closely related to *T. vaginalis* than to *T. gallinae* strains.

1.11. *In vitro* characterization

1.11.1. Growth in different culture media

Different culture media could be used for the *in vitro* propagation of the parasite. However, few studies have determined the best media for the obtention of high yields of protozoa or the maximum survival time for clinical isolates.

Amin *et al.* (2010) reviewed six different media (M199, TYM, TYI-S-33, HF, TV and modified TV) and two different incubation temperatures (37 ºC and 40 ºC). The authors

observed that the highest trophozoite yields were obtained in an optimized HF medium at 37 ºC of incubation for *T. gallinae* axenic clones.

Sansano-Maestre (2009) detected differences in the growth of trophozoites in TYM medium, according to the genotype of the parasite. This author reported that strains of genotype B for the ITS1/5.8S rRNA/ITS2 region had a lower growth in comparison with genotype A, although a low number of isolates were analyzed.

1.11.2. Virulence factors

One of the major research interests within avian trichomonosis is the description of the virulence factors that could be present and determine the development of gross lesions on the birds. First attempts to clarify this issue were the performance of experimental infections and immunologic analysis on affected birds (Stabler and Kihara, 1954; Kocan, 1969a; Kocan, 1970; Kocan and Knisley, 1970; Stabler and Braun, 1979). Cell culture infections were carried out by Honigberg *et al.* (1964) on chicken liver cells dispersed with trypsin. Differences were seen between a pathogenic and a non pathogenic strains (Jone's Barn and Lahore, respectively). These authors also tested cell-free filtrates but observed minor effects.

More recently, Amin *et al.* (2012a) compared the effects of *T. gallinae* clonal cultures with different genotype of high (P121) and low passage number (P10) in a permanent chicken liver (LMH) and quail fibroblast (QT35) cell lines. Distinct degrees of monolayer damage were detected according to the genotype of the parasite and cell-free filtrates produced a similar cytotoxic effect.

The haemolytic activity of the parasite has been also investigated, although the experiments were not conclusive. De Carli *et al.* (1996) demonstrated that *T. gallinae* isolates produced haemolysis of blood from seven different animal species (human, rabbit, rat, chicken, horse, bovine and sheep). This phenomenon was visualized by scanning electron microscopy (SEM) by De Carli and Tasca (2002). *T. gallinae* trophozoites adhered to the erythrocytes and internalized the cells by phagocytosis, although no hemolysins were identified. Tasca and De Carli (1999) and Gerhold *et al.*

(2009a) found evidence of haemolysis, although no correlation with strain pathogenicity could be established.

1.11.3. RNA viruses

The discovery of double-stranded RNA viruses in *T. vaginalis* and virus-like particles in *T. foetus* (Wang and Wang, 1985; Benchimol *et al.*, 2002a, b and c; Alderete *et al.*, 2003; Gomes-Vancini and Benchimol, 2005) lead to the investigations of these particles in *T. gallinae*.

In *T. vaginalis*, viral infections have been related to the expression of surface immunogenic proteins (Wang *et al.*, 1987) and an increased cytopathogenecity (Champney *et al.*, 1995). Recently, an association between infections by *T. vaginalis* strains containing these viral particles and severe clinical manifestations in patients has been also found (Fraga *et al.*, 2007 and 2012; El-Gayar *et al.*, 2016).

In *T. foetus*, virus-like particles are linked to drug treatment and its relation with the pathogenicity of the parasite is still unknown (Gomes-Vancini and Benchimol, 2005). Investigations in *T. gallinae* isolates have not identified these particles so far (Gerhold *et al.*, 2009b).

1.12. Proteomic characterization

The identification of new proteins from *T. gallinae* provides a precious insight that will aid in the complex process for the design of new therapeutic agents. González-Díaz *et al.* (2011) provided a public web server for the analysis of drug-target pairs that enabled the 3D structure reconstruction of peptides, including some components of the *T. gallinae* proteome.

The interaction between the parasite and host's cells has been also studied at the proteomic level. Consequently, Amin *et al.* (2012b) characterised the secreted products from an *in vitro* cell culture infection of chicken hepatocytes. These authors described cathepsin L-like cysteine peptidases that were involved in the cytophatogenic effects. Moreover, gene coding sequences from four peptidases were identified by the use of

PCR degenerated primers. In the study of the human pathogen *T. vaginalis*, a substantial amount of investigations regarding the proteome of the parasite have been performed.

A cornerstone in the study of *T. vaginalis* was the publication of its draft genome by Carlton *et al.* (2007). The data obtained from the first sequenced parabasalid genome identified approximately 800 possible surface proteins that could be involved in pathogenic mechanisms of the parasite, such as the adhesion to the host cells. In fact, more than 650 BspA-like proteins, that have been characterized in the adhesion and aggregation processes of pathogenic bacteria, were detected and may possibly regulate the invasion of the host cells. In addition, more than 75 leishmanolysins homolog proteins were found, the GP63-like proteins. In *Leishmania major*, this type of molecules participate in the pathogenicity of the parasite. Cytolytic candidate genes were also recognized, with saposin-like pore-forming domains (SAPLIP), which could be associated with the contact-dependent hemolytic activity of the parasite. Remarkably, more than 400 peptidases were revealed, proteins that are implicated with the clinical manifestations of the infection as virulence factors.

Adhesins, cysteine proteinases, the P270 immunogenic family proteins and other families with similar structures to proteins recognized in pathogenic microorganisms, are the major *T. vaginalis* surface proteins of interest that could aid in the development of new therapeutic strategies (Hirt *et al.*, 2007).

Cysteine proteinases, recognized virulence factors expressed on the plasmatic membrane of *T. vaginalis*, have demonstrated their role in the adherence, cytopathogenicity, haemolytic activity and immune evasion of the parasite in several studies (Neale and Alderete, 1990; Provenzano and Alderete, 1995; Álvarez-Sánchez *et al.*, 2000; Mendoza-López *et al.*, 2000; Hernández-Gutiérrez *et al.*, 2003 and 2004; Yadav *et al.*, 2007; Carvajal-Gámez *et al.*, 2014; Hernández *et al.*, 2014).

Other authors have investigated the influence of iron in *T. vaginalis* virulence. Surface adhesins, other proteins known as virulence factors, had contact and iron-dependent expression (García *et al.*, 2003). Morphological changes in the trophozoites are also highly regulated by this element, its absence leading to the development of pseudocysts

(De Jesús *et al.*, 2007). In *T. foetus* a lower expression of cysteine proteases and cytophatogenicity were also reported in parasites grown on iron-depleted medium (Melo-Braga *et al.*, 2003).

Cuervo *et al.* (2008) investigated the soluble protein fraction of two low and high virulent *T. vaginalis* isolates. A marked adhesion capacity to vaginal epithelial cells was present in the high virulent strain. The proteomic profile revealed a differential expression, possibly related to their marked different adherence.

2. OBJECTIVES

The general objective of this Doctoral Thesis is to elucidate some aspects of the oropharyngeal avian trichomonads present in wild birds, especially those related with genetic diversity and virulence.

The specific objectives to be fulfilled are:

1. To explore and describe the presence of trichomonads of the upper digestive tract in different hosts, including not previously reported species.

2. To analyze the risk factors associated with the development of gross lesions caused by avian trichomonosis in wild birds.

3. To describe the new variants of oropharyngeal avian trichomonads by genetic and morphometric analysis.

4. To determine the virulence of the most frequent genetic variants of oropharyngeal avian trichomonads by *in vitro* infections on cell cultures.

5. To identify and quantify the differences in protein composition of the plasmatic and organelle membranes from two clonal *T. gallinae* cultures with different genotype and pathogenicity.

3. MATERIAL AND METHODS

3.1. Samples of oropharyngeal trichomonads from wild birds

3.1.1. Study design

A cross-sectional epidemiological study was performed in different wild bird species. The first objective was to evaluate the presence of the parasite in non-frequent reservoirs. As samples from wild animals were difficult to obtain and subsequent evaluation of the birds is almost impossible, wildlife recovery centers (WRCs) were selected as the major source of samples, although other options were also employed.

3.1.2. Origin of the samples

3.1.2.1. Wildlife recovery centers and breeding centers

A formal petition of samples from birds with lesions of trichomonosis was submitted to the WRCs with the highest number of admissions (i.e., around 1,500 per year in Santa Faz, Alicante, 3,000 in GREFA, Madrid and 5,500 in Torreferrussa, Barcelona, table 1). Besides, a systematic sampling of all the bird entries was carried out in centers that were located close to the research laboratories of the CEU Cardenal Herrera University (Valencia, Spain) and the University Complutense of Madrid (Madrid, Spain). This systematic sampling was performed at the WRC of La Granja de El Saler (Valencia, Spain) and the wildife veterinary hospital of GREFA (Madrid, Spain), respectively. Centers that collaborated on this study are detailed on table 1.

Table 1. Wildlife recovery centers that have collaborated in the study.

Province	Location	Name	Center
Alicante	Santa Faz	Santa Faz	WRC
Badajoz	Almendralejo	Defensa y Estudio del Medio Ambiente – DEMA	Captive breeding center of lesser kestrel (*Falco naumanni*)
Barcelona	Santa Perpètua de Mogoda	Torreferrussa	WRC
Cuenca	Albendea	El Ardal	WRC
Madrid	Majadahonda	Wildlife veterinary hospital of Grupo de Rehabilitación de la Fauna Autóctona y su Hábitat – GREFA	WRC and captive raptor breeding center
Murcia	La Alberca	El Valle	WRC
Toledo	Sevilleja de la Jara	Centro de Estudios de Rapaces Ibéricas – CERI	WRC
Valencia	Valencia	Centro municipal de recogida de avifauna urbana y animales exóticos	WRC
	El Saler	La Granja de El Saler	WRC
Zaragoza	Zaragoza	La Alfranca	WRC

3.1.2.2. Scientific ringing campaings

Other centers were chosen for their convenient accessibility or number of animals present. For this reason, the captive breeding center of lesser kestrel from the non-governmental organisation "Defensa y Estudio del Medio Ambiente – DEMA" in Almendralejo (Badajoz, Spain), the Experimental Station of "La Hoya" from the Spanish National Research Council (Almería, Spain), several bird ringing scientists, wardens and researchers collaborated also in the sampling (table 2). Specific and detailed instructions of the sampling procedure and conservation of culture media were given to each center or collaborator, if the samples were not taken by the author.

Also, sampling of nestlings from endangered or protected birds of prey studied in other research projects were carried out, with permission. The official rings were placed on the birds, adding the sampling for oropharyngeal trichomonads to the standard procedures. These cases were the following ones: goshawks (*Accipiter gentilis*) from Murcia in 2011 and 2012, Eurasian eagle owls (*Bubo bubo*) from Alicante and Murcia in 2012 and 2013, common kestrels (*Falco tinnunculus*) from Madrid and Valencia from 2011-2014, Bonelli's eagles (*Aquila fasciata*) from Alicante, Almería, Castellón, Granada, Guadalajara, Jaén, Madrid, Málaga, Toledo and Valencia in 2015.

Some of the sampled birds belonged to conservation projects from the European LIFE program. These animals were lesser kestrels from the captive breeding center of DEMA (LIFE11 NAT/BG/000360-Lesser kestrel recovery) and Bonelli's eagles from GREFA (LIFE12 NAT/ES/000701-Integral recovery of Bonelli's eagle population in Spain).

Besides, at the same time, a special campaign that involved the monitorization of wild nestlings of Bonelli´s eagles took place in the Valencian Community by the local Government ("Conselleria de Agricultura, Medio Ambiente, Cambio Climático y Desarrollo Rural, Servicio de Vida Silvestre, Comunidad Valenciana, Generalitat Valenciana"). The Public Administration showed its interest in testing for *T. gallinae* infection and asked our team to analyze the samples obtained from these birds.

Additionally, another researcher from University of Valencia was performing a project about the movement patterns of adult Bonelli's eagles in the province of Castellón (Professor Dr. Pascual López López). For this purpose, adult birds were captured and a satellite navigation telemetry device was placed in the back of the birds, secured by a special harness (figure 6). Oropharyngeal swabs for the culture of *T. gallinae* were also obtained from these birds.

Figure 6. Adult of Bonelli's eagle (*Aquila fasciata*) with harness for satellite telemetry monitoring and collection of sample.

3.1.2.3. Bird control programmes and game wardens

Samples from columbiformes were also obtained from bird control programmes carried out by the Public Administration in the provinces of Almería and Valencia. In addition, private game wardens were also contacted in Valencia province in order to obtain samples from European turtle doves, a cynegetic species (figure 7 and table 2).

Figure 7. European turtle dove (*Streptopelia turtur*).

Table 2. Other collaborators for the sampling of the study.

Province	Personnel	Occupation
Alicante	Dr. Juan Manuel Pérez García	Researcher, bird ringing scientist
Madrid	Dr. Carlos Ponce	Researcher, bird ringing scientist
	Ignacio Otero Cañas	Bird ringing scientist
Murcia	Ángel Guardiola Gómez	Bird ringing scientist
	Fco. Alberto García Castellanos	Bird ringing scientist
	Dr. Mario León Ortega	Researcher, bird ringing scientist
Valencia	Antonio Maicas Ventura	Private game warden
	Antonio Polo Aparici	Bird ringing scientist
	Francisco Javier García Gans	Bird ringing scientist
	Pablo Vera García	Bird ringing scientist
	Dr. Pascual López López	Researcher

3.1.3. Sampling procedure and collection of data

Oropharyngeal trichomonads were recovered by culture in TYM medium (table 3). All solid ingredients were mixed in purified water and pH adjusted to 6.5. The medium was sterilized by filtration through 0.22 µm (Millipore, Billerica, Massachusetts, USA) in a sterile cabinet of biosecurity level class II. Inactivated fetal bovine serum and the supplement of antibiotics and antifungals were added later. Fetal bovine serum was inactivated at 56 ºC for 30 minutes. The antibiotic supplement consisted of 36 mg/l each of ticarcillin, vancomycin and ceftiofur (Sigma-Aldrich, St. Louis, Missouri, USA) and 24 ml of nystatin per liter (10,000 IU/ml, Sigma-Aldrich, St. Louis, Missouri, USA).

The medium was stored at -20 ºC until use, but never more than 6 months. For the sampling of the birds in the field, 5 ml of medium with antibiotics and antifungal supplement were aliquoted in polystyrene sterile tubes with screwed caps. Tubes were thawed at 4-8 ºC prior to use.

For the obtention of samples, the culture medium was brought to room temperature and warmed by holding between the hands. Two different sizes of sterile cotton swabs were used, according to the species of bird (figure 8). The smallest was applied in passeriformes with a similar or smaller size as house sparrows (*Passer domesticus*). The swab was moistened in the culture medium before use. Afterwards, it was introduced until the crop of the bird, with the exception of nocturnal birds of prey that lack this organ, and rubbed gently against the crop epithelium and/or mucosa of the oropharyngeal cavity. Tubes containing medium were inoculated immediately afterwards with the oral swabs. The swab was introduced to the bottom of the tube, agitated and scratched against the internal walls several times in order to leave all the attached material. Swabs were discarded after inoculation. The caps were tightly closed and tubes were protected against sun exposure and transported as soon as possible, in vertical position, for subsequent incubation at the laboratory.

If gross lesions were present in the oropharyngeal cavity, the peripheral area of the lesion was softly wiped with the swab.

Table 3. TYM medium composition for 1 l in distilled water.

Ingredient	Quantity
Trypticase	20 g
Yeast extract	10 g
D(+)-maltose	10 g
L-cysteine	1 g
Ascorbic acid	0.1 g
Inactivated fetal bovine serum	100 ml

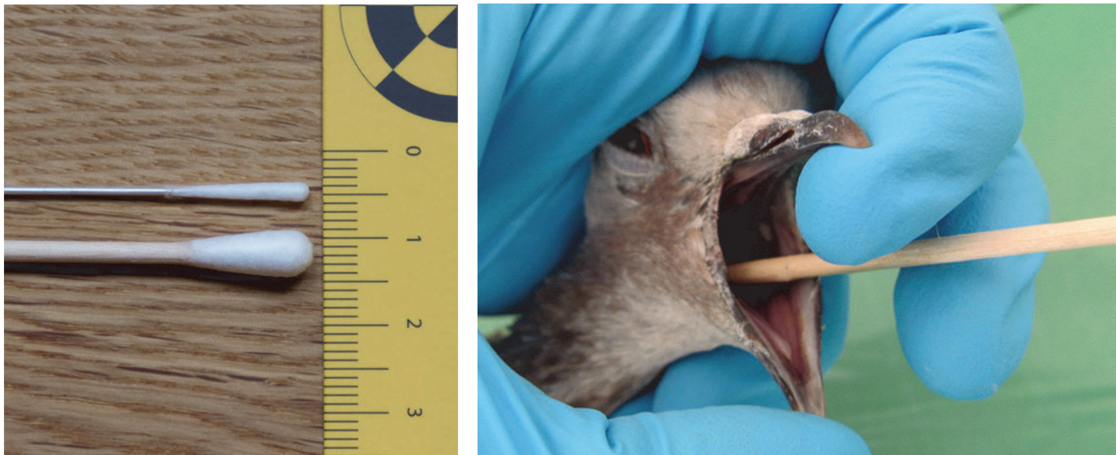


Figure 8. From left to right: types of sterile cotton swabs and collection of samples from the oropharyngeal cavity and crop of a rock pigeon (*Columba livia*).

Information of the bird species, age, sex, location, body score condition, clinical signs compatible with avian trichomonosis infection and presence of gross lesions at the oropharyngeal cavity were collected. Complete necropsies were performed from those birds sampled as carcasses.

Protocols of the sampling procedure and collection of data were submitted to each centre that collaborated in the study. For those centers that were located more than one hour in distance by car, the protocol included an incubation of 48 hours prior to the submission of the sample to the laboratory. This modification was included shortly after the initiation of the study because some of the remitted samples were determined as false negatives after a positive PCR from the sediment of the tube. This initial incubation period favored the adaptation of the parasite and assessed its growth during the first critical hours. In addition, the InPouch™ TF (BioMed Diagnostics, White City, Oregon, USA) diagnostic test was used in some of those centers.

Whenever possible, a cytological smear was performed before the oropharyngeal swab was discarded. This cytology was fixed in methanol and stained with Diff-Quick for a posterior analysis by optical microscopy.

3.1.4. Proposed classification of oropharyngeal lesions compatible with avian trichomonosis

A subset of 77 animals with different types of gross lesions at the oropharyngeal cavity or crop were selected for further clinical evaluation. Crop was examined by palpation and visual inspection for detection of large lesions. If birds were admitted as carcasses or died shortly after admission necropsies were performed. All the animals were screened for the presence of oropharyngeal trichomonads by culture in TYM medium.

Photographs from cases were collected or submitted by the different wildlife recovery centers and collaborators. These images were used to establish a classification system of lesions compatible with avian trichomonosis into four grades, attending to several criteria: number (focal or multifocal distribution), size, type of lesions (appeareance), if present; and affected structures (figure 9 and table 4).

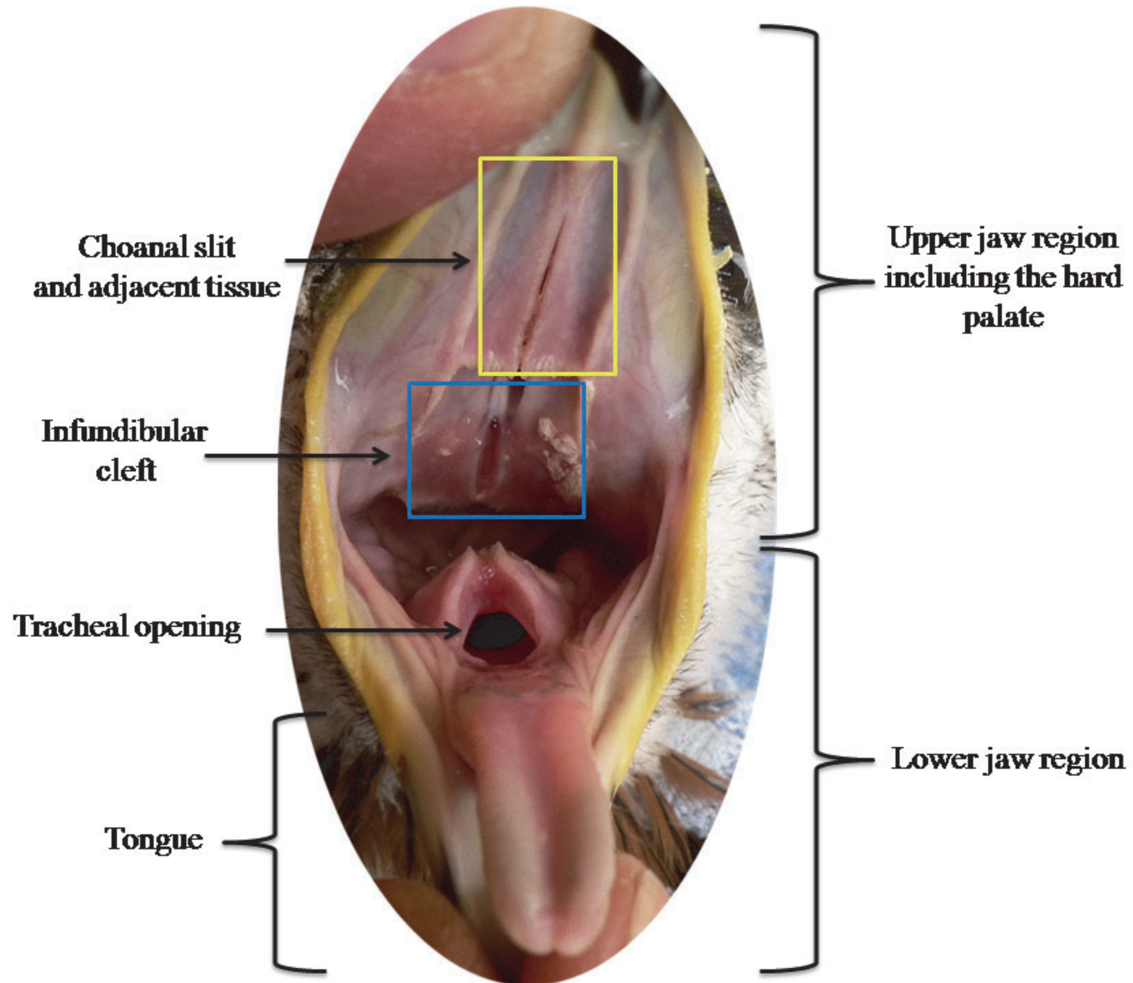


Figure 9. Anatomical regions of the oropharyngeal cavity of a nestling of Bonelli's ealge (*Aquila fasciata*, case R3-15, see section 4.4. for further information).

Grade 0 is composed of healthy animals without gross lesions at the mucosa of the oropharyngeal cavity, no clinical signs compatible with trichomonosis and a good body condition, i.e. body score of ≥ 3, will be present (figure 10).

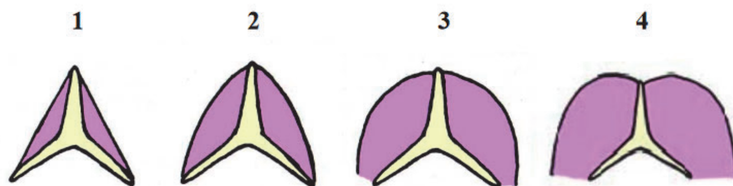


Figure 10. Avian body score condition used as reference in this study. The image represents a cross section of the pectoral muscles and the keel bone of a bird. From Heatley, 2015.

Grade 1 comprised animals with a small ulcer of less than 0.5 cm in length in the mucosa of the oropharyngeal cavity. The opening of the trachea was selected for the relative comparison of the dimensions of the lesions and it was proposed as a reference structure. No signs of regurgitation at the feathers surrounding the beak were detected and a normal body score condition would be expected (score 2 or 3).

Grade 2 corresponded to lesions up to one cm in length, smaller or with the same dimensions of the tracheal opening, but with a multifocal distribution. The body score condition would be between 2 or 3.

Finally, grade 3 was applied to nodular lesions of more than two cm in length, larger than the tracheal opening, with focal or multifocal distribution. Wet feathers surrounding the bill due to regurgitation or sialorrhea were also present. Poor body condition (score 1) with noticeable weight loss and, in extreme cases, cachexia, would be expected with this grade due to the generated inflammatory response, associated pain and potential blockage of the alimentary cannal by the large size of the lesions.

Nevertheless, proper differential diagnosis should be taken into account as other pathological processes could generate similar lesions, specially in grades 1 and 2.

Table 4. Criteria for the classification of oropharyngeal lesions compatible with avian trichomonosis infection employed in this study.

Classification	Lesion	Distribution	Length (cm) and size reference	Tissues affected	Wet feathers around the beak	Body score
GRADE 0	Absent	None	No	None	No	≥ 3
GRADE 1	Present	Focal	< 0.5, smaller than tracheal opening	Oropharyngeal cavity	No	≥ 3
GRADE 2	Present	Multi-focal	< 1, smaller or equal than tracheal opening	Oropharyngeal cavity	Yes	2 or 3
GRADE 3	Present	Focal or multi-focal	≥ 2, larger than the tracheal opening	Oropharyngeal cavity and/or crop	Yes	≤ 2

The proposed classification system will help to reach a common agreement among wildlife-related professionals, promoting a unique standardization process for the evaluation of lesions, determining the prognosis of the bird and the requirement or not of urgent treatment, if needed.

3.1.5. Culture and preservation of the parasites

Culture tubes were monitored every 48 hours post inoculation during 15 days by optical microscopy using an inverted microscope. Positive cultures were transferred into new fresh TYM medium with antibiotics and antifungals and passages were performed every 48-72 hours. Approximately one hundred µl of culture were transferred into a new culture tube with 5 ml of fresh culture medium at 37 ºC. The quantity of inoculum varied according to the state of the original culture, depending on the number of cells observed and their morphology.

One ml aliquots from cultures were kept for DNA extraction during the first hours of *in vitro* subcultivation.

Cultures were passaged until bacterial and fungal contamination was controlled; this condition occurred after three to six passages, generally. Contamination was monitored with nutritive and Sabouraud agars. One hundred µl of culture were distributed into each agar plate. Nutritive agars were incubated at 37 ºC for 24 hours, while Sabouraud agars were maintained at 22 ºC for 15 days.

Cryovials were prepared by the addition of 5% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, Missouri, USA) to the culture medium. Long term storage was initiated by the placement of the cryovials containing the isolates in a freezing container that provided a slow cooling rate (-1 ºC per minute) inside a -80 ºC freezer.

3.1.6. Statistical analysis

For the calculation of prevalence of infection, cultures that were contaminated by fungi or bacterial overgrowth were excluded.

A risk factor analysis was performed using Win Episcope software version 2.0 (http://www.clive.ed.ac.uk/winepiscope/). Categorical data of type of host (predator /prey), presence of gross lesions compatible with avian trichomonosis, ITS1/5.8S rRNA/ITS2 genotype of the isolate and diet of predator bird (ornitophagous/non-strict ornitophagous) were employed in the analysis. Goshawks, Eurasian sparrowhawks and peregrine falcons were considered as strict ornithophagous species. Interaction and confounding variables were analyzed by stratification of the data. Odds ratios were calculated for each stratum and adjusted by the Mantel-Haenszel test (OR_{MH}).

3.2. Genetic characterization of the parasites

3.2.1. DNA isolation

DNA extraction was performed from 1 ml of parasite culture. These aliquots were obtained from the initial culture tube or with a passage number lower than 3, for the majority of the samples. A silica-based column extraction kit was employed for this task (DNeasy blood and tissue extraction kit, QIAGEN, Valencia, California, USA). Extracted genomic DNA was stored at -20 ºC until use.

3.2.2. MLST analysis

3.2.2.1. ITS1/5.8S rRNA/ITS2 sequence

For the genetic characterization of the isolates, the ITS1/5.8S rRNA/ITS2 sequencing was selected as a primary analysis. This multicopy ribosomal genetic marker has been validated in a high number of scientific studies of parasitic trichomonads (Felleisen, 1997; Kleina *et al.*, 2004; Gaspar da Silva *et al.*, 2007; Gerhold *et al.*, 2008; Grabensteiner *et al.*, 2010; Lawson *et al.*, 2011; Chi *et al.*, 2013; Ganas *et al.*, 2014; McBurney *et al.*, 2015). This region contains internal transcribed spacers (ITS) that had a higher rate of nucleotide variations in comparison with the 5.8S rRNA sequence and are used for phylogenetic analysis among species within the same genus (Hillis and Dixon, 1991).

The protocol of Felleisen (1997) was used for the amplification of this region. The primers used had the following sequence: TFR1 (5′-TGCTTCAGCTCAGCGGGTCTTCC-3′) and TFR2 (5′-CGGTAGGTGAACCTGCCGTTGG-3′). For the PCR, 50 µl were prepared as follows:

5 µl of 10x PCR buffer, 1.5 mM of $MgCl_2$, two mM of dNTP, 2 µM of each primer, 2.5 IU of Taq polymerase (MP, Thomas Scientific, Swedesboro, New Jersey, USA) and 5 µl of genomic DNA. Temperature cycles started with an initial denaturation step of 9 minutes at 95 ºC, followed by 40 cycles of denaturation for 30 seconds at 94 ºC, annealing for 30 seconds at 66 °C, extension for 30 seconds at 72 °C and a final extension step for 15 minutes at 72 °C. Positive and negative controls were added in each PCR reaction. A GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, California, USA) was employed. Amplicons were visualized in a UV transilluminator after their electrophoresis at 80 V, 400 mA, for 35 minutes in 1.5% agarose gels (Sigma-Aldrich, St. Louis, Missouri, USA) stained with ethidium bromide (Sigm-Aldrich, St. Louis, Missouri, USA).

Amplicons were purified with a commercial kit (MinElute PCR Purification kit, QIAGEN, Valencia, California, USA) and submitted for Sanger sequencing to an external laboratory (Sistemas Genómicos, S. A., Paterna, Valencia, Spain). An automatic sequencer (3730XL DNA Analyzer, Applied Biosystems, Foster City, California, USA) with the ABI PRISM© BigDye© Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) was employed for this purpose.

3.2.2.2. Small subunit of rRNA

The small subunit of rRNA provides useful information as a multicopy sequence that is highly conserved (Gerhold *et al.*, 2008; Grabensteiner *et al.*, 2010; Lawson *et al.*, 2011; Ganas *et al.*, 2014; Zu Ermgassen *et al.*, 2016).

The amplification reaction was completed following the protocol of Ganas *et al.* (2014). Oligonucleotides used as primers were: Hm long f (5′-AGGAAGCACACTATGGTCATAG-3′) and Hm long r (5′-CGTTACCTTGTTACGACTTCTCCTT-3′).

The PCR was performed in 25 µl of: 12.5 µl of HotStarTaq Master Mix Kit polymerase (QIAGEN, Hilden, Germany), 8 µl of PCR water (QIAGEN, Hilden, Germany), 1 µl of each primer at 10 µM and 2.5 µl of genomic DNA. The temperature profile was as

follows: 15 minutes at 95 ºC for initial denaturation, 40 cycles of 30 seconds at 94 ºC, 1 minute at 55 ºC, 2 minutes at 72 ºC and a final extension step of 10 minutes at 72 ºC.

All reaction results were visualized and processed as described previously for the ITS1/5.8S rRNA/ITS2 sequences.

3.2.2.3. Fe-hydrogenase gene

The single-copy gene of the Fe-hydrogenase enzyme has a superior discrimination capacity due to its higher variation rate (Lawson *et al.*, 2011; Girard *et al.*, 2013; Ganas *et al.*, 2014; McBurney *et al.*, 2015; Sansano-Maestre *et al.*, 2016; Zu Ermgassen *et al.*, 2016).

For this genetic marker, the same PCR reaction mix was prepared with the following primers: TrichhydFOR (5´-GTTTGGGATGGCCTCAGAAT-3´) and TrichhydREV (5´-AGCCGAAGATGTTGTCGAAT-3´). The thermal cycler was programmed for: 15 minutes at 95 ºC for initial denaturation, 40 cycles of 30 seconds at 94 ºC, 1 minute at 58 ºC, 2 minutes at 72 ºC and lastly, 10 minutes at 72 ºC.

Amplicons were prepared as mentioned before for the ITS1/5.8S rRNA/ITS2 sequences. Tables 5 and 6 summarize the different primers and PCR conditions of the MLST study.

Table 5. Nucleotide sequence of the different primers employed for the MLST study of oropharyngeal trichomonad parasites.

PCR	Primer name	Primer sequence
ITS1/5.8S rRNA/ITS2	TFR1	5′-TGCTTCAGCTCAGCGGGTCTTCC-3′
	TFR2	5′-CGGTAGGTGAACCTGCCGTTGG-3′
Small subunit of rRNA	Hm long f	5′-AGGAAGCACACTATGGTCATAG-3′
	Hm long r	5′-CGTTACCTTGTTACGACTTCTCCTT-3′
Fe-hydrogenase	TrichhydFOR	5´-GTTTGGGATGGCCTCAGAAT-3´
	TrichhydREV	5´-AGCCGAAGATGTTGTCGAAT-3´

Table 6. Thermal cycler conditions used for the MLST DNA amplification from oropharyngeal trichomonad parasites. ITS: ITS1/5.8S rRNA/ITS2. SSU: small subunit of rRNA. Fe: Fe-hydrogenase gene.

Loci	PCR phase		Temperature	Time
ITS	Separation		95 ºC	9 min
	40 Cycles	Denaturation	94 ºC	30 sec
		Primer annealing	66 ºC	30 sec
		Extension	72 ºC	30 sec
	Final extension		72 ºC	15 min
SSU	Separation		95 ºC	15 min
	40 Cycles	Denaturation	94 ºC	30 sec
		Primer annealing	55 ºC	1 min
		Extension	72 ºC	2 min
	Final extension		72 ºC	10 min
Fe	Separation		95 ºC	15 min
	40 Cycles	Denaturation	94 ºC	30 sec
		Primer annealing	58 ºC	1 min
		Extension	72 ºC	2 min
	Final extension		72 ºC	10 min

3.2.3. Sequence analysis

Sequence chromatograms were manually checked and assembled with the Lasergene SeqMan software version 7.0.0 (DNASTAR, Madison, Wisconsin, USA).

3.2.4. Construction of phylogenetic trees

Multiple sequence alignments and phylogenetic tree reconstructions were carried out by nucleotide BLAST online resource (NCBI, 2016) and MEGA software version 6.06 (Tamura *et al.*, 2013). All possible codon positions were included (1st+2nd+3rd+non-coding). Overall mean distance was calculated with the Jukes-Cantor method prior to the construction of Neighbor-Joining trees. The nucleotide substitution model with the lowest Bayesian information criterion score was selected (Evans and Sullivan, 2011; Park and Nakhleh, 2012). Bootstrap analysis with 2,000 replicates was employed as statistical test.

3.3. Establishment of clonal cultures

Clonal generation of cultures could be performed by two basic methodologies, serial dilution o micromanipulation. At the present study, micromanipulation was selected as it had been previously used for the obtention of *T. gallinae* clones with good results

by Hess *et al.* (2006) and Grabensteiner *et al.* (2010). Moreover, a complete visual monitorization of the whole process is achieved by this technique as single cells are assured to be isolated for their clonal development. Isolates with a mixed infection (ITS1/5.8S rRNA/ITS2 genotypes *T. gallinae*-1 and *T. gallinae*-2; genotype groups according to Martínez-Herrero *et al.*, 2014) were selected for the establishment of clonal cultures. Strains from columbiformes and birds of prey, with and without gross lesions compatible with avian trichomonosis, were included. These isolates were genotyped with a low passage (lower or equal to six) and the micromanipulation procedure was performed at the Clinical Unit for Poultry Medicine of the University of Veterinary Medicine (Vetmeduni, Vienna, Austria), in collaboration with the research group of Professor Dr. Michael Hess (Diplomate of the European College of Poultry Veterinary Science).

3.3.1. Adaptation of parasites for micromanipulation

Isolates were subcultured in medium 199 (M199) with Earle's salts, L-glutamine (100 mg/ml), 25 mM of HEPES and L-amino acids (Gibco™, Thermo Fisher Scientific, Vienna, Austria). This medium was supplemented with 15% of inactivated fetal bovine serum (Gibco™, Thermo Fisher Scientific, Vienna, Austria), 0.22% of rice starch that was previously sterilized by dry heat at 180 ºC for one hour (Carl-Roth, Karlsruhe, Germany) and 0.5 ml of *Escherichia coli* DH5α-T1 bacterial culture incubated in agitation at 37 ºC for 24 hours in nine ml of supplemented M199. Cultures were passaged every 48-72 hours with an inoculum of 1,000 or 500 µl into nine ml of a temperated complete M199 (with the mentioned supplements and bacterial culture).

3.3.2. Micromanipulation procedure

One day previously to the micromanipulation procedure, 0.5 ml of culture were passaged into 9 ml of complete M199. At 24 hours post inoculation, 20 µl of these cultures were carefully placed at the centre of glass slides and diluted with M199 without rice starch. This step was done in order to have a clear suspension of trophozoites for the micromanipulation technique.

Once an adequate trophozoite density was observed by optical microscopy, the micromanipulation process began. An inverted microscope (Diaphot 300, Nikon,

Austria) with Narishige micromanipulators (Narishige, Japan) was employed. Single active trophozoites were visualized and retained by negative pressure with an aspiration micropipette (figure 11). The trophozoite was deposited in a drop of M199 without rice starch. Temperature of all the reagents used in this procedure was maintained at 37 ºC to avoid a decrease in the metabolic activity of trophozoites, detected by a reduced pattern of movement, that could negatively impact on the subsequent growth of clonal cultures.

Right after their selection, the drops of M199 containing single trophozoites were collected with a micropipette and added to Eppendorf tubes with 0.5 ml of complete M199.

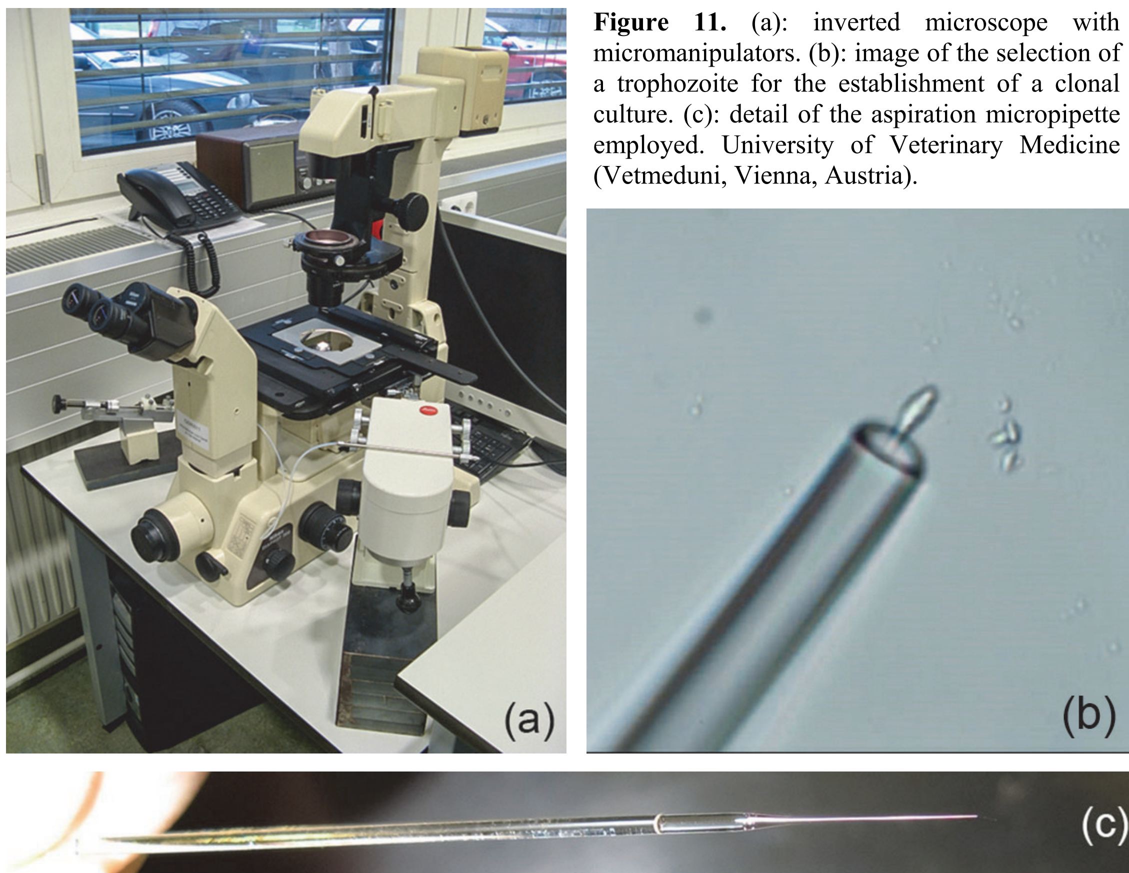


Figure 11. (a): inverted microscope with micromanipulators. (b): image of the selection of a trophozoite for the establishment of a clonal culture. (c): detail of the aspiration micropipette employed. University of Veterinary Medicine (Vetmeduni, Vienna, Austria).

Eppendorf tubes were incubated for five days at 37 ºC and examined every 48 hours for trophozoite growth. An aliquot of 15 µl was taken from the bottom of the Eppendorf tube and examined by optical microscopy. Growth was easily determined by trophozoite movement and also the pH indicator (phenol red) of the culture medium usually changed to an acid indicator (yellow colouration). Positive clonal cultures where growth

was detected were transferred into nine ml of complete M199, performing their subcultivation as previously mentioned.

Aliquots of 1 ml from the clones were taken for DNA extraction to be used in further analysis and cryovials were also prepared. Cryopreservation was made from 500 µl of cultures by the addition of 5% of DMSO (Sigma-Aldrich, St. Louis, Missouri, USA) in M199 without rice starch supplemented with 10% of inactivated fetal bovine serum. The freezing process was initiated with the placement of the cryovials in an special container (Mr. Frosty™, ThermoFisher Scientific, Vienna, Austria) with isopropyl alcohol that assured a slow cooling rate (-1 ºC per minute). The container was located in a -80 ºC freezer for 24 hours and later changed into a -150 °C for long term preservation.

3.3.3. Genetic characterization of clonal cultures

DNA extraction and genotyping of the ITS1/5.8S rRNA/ITS2 region of clonal cultures was performed as it was described earlier in section 3.2. for the genetic characterization of the parasites.

The results of the ITS1/5.8S rRNA/ITS2 sequences were used as criteria to select certain clonal cultures for further analysis, namely, virulence assays in LMH cells and the description of new variants of oropharyngeal trichomonads. Thereupon, these selected clonal cultures were further genetically characterized by the analysis of other two genetic markers, the small subunit of rRNA and the Fe-hydrogenase gene.

3.4. Morphologic analysis of new genetic variants

3.4.1. Selection of clones for the description of new variants

As a consequence of the epidemiological study of oropharyngeal trichomonads, new genetic variants of the ITS1/5.8S rRNA/ITS2 region were detected. In order to complete the characterization of these novel variants, isolates which underwent the micromanipulation procedure and therefore, had different clonal cultures available, were selected. In this case, the genetic variant from European turtle doves (*T. canistomae*-like-1) fulfilled these criteria.

Two other clonal cultures from the most widely reported *T. gallinae* genotypes from a multitude of avian host species in the literature (corresponding to the genetic groups *T. gallinae*-1 and *T. gallinae*-2 of Martínez-Herrero *et al.*, 2014), were included to use as reference organisms. These specimens were a *T. gallinae*-1 type clone isolated from a bird of prey with lesions and a *T. gallinae*-2 type clone from a columbiforme without lesions (table 7). In addition, one clone of genotype *T. tenax*-like-1 from a columbiforme with a clinical infection was added due to its different genetic profile. This clone was gently submitted by the research group of Professor Michael Hess (Vetmeduni, Vienna, Austria).

A MLST analysis was completed from the selected clonal cultures by the addition of the small subunit of rRNA and Fe-hydrogenase genes, following the methodology that has been aforementioned. Besides, a morphologic analysis of the variants was done by optical as well as scanning electron microscopy (SEM) methodologies.

Table 7. Clonal cultures selected for further genetic and morphometric characterization. Genetic groups according to Martínez-Herrero *et al.* (2014). ITS: ITS1/5.8S rRNA/ITS2 sequence.

ITS genotype	Clone	Lesion	Host
T. gallinae-1	R17-12 C1	Yes	*Bubo bubo*
T.gallinae-2	P178-13 C7	No	*Columba palumbus*
T. tenax-like-1	7895-06 C2	No	*Columba livia*
T. canistomae-like-1	P196-13 C20	No	*Streptopelia turtur*

3.4.2. Morphometric analysis of new variants

3.4.2.1. Axenization of clonal cultures

In order to analyze the morphology of the trophozoites, clonal cultures were axenized. During this process, bacterial and fungal contamination was examined and eradicated.

Clonal cultures were initially thawed in complete M199 with the *E. coli* DH5α-T1 strain. Then, new passages were done in M199 without the bacterial strain and later, with the addition of antibiotics. Penicillin (200 IU/ml) and streptomycin (200 µg/ml) were used to remove the remaining *E. coli* DH5α-T1 bacteria.

Nutritive and Sabouraud agars were employed to monitor bacterial and fungal contamination in each culture passage. One hundred µl of culture were disposed into each agar plate to test for contamination. Nutritive agars were incubated at 37 ºC for 24 hours. Sabouraud agars were kept protected from light at 22 ºC for 15 days. Once clonal cultures were axenized, M199 was changed to TYM medium and samples were prepared for the morphometric study by optical microscopy and the SEM structural analysis.

3.4.2.2. Study by optical microscopy

Fresh mount smears were prepared by the following methodology: one ml of culture that was passaged 24 hours previously was transferred into an Eppendorf tube. Trophozoites were sedimented by centrifugation (1,500 rpm for 3 minutes) and the medium was removed. The pellet was resuspended in 50 µl of RPMI medium with L-glutamine (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10% of inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, Missouri, USA). Ten µl of this suspension were deposited in clean glass slides for microscopy, extending the liquid in a circular shape of 1.5 cm of diameter. The slides were left to air dry and stained with Diff-Quick by 30 second immersion in each reagent (Medion Diagnostics, AG, Düdingen, Switzerland). A mounting medium (Depex, SERVA Electrophoresis, GmbH, Heidelberg, Germany) was used to add a cover slide for long term preservation.

An optical microscope (Zeiss Axioskop 20, Carl Zeiss, Germany) with a CMEX 10.0 (Euromex Microscopen BV, Arnhem, The Netherlands) digital camera was used for the obtention of photographs from 30 trophozoites of each clonal culture. The software ImageFocus 4, version 2.6 (Euromex Microscopen BV, Arnhem, The Netherlands) was employed for measurements.

Different measures were recorded to analyse if morphometric variations were present among the clonal cultures. These criteria were: length, width, perimeter and surface of the cell and nucleus; length of anterior flagella, recurrent flagellum and costa and total length of the axostyle and axostyle projection.

3.4.2.3. Statistical analysis

A multivariate analysis of variance (MANOVA) was performed to assess if there were differences between the measured variables depending on the clonal culture. In order to avoid collinearity, highly correlated variables ($r \geq 0.7$, p-value < 0.01) were identified by calculating the Pearson's correlation coefficient and discarded for the analysis.

Variables showing statistical differences among the clones were detected by univariate ANOVA tests. The Scheffé post hoc test was employed to identify their possible grouping. P values of ≤ 0.01 were considered statistically significant. The analysis was performed with the IBM SPSS Statistics software version 21 (IBM Corp., Armonk, New York, USA).

3.4.2.4. Structural analysis by scanning electron microscopy

Electron microscopy has superior magnification and resolution, which is not achieved by any other technique up to date, allows for a detailed analysis of superficial structures, although the sample specimen is destroyed during the procedure. However, SEM images have a maximal resolution of the external surface of the sample, giving the impression of three dimensional objects. Indeed, both types of electron microscopy, TEM and SEM, have been performed in *T. gallinae* isolates and other trichomonads such as *T. gallinarum* with interesting findings (Tasca and De Carli, 2003; Mariante *et al.*, 2004; Mehlhorn *et al.*, 2009; Martínez-Díaz *et al.*, 2015).

Images by SEM of this study were obtained at the National Center of Electron Microscopy (CNME, University Complutense of Madrid, Madrid, Spain), using a JEOL JSM 6400 scanning electron microscope (figure 12).

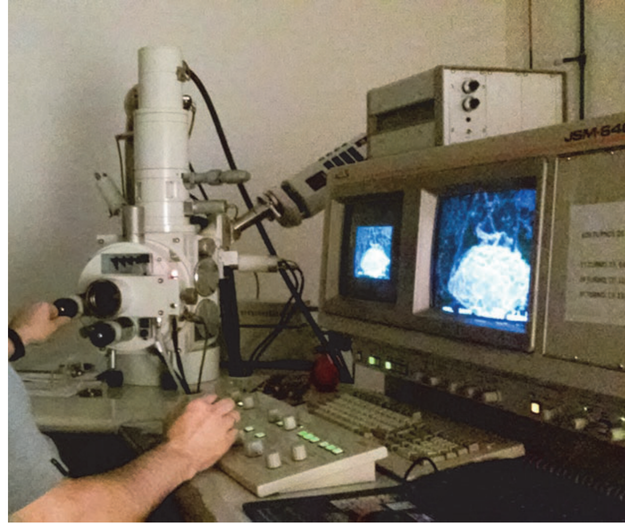


Figure 12. JEOL JSM 6400 scanning electron microscope at the National Center of Electron Microscopy (CNME, University Complutense of Madrid, Madrid, Spain).

Firstly, trophozoites were washed twice in sterile PBS (pH 7.2) for 15 minutes and centrifuged at 1,000 rpm for one minute. Pellets were fixed with a sterile PBS solution containing 2.5% glutaraldehyde and 4% paraformaldehyde for 24 hours at least. Secondly, cells were washed three times for 10 minutes in PBS (pH 7.2) and centrifuged at 700 rpm for 10 minutes, to remove the fixing solution. Then, trophozoites were transferred to cellulose filters with a syringe and dehydrated by immersion for 5 minutes in a gradient of increasing concentration of acetones (30%, 50%, 70%, 80%, 90% and 100%). Critical point drying and sputter coating with an ultra-thin layer of gold were the last steps of sample preparation performed at the CNME before the analysis of the specimens by SEM.

3.5. Virulence assays of *T. gallinae* clonal cultures in LMH cells

As Amin *et al.* (2012a and b) demonstrated, the cell line from a chicken hepatocellular carcinoma (LMH) provides a suitable system for the *in vitro* pathogenicity testing of *T. gallinae* clonal cultures. The same cell culture line was selected for this study. Moreover, these experiments were even performed at the same laboratory, in collaboration with the research group of Professor Michael Hess (Vetmeduni, Vienna, Austria).

For the purpose of determining the virulence capacity of oropharyngeal trichomonad isolates obtained from wild birds with clinical infections, that is, displaying gross lesions compatible with avian trichomonosis, clonal cultures were developed. This method avoided the use of possibly mixed isolates, i.e. with more than one genotype present, which will invalidate the results of experimental infections and further investigations. Clones derived from isolates of birds without gross lesions were also included for comparison (table 8). These clones were genetically characterized by MLST, following the procedures described previously (section 3.2.2.).

Table 8. Clonal cultures selected for virulence assays in LMH cells. Genetic groups according to Martínez-Herrero *et al.* (2014), Chi *et al.* (2013) or with GenBank acc. n. ITS: ITS1/5.8S rRNA/ITS2 sequence. SSU: small subunit of rRNA. Fe: Fe-hydrogenase gene.

<table>
<tr><th>ITS genotype</th><th>Clone</th><th>Lesion</th><th>Host (common name)</th></tr>
<tr><td rowspan="4">T. gallinae-1</td><td>P349-12 C11</td><td>Yes</td><td>Columba palumbus (wood pigeon)</td></tr>
<tr><td>P95-13 C3</td><td>No</td><td>Streptopelia decaocto (Eurasian collared dove)</td></tr>
<tr><td>R17-12 C1</td><td rowspan="2">Yes</td><td>Bubo bubo (eagle owl)</td></tr>
<tr><td>R193-13 C3</td><td>Falco tinnunculus (common kestrel)</td></tr>
<tr><td rowspan="3">T. gallinae-2</td><td>P178-13 C7</td><td rowspan="3">No</td><td>Columba palumbus (wood pigeon)</td></tr>
<tr><td>R24-12 C10</td><td>Falco tinnunculus (common kestrel)</td></tr>
<tr><td>R44-12 C5</td><td>Accipiter gentilis (goshawk)</td></tr>
</table>

3.5.1. Cultivation of LMH cells

LMH cells were grown at 37 ºC, with 85-90% humidity and 5% CO_2, in RPMI-1640 medium supplemented with 10% of inactivated fetal bovine serum and 0.5% of an antibiotic supplement of penicillin (40,000 IU/ml) and streptomycin (40 mg/ml) (Gibco™, Thermo Fisher Scientific, California, USA).

A total of 1,400,000 cells were seeded in a final volume of seven ml of medium and split every three or four days. Flasks of 25 cm^2 with filtered caps were employed (Nunc™, Thermo Fisher Scientific, California, USA). For cell splitting, the medium was removed and discarded. Five ml of sterile PBS (pH 7.2) at 37 ºC were used to wash the cells to avoid a lower trypsin efficacy by the remains of inactivated fetal bovine serum from the growth medium. Two ml of a trypsin-EDTA solution (0.05% of trypsin in 0.53 mM EDTA, Thermo Fisher Scientific, California, USA) were added to the flask and incubated for 3-5 minutes at 37 ºC in order to detach the cells from the flask surface. The flask was gently tapped and detachment was assessed by

observation in an inverted microscope. Trypsin and cells were placed in a 50 ml Falcon tube with 5 ml of growth medium to inactivate the effect of trypsin. The tube was centrifuged at 1,300 rpm for 5 minutes. The supernatant was discarded and cells were resuspended in 10 ml of fresh growth medium at 37 ºC. Fifty µl of the cell suspension were stained with 50 µl of trypan blue (Sigma-Aldrich, St. Louis, Missouri, USA) to assess their viability. Live cells were counted and inoculums of 1,400,000 live cells were selected to seed new flasks.

Cells were used only when a complete monolayer with 95% confluence was obtained (figure 13). Prior to the infection with trophozoites, RPMI-1640 medium was removed and cells were washed in a temperated sterile PBS (pH 7.2). A final volume of 6.5 ml of pre-warmed M199 supplemented with 10% of inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, Missouri, USA), 10% of tryptose phosphate broth (TPB, Gibco™, Thermo Fisher Scientific, California, USA) and 0.5% of a solution containing penicillin (40,000 IU/ml) and streptomycin (40 mg/ml) (Gibco™, Thermo Fisher Scientific, California, USA) was added for the virulence assays, according to the protocol described by Amin *et al.* (2012a).

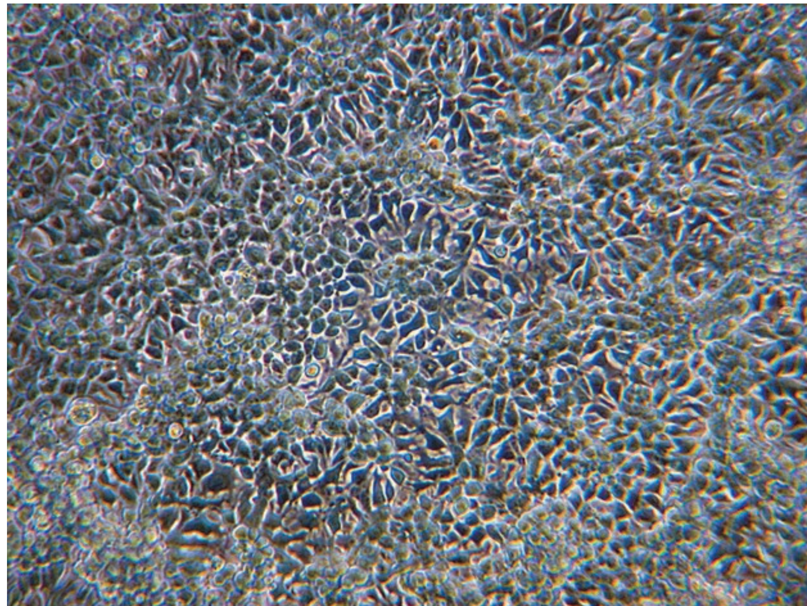


Figure 13. Monolayer of LMH cells used in this experiment.

3.5.2. Axenization of trichomonad clonal cultures and inoculation of LMH cells

Clonal cultures were cryopreserved in M199 supplemented with *E. coli* DH5α-T1. Therefore, the axenization procedure of parasites needed to be performed in order to infect LMH cells. Clones were subcultured in M199 without the *E. coli* DH5α-T1 and antibiotics were added to the medium according to the protocol of Amin *et al.* (2010): 200 IU/ml of penicillin, 200 µg/ml of streptomycin and 6 µg/ml of meropenem (Sigma-Aldrich, St. Louis, Missouri, USA).

Tryptose-soy-agar (TSA) and TPB were used to monitor bacterial and fungal contamination (Scharlab, S. L., Barcelona, Spain). TPB with 0.2% of agar was employed for the detection of aerobic, microaerophilic and anaerobic bacteria. One hundred µl of culture were taken for both agar and broth inoculation. Ten µl sterile inoculation loops were used in TSA plates to distribute the sample through the entire surface. Tubes with 5 ml of semi solid TPB were inoculated using the stab method. Positive and negative controls were used to test each batch of TSA and TPB media. Both, TSA plates and TPB tubes were incubated 24 hours at 37 ºC before examination.

Once trophozoites were growing without other microorganisms present, cultures were transferred to HF medium. The medium was prepared according to Amin *et al.* (2010). Briefly, all solid reagents were dissolved in purified water and pH adjusted to 7.2. The medium was sterilized in an autoclave at 121 ºC for 15 minutes. Inactivated fetal bovine serum was added later in a sterile cabinet. Medium composition is listed in table 9.

Table 9. Hollander fluid medium composition for 1 l.

Ingredient	Quantity
Trypticase	20 g
Yeast extract	10 g
D(+)-maltose	5 g
Agar	1 g
Ascorbic acid	1 g
KCl	1 g
$KHCO_3$	1 g
KH_2PO_4	1 g
K_2HPO_4	0.5 g
$FeSO_4$	0.1 g
Inactivated fetal bovine serum	100 ml

Initial trials with 100,000 trophozoites were tested in LMH cells in order to determine an adequate parasite inoculum for virulence assays. A lower inoculum of 10,000 trophozoites was finally selected as a slower parasite growth curve and damage to the LMH cell monolayer was produced.

Trophozoites were passaged 24 hours before the infection and cell viability was determined by trypan blue staining (Sigma-Aldrich, St. Louis, Missouri, USA) before the preparation of inoculums for LMH cell infection. The desired number of live trichomonad cells was resuspended in 0.5 ml of M199 with the supplements mentioned before on section 3.5.1.

LMH cell flasks were infected in triplicate, with positive and negative controls that were incubated in the same conditions (37 ºC, 85-90% humidity and 5% of CO_2). Virulence assays were maintained for five days.

3.5.3. Determination of cytopathogenic effect

All infected flasks plus negative and positive controls were daily monitored in an inverted microscope for the recording of the cytopathogenic effect score.

Trophozoites from the triplicated infected flasks were counted at 24, 48, 72, 96 and 120 hours post-infection. The contents of the flask were transferred into a 50 ml sterile Falcon tube. Flasks were washed twice with 5 ml of a temperated PBS (pH 7.2) and these washings were added to the 50 ml Falcon tube. LMH cells were detached from the flask, if it was needed, by the incubation in 2 ml of a trypsin-EDTA solution (0.05% of trypsin in 0.53 mM EDTA, Thermo Fisher Scientific, California, USA) at 37 ºC for three to 5 minutes. Trophozoites were stained with trypan blue and live parasites were counted by optical microscopy in a Neubauer hemocytometer chamber.

3.5.4. Statistical analysis

An analysis of variance was performed with continuous data of trophozoite counts and categorial data of CPE scores.

The results of average viable trophozoites counts in positive control flasks and flasks containing LMH cells were compared for each clonal culture tested. The Wilcoxon signed rank test, a non parametric equivalent of the paired t-test, was employed for this objective. *P* values of < 0.05 were considered statistically significant.

The values of CPE score on day 1 and day 2 post-infection and the ITS1/5.8S rRNA/ITS2 genetic group of the clonal culture were analysed to detect if there were differences among groups. Non parametric Chi-square tests for association were employed for this purpose. *P* values of < 0.01 were considered statistically significant. The IBM SPSS Statistics software package version 22 was employed for these analysis (IBM Corp., Armonk, New York, USA).

3.6. Analysis of membrane proteins in two *T. gallinae* clonal cultures with different genotype by RP-LC-MS/MS

3.6.1. Selection of clones, axenization and growth

Clonal cultures of *T. gallinae* were selected attending to their ITS genotype and presence of gross lesions in the host (table 10).

Proteins from the plasmatic membrane were the chosen fraction for the proteomic analysis of the clones. Some of these molecules would be implicated in virulent mechanisms of the parasite, such as adherence to the host cells, presence of peptidases, hemolysins or proteins related to vesicle-mediated transport that will reflect the exocytosis of molecules from the trophozoite. This experiment will identify the proteins of this fraction by liquid chromatography tandem mass spectrometry (LC-MS/MS) and determine their abundance by their relative intensity.

Table 10. Information of the clonal cultures selected for the proteomic analysis. ITS: ITS1/5.8S rRNA/ITS2 sequence type. SSU: small subunit of rRNA. Fe: Fe-hydrogenase gene. Genotype nomenclature according to table Martínez-Herrero *et al.* (2014)[1], Gerhold *et al.* (2008)[2], Grabensteiner *et al.* (2010)[3] and Chi *et al.* (2013)[4].

Clone code	Host	Age	Lesion	Location	ITS	SSU	Fe
P178-13 C7	*Columba palumbus*	Juvenile	No	Bétera, Valencia	*T. gallinae*-2-[1]	EU215373[2]	C4[4]
R17-12 C1	*Bubo bubo*	Nestling	Yes	Fuente Álamo, Murcia	*T. gallinae*-1[1]	VI[3]	A1[4]

Clones were thawed in 50 ml Falcon tubes with conical bottom that contained 9 ml of complete M199 temperated at 37 ºC. The procedure of growing, axenization and adaptation to TYM was done as it has been described previously (section 3.4.2.1). When no contamination was observed, trophozoites were inoculated in 20 Falcon tubes of 50 ml with 40 ml of TYM medium without antibiotics and antifungals.

Growth and cell viability was monitored at 24 and 48 hours post-inoculation in one tube from each clonal culture. Trypan blue was used as vital stain. Trophozoites were harvested at 48 hours post-inoculation by centrifugation at 2,000 rpm for 5 minutes. Trichomonad cells were washed three times with sterile PBS (pH 7.2) to remove growth medium and were immediately frozen at -80 ºC until subsequent processing could be performed.

The analysis required three biological replicates from each clone to validate the results. Besides, as a standard protocol in the proteomics laboratory, biological replicates were analysed in duplicate, i. e. two technical replicates were obtained from each sample (figure 14).

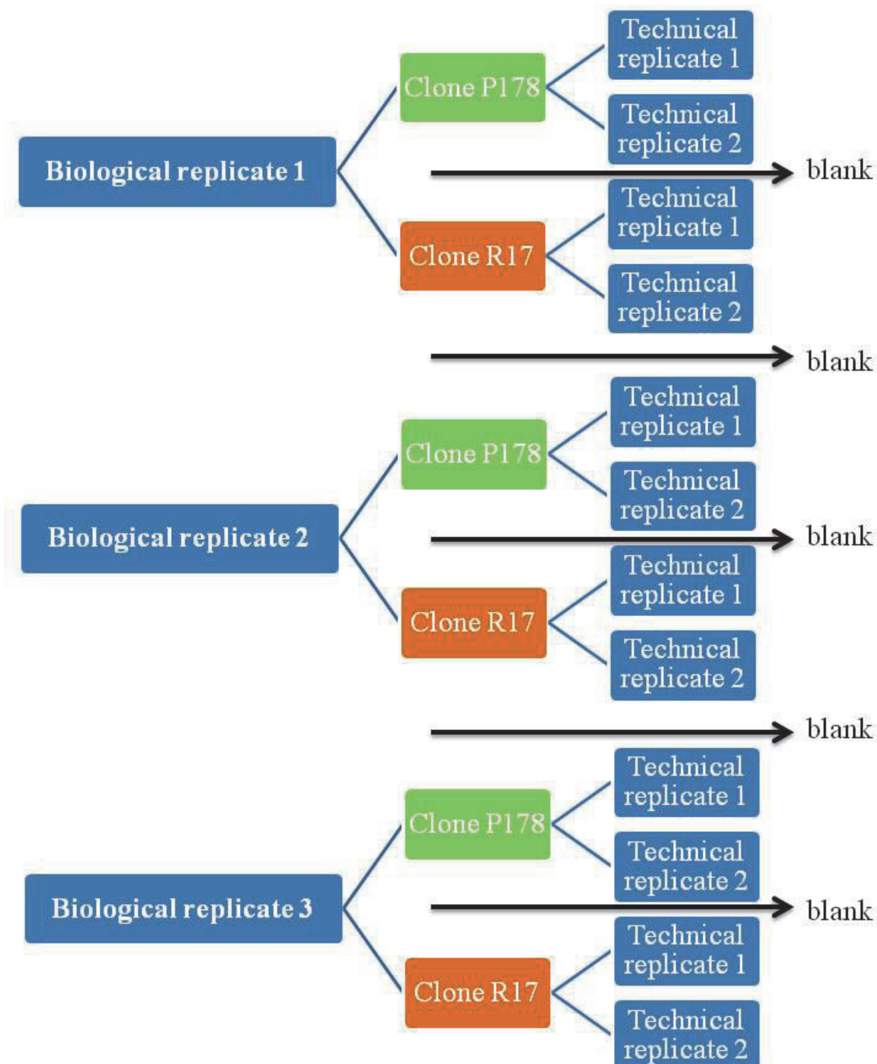


Figure 14. Study design for the proteomic analysis of plasmatic membrane and organelle membrane fraction from two clonal cultures of *T. gallinae* with different genotype. Clone P178: P178-13 C7. Clone R17: R17-12 C1.

3.6.2. Subcellular fractionation and protein extraction

A commercial subcellular fractionation kit was employed for this purpose: ProteoExtract® Subcellular Proteome Extraction kit (Calbiochem®, Merck KGaA, Darmstadt, Germany).

Cells were slowly thawed from -80 ºC to 4 ºC and later at room temperature (approximately 20 ºC) to avoid protein degradation and kit manufacturer's instructions were followed. Briefly, cells were resuspended with protease inhibitor cocktails and a series of extraction buffers that dissolved different proteome fractions were applied.

Firstly, soluble proteins corresponding to the cytosolic fraction were extracted. Secondly, membrane and organelle membrane proteins were obtained. Thirdly, nuclear proteins and, finally, the cytoskeletal fraction was recovered. Cells were kept on ice for all this procedure in order to avoid protein degradation. Incubation times were done in mechanical agitators to assess the correct distribution and solubilisation of the extraction buffers. The several fractions were obtained after cell centrifugation and kept at -80 ºC for further applications.

3.6.3. Protein precipitation and quantification

The membrane and organelle membrane protein fraction was gradually thawed from -80 ºC to 4ºC and kept on ice for acetone precipitation. Six volumes of acetone at -20 ºC were added to one volume of sample. The tube was mixed by inversion for three times and incubated at -20 ºC for four hours. Samples were centrifuged at 8,000 rpm for 10 minutes. Acetone was carefully poured from the tube and the pellet was kept at -80 ºC. Samples were resuspended in 100 µl of urea 8M. Quantification was done by the Bradford method (RC DC™, Bio-Rad Laboratories, Hercules, California, USA). This technique is based in colorimetric spectroscopy to determine the quantity of protein.

3.6.4. SDS gel electrophoresis

Ten µg of protein from each replicate were loaded in a 12% sodium dodecyl sulfate polyacrylamide gel for the electrophoretic separation (SDS-PAGE) of proteins according to their molecular weight. This gel was done as a quality control, to assess that the quantity of protein determined with the Bradford technique was correct. These analyses were carried out at the Proteomics Facility of University Complutense of Madrid (Madrid, Spain) that belongs to the ProteoRed network (PRB2-ISCIII, grant PT13/0001).

3.6.5. Protein digestion

For this procedure, 30 µg of sample from each replicate were reduced with 10 mM of dithiothreitol (DTT) at 37 ºC for 30 minutes. Proteins were alkylated with 55 mM of iodacetamide for 20 minutes and preserved from light exposure. For protein digestion, 1/30 (w/w) ratio of trypsin was added to each sample in a final volume of 50 µl (final trypsin concentration of 0.02 µg/µl) that were left at 37 ºC overnight (recombinant

trypsin sequencing grade, Roche). Peptides were desalted and concentrated by C18 reverse phase chromatography (C18 OMIX pipette tips, Agilent Technologies, Germany). A solution of 50% acetonitrile (ACN) and 0.1% of trifluoroacetic acid was used for peptide elution. The samples were freeze-dried in a Speed-vac and stored at -20 ºC until further analyses were made. Samples were resuspended in 0.1% of formic acid (FA) prior to the Nano LC-MS/MS analysis.

3.6.6. iTRAQ preliminar analysis

Originally, an iTRAQ quantification was the selected methodology for the proteomic analysis. However, due to the highly different protein profile revealed by the SDS gel electrophoresis, a label-free identification method was performed instead.

3.6.7. Label-free peptide ionization

Samples were analysed by reverse phase nano liquid chromatography (Easy-nLC II, Thermo Fisher Scientific, California, USA) connected with an ion trap mass spectrometer (LTQ-Orbitrap-Velos-Pro, Thermo Fisher Scientific, California, USA).

Two technical replicates were used with every one of the three biological replicates of each different clonal culture. Peptides were concentrated online by reversed-phase chromatography in a 0.1 x 20 mm pre-column and 0.075 x 250 mm column with 0.3 µl/minute flux (C18 RP, Thermo Fisher Scientific, California, USA). Peptide elution was performed with a 5-40% gradient of 0.1% FA in 80% ACN in water as buffer solution for 180 minutes. For peptide ionization (ESI) a nanoelectrospray source and 30 µm capillary emitters were employed (Nano-bore emitters stainless steel ID 30 µm, Proxeon, Thermo Fisher Scientific, California, USA).

The mass spectrometer was runned in data-dependent positive ionization for automatically detection from MS to MS/MS spectra (LTQ-Orbitrap-Velos-Pro, Thermo Fisher Scientific, California, USA). Peptides were detected with a 30,000 resolution in full scan MS mode with 400-1,600 atomic mass units (amu). A maximum of fifteen dependent MS/MS precursor scans were selected according to their intensity. Thirty seconds of dynamic exclusion were used and ± 2 mass-to-charge ratio units (m/z) of

isolation width. Peptides were fragmented by collision induced dissociation, with a normalized collision energy of 35%. MS/MS spectra were acquired in positive mode.

3.6.8. Relative peptide quantification and identification

Mass spectra were examined with MASCOT version 2.3 of Proteome Discoverer™ version 2.1.4 (Thermo Fisher Scientific, California, USA). The non-redundant NCBI protein database, family Trichomonadidae, with 103,432 protein sequences on date 27/01/2016, was selected for subsequent analysis (NCBInr, 2016).

The search was optimized with the following criteria: 20 ppm of tolerance for precursor peptide, 0.8 Da of fragment tolerance, two points of breakage for trypsin, carbamidometil-cysteine as fixed modification and metionin oxidation as variable modification. In all cases, the Decoy option was used for false discovery rate (FDR) estimation. MASCOT scores were adjusted with the percolator algorithm that has superior discrimination power between true and false identifications of the spectra (FDR < 1%). Correctly identified proteins were considered those with a FDR < 1% and at least one peptide identified with a confidence interval > 95% (p-value < 0.01).

Progenesis QI software version 4.1 (Nonlinear Dynamics, Waters company, Newcastle upon Tyne, UK) was employed for the relative quantification of the identified proteins. This program detects and quantifies all the peptide ions produced in the LC-MS/MS and analyses protein expression. The abundance of a specific protein was calculated as the average normalized abundance of its unique peptides in each run. Differential expressed proteins were considered those with a fold change ≥ 3 for their average value of normalized intensity ratio.

Predicted protein functions were assigned using the UniProtKB database which includes Swiss-Prot (551,987 protein sequences) and TrEMBL (66,905,753 proteins) (UniProt, 2016). BLAST analysis was performed and Gene Ontology (GO) information entries were used for the inference of biological functions.

4. RESULTS

4.1. Presence of oropharyngeal trichomonads on wild birds

A total of 1,688 wild birds were screened for oropharyngeal trichomonads from 2011 to 2013, including different provinces of Spain (table 11 and figure 15). Fifty three different host species were sampled, belonging to eleven avian orders: Accipitriformes, Charadriiformes, Ciconiiformes, Columbiformes, Falconiformes, Galliformes, Gruiformes, Passeriformes, Pelecaniformes, Psittaciformes and Strigiformes.

The overall prevalence of infection was 20.3%. Positive birds were detected in host species from orders Accipitriformes, Columbiformes, Falconiformes, Passeriformes and Strigiformes. Twenty three different avian host species were infected with oropharyngeal trichomonads and from 16 of them new submissions of parasite genetic sequences were sent to GenBank public database (GenBank, 2016): common buzzards (*Buteo buteo*), eagle-owls, Egyptian vultures (*Neophron percnopterus*), Eurasian scops owls (*Otus scops*), Eurasian sparrowhawks, European honey-buzzards (*Pernis apivorus*), European turtle doves, goshawks, hen harriers (*Circus cyaneus*), lesser kestrels (*Falco naumanni*), long-eared owls (*Asio otus*), magpies (*Pica pica*), marsh harriers (*Circus aeruginosus*), peregrine falcons, tawny owls and wood pigeons.

The maximum levels of infection were obtained in budgerigars (100.0%), European turtle doves (100.0%), rock pigeons (79.4%), goshawks (74.5%), wood pigeons (70.0%), Eurasian collared doves (*Streptopelia decaocto*, 57.8%) and Egyptian vultures (50.0%).

In contrast, the lowest values of infection were detected in golden eagles (*Aquila chrysaetos*, 4.0%), peregrine falcons (4.2%), lesser kestrels (4.4%), eagle owls (5.3%), barn owls (5.6%), Eurasian scops owls (8.3%), common buzzards (8.8%), common kestrels (*Falco tinnunculus*, 14.2%), tawny owls (16.7%), marsh harriers (18.2%), booted eagles (*Aquila pennata*, 21.1%), European honey-buzzards (25.0%), magpies (25.0%), long-eared owls (27.3%), Eurasian sparrowhawks (27.8%) and hen harriers (28.6%).

Finally, the parasite was not detected (during the sampling period of this study) in the following species: black kites (*Milvus migrans*), blackbirds (*Turdus merula*), blue tits (*Cyanistes caeruleus*), Bonelli's eagles, common chaffinches, common quails (*Coturnix coturnix*), Eurasian jays (*Garrulus glandarius*), European serins (*Serinus serinus*), goldfinches (*Carduelis carduelis*), great tits (*Parus major*), greenfinches (*Carduelis chloris*), grey herons (*Ardea cinerea*), griffon vultures (*Gyps fulvus*), hobbies (*Falco subbuteo*), house sparrows, lesser black-backed gulls (*Larus fuscus*), little bitterns (*Ixobrychus minutus*), little owls (*Athene noctua*), merlins (*Falco columbarius*), monk parakeets (*Myiopsitta monachus*), monk vultures (*Aegypius monachus*), Montagu's harriers (*Circus pygargus*), moorhens (*Gallinula chloropus*), red kites (*Milvus milvus*), reed buntings (*Emberiza schoeniclus*), short-eared owls (*Asio flammeus*), short-toed eagles (*Circaetus gallicus*), Spanish imperial eagles (*Aquila adalberti*), spotless starlings (*Sturnus unicolor*) and white storks (*Ciconia ciconia*). However, some of these species were found positive in posterior samplings, like Bonelli´s eagle (data on section 4.2.) or monk vultures (Martínez-Díaz *et al.*, 2015; see appendix V: other publications).

Gross lesions at the oropharyngeal cavity were detected in species of the orders Accipitriformes, Columbiformes, Falconiformes, Passeriformes, Psittaciformes and Strigiformes. In the first order, the species with the highest number of birds presenting lesions were Eurasian sparrowhawks (27.8%). Lesions were also detected in booted eagles (21.1%), peregrine falcons (12.5%), common buzzards (11.8%), red kites (11.1%), common kestrels (9.4%), marsh harriers (9.1%), black kites (9.1%), golden eagles (8.0%), Bonelli's eagles (4.8%), griffon vultures (2.0%) and lesser kestrels (0.8%). Subclinical infections, with no clinical signs and no lesions, were found in: European honey-buzzards, Egyptian vultures, goshawks and hen harriers.

In species from the order Columbiformes, lesions were detected in wood pigeons (60.0%, 6/10) with the maximum number of birds registered. Eurasian collared doves presented a minimal rate of birds with lesions (1.0%, 3/289). Positive birds without gross lesions were found in Eurasian turtle doves and rock pigeons.

In the orders Passeriformes, Psittaciformes and Strigiformes, host species from which a positive culture was detected presented macroscopical lesions at their oropharyngeal

cavity compatible with avian trichomonosis. Subclinical infections, i.e. without clinical signs or lesions, were not detected.

Magpies were the unique species of the order Passeriformes with positive results and which had gross lesions of avian trichomonosis in 25.0% (1/4) of the sampled birds.

From the order Psittaciformes, the sampled budgerigar had a positive culture (100.0%, 1/1) and clinical signs of trichomonosis, namely regurgitation, weight loss, sialorrhea with evident wet feathers around the beak were observed. The bird was unable to feed.

Finally, in the case of the order Strigiformes, tawny owls had the highest percentatge of birds with lesions (44.4%, 8/18), continuing with long-eared owls (27.3%, 3/11), barn owls (8.3%, 3/36), Eurasian scops owls (8.3%, 1/12) and eagle owls (5.3%, 3/57).

Figure 15. Map of Spain with shaded provinces where samples were obtained. Numbers indicate the wildlife recovery center locations: **1.-** DEMA (Almendralejo, Badajoz), **2.-** CERI (Sevilleja de la Jara, Toledo); **3.-** GREFA (Majadahonda, Madrid); **4.-** El Ardal (Albendea, Cuenca); **5.-** La Alfranca (Pastriz, Zaragoza); **6.-** Torreferrusa (Santa Perpétua de Mogoda, Barcelona); **7.-** La Granja (El Saler, Valencia); **8.-** Santa Faz (Santa Faz, Alicante); **9.-** El Valle (La Alberca, Murcia).

Table 11. Number of samples and prevalence values of infection and gross lesions compatible with avian trichomonosis. n: total number of birds sampled. Prevalence %: birds with a positive culture in TYM medium. Lesion %: birds with lesions from the total number of birds sampled by host species. O: ornithophagous species. NO: non-strict ornithophagous species. * indicates first parasite DNA sequence obtained from the host species. Nd: not determined.

	Host species (common name)	n	Prevalence		Lesion		Diet
			n	%	n	%	
Predator	*Accipiter gentilis* (goshawk)*	55	41	74.5	0	0	O
	Accipiter nisus (Eurasian sparrowhawk)*	18	5	27.8	5	27.8	O
	Aegypius monachus (monk vulture)	30	0	0	0	0	NO
	Aquila chrysaetos (golden eagle)	25	1	4	2	8.0	NO
	Aquila fasciata (Bonelli's eagle)	21	0	0	1	4.8	NO
	Ardea cinerea (grey heron)	1	0	0	0	0	NO
	Asio flammeus (short-eared owl)	2	0	0	0	0	NO
	Athene noctua (little owl)	80	0	0	0	0	NO
	Bubo bubo (eagle owl)*	57	3	5.3	3	5.3	NO
	Ciconia ciconia (white stork)	1	0	0	0	0	NO
	Circaetus gallicus (short-toed eagle)	3	0	0	0	0	NO
	Circus aeruginosus (marsh harrier)*	11	2	18.2	1	9.1	NO
	Circus cyaneus (hen harrier)*	7	2	28.6	0	0	NO
	Falco columbarius (merlin)	3	0	0	0	0	NO
	Falco naumanni (lesser kestrel)*	249	11	4.4	2	0.8	NO
	Falco peregrinus (peregrine falcon)*	24	1	4.2	3	12.5	NO
	Falco subbuteo (hobby)	2	0	0	0	0	NO
	Falco tinnunculus (common kestrel)	373	53	14.2	35	9.4	NO
	Garrulus glandarius (Eurasian jay)	2	0	0	0	0	NO
	Gyps fulvus (griffon vulture)	50	0	0	1	2.0	NO
	Ixobrychus minutus (little bittern)	1	0	0	0	0	NO
	Milvus migrans (black kite)	11	0	0	1	9.1	NO
	Milvus milvus (red kite)	9	0	0	1	11.1	NO
	Neophron percnopterus (Egyptian vulture)*	2	1	50.0	0	0	NO
	Otus scops (Eurasian scops owl)*	12	1	8.3	1	8.3	NO
	Pernis apivorus (European honey-buzzard)*	4	1	25.0	0	0	NO
	Pica pica (magpie)*	4	1	25.0	1	25.0	NO
	Strix aluco (tawny owl)*	18	3	16.7	8	44.4	NO
	Tyto alba (barn owl)	36	2	5.6	3	8.3	NO
	Aquila adalberti (Spanish imperial eagle)	3	0	0	0	0	NO
	Aquila pennata (booted eagle)	19	4	21.1	4	21.1	NO
	Asio otus (long-eared owl)*	11	3	27.3	3	27.3	NO
	Buteo buteo (common buzzard)*	34	3	8.8	4	11.8	NO
	Circus pygargus (Montagu's harrier)	35	0	0	0	0	NO
	Larus fuscus (lesser black-backed gull)	1	0	0	Nd	Nd	NO
	Total predator hosts	**1,214**	**138**	**11.3**	**79**	**6.5**	

Table 11. (Cont.)

	Host species (common name)	n	Prevalence		Lesion	
			n	%	n	%
Prey	*Carduelis carduelis* (goldfinch)	5	0	0	0	0
	Chloris chloris (greenfinch)	20	0	0	0	0
	Columba livia (rock pigeon)	34	27	79.4	0	0
	Columba palumbus (wood pigeon)*	10	7	70.0	6	60.0
	Coturnix coturnix (common quail)	1	0	0	0	0
	Cyanistes caeruleus (blue tit)	1	0	0	0	0
	Emberiza schoeniclus (reed bunting)	4	0	0	0	0
	Fringilla coelebs (common chaffinch)	3	0	0	0	0
	Gallinula chloropus (moorhen)	1	0	0	0	0
	Melopsittacus undulatus (budgerigar)	1	1	100.0	1	100.0
	Myiopsitta monachus (monk parakeet)	3	0	0	0	0
	Passer domesticus (house sparrow)	68	0	0	0	0
	Serinus serinus (European serin)	15	0	0	0	0
	Streptopelia decaocto (Eurasian collared dove)	289	167	57.8	3	1.0
	Streptopelia turtur (European turtle dove)*	3	3	100.0	0	0
	Sturnus unicolor (spotless starling)	2	0	0	0	0
	Turdus merula (blackbird)	12	0	0	0	0
	Parus major (great tit)	2	0	0	0	0
	Total prey hosts	**474**	**205**	**43.2**	**10**	**2.1**
	Total (predator and prey hosts)	**1,688**	**343**	**20.3**	**89**	**5.3**

4.1.1. Genetic characterization of the parasites

A classification of ITS1/5.8S rRNA/ITS2 region genotypes reported by our group and other authors was performed according to their phylogenetic similarity by BLAST analysis. Genetic groups were established in accordance with these results, using a 99% of sequence identity as minimal criteria for their inclusion (table 12).

Table 12. Genetic groups for the ITS1/5.8S rRNA/ITS2 locus of oral *Trichomonas* sp. isolated from birds reported in GenBank database. *Associated with gross lesions. Genetic group indicated as: ITS-OBT-X-n. OBT: Oropharyngeal Bird Trichomonad. X: organism BLAST homology (*Simplicimonas* sp.; Tcl: *T. canistomae*-like; Tg: *T. gallinae*; Ts: *T. stableri*; *Trichomonadida* sp.; Ttl: *T. tenax*-like; Tvl: *T. vaginalis*-like). n: subgroup number (new numbers were used when homology was less than 99%). Authors references: Anderson *et al.* (2009), Chi *et al.* (2013), Ecco *et al.* (2012), Felleisen (1997), Ganas *et al.* (2014), Gaspar da Silva *et al.* (2007), Gerhold *et al.* (2008), Girard *et al.* (2013), Grabensteiner *et al.* (2010), Kelly-Clark *et al.* (2013), Kleina *et al.* (2004), Reinmann *et al.* (2012), Robinson *et al.* (2010) and Sansano-Maestre *et al.* (2009).

Genotype						
Gerhold *et al.*	**Grabensteiner *et al.***	**Chi *et al.***	**Sansano-Maestre *et al.***	**GenBank acc. n. (author)**	**Organism sequence homology (% of BLAST identity, E-value)**	**Genetic group (ITS-OBT)**
	IV*	A*	B*	AY349182 (Kleina)	*T. gallinae* AY349182 (Kleina) (100%, 1e-154)	*T. gallinae*-1
				EF208019 (Gaspar Da Silva)	*T. gallinae* AY349182 (Kleina) (100%, 4e-108)	
A*				EU215369 (Gerhold)*	*T. gallinae* EU881911 (Sansano) (100%, 9e-146)	
				EU290649 (Anderson)*	*T. gallinae* EU881911 (Sansano) (100%, 1e-159)	
				FN433476 (Grabensteiner)*	*T. gallinae* AY349182 (Kleina) (100%, 1e-154)	
				GQ150752 (Robinson)*	*T. gallinae* AY349182 (Kleina) (100%, 5e-107)	
				HG008050 (Ganas)*	*T. gallinae* AY349182 (Kleina) (100%, 1e-138)	
				KC215387 (Girard)*	*T. gallinae* JN007005 (Reimann) (100%, 0.0)	
B*				EU215368 (Gerhold)*	*T. gallinae* KC215387 (Girard) (99%, 1e-170)	
				EU881911,13*,15,16* (Sansano)	*T. gallinae* KC215387 (Girard) (99%, 0.0; 100%, 3e-171; 100%, 3e-171; 100%, 3e-171)	
D, E	I	C	A	EU215363-64 (Gerhold)	*T. gallinae* U86614 (Felleisen) (100%, 3e-125; 100%, 4e-154)	*T. gallinae*-2
				FN433475 (Grabensteiner)	*T. gallinae* U86614 (Felleisen) (100%, 4e-154)	
C				EU215362 (Gerhold)	*T. gallinae* U86614 (Felleisen) (99%, 0.0)	
				EU881912, 14, 17 (Sansano)	*T. gallinae* U86614 (Felleisen) (99%, 0.0); EU215362 (Gerhold) (99%, 5e-169; 99%, 5e-169)	
	V			FN433477 (Grabensteiner)	*T. gallinae* EU881912 (Sansano) (99%, 9e-151)	
				U86614 (Felleisen)	*T. gallinae* EU881912 (Sansano) (99%, 0.0)	

Table 12. (Cont.)

Genotype						
Gerhold *et al.*	**Grabensteiner *et al.***	**Chi *et al.***	**Sansano-Maestre *et al.***	**GenBank acc. n. (author)**	**Organism sequence homology (% of BLAST identity, E-value)**	**Genetic group (ITS-OBT)**
	II*			FN433474 (Grabensteiner)*	*T. tenax* (KF164607) (99%, 2e-147)	*T. tenax*-like-1
				KF993705 (this study)	*T. canistomae* (AY244652) (97%, 1e-144)	*T. canistomae*-like-1
				KF993706 (this study)	*T. canistomae* (AY244652) (97%, 1e-134)	*T. canistomae*-like-2
	III*			FN433473 (Grabensteiner)*	*T. canistomae* (AY244652) (91%, 3e-110)	*Trichomonas* sp.-1
				KC529665 (Chi)	*T. gallinae* FN433473 (Grabensteiner) (99%, 4e-150)	*Trichomonas* sp.-2
I				EU215361 (Gerhold)	*T. vaginalis* (U86613) (Felleisen) (99%, 0.0)	*T. vaginalis*-like-1
L				EU215366 (Gerhold)	*T. vaginalis* (U86613) (Felleisen) (99%, 2e-167)	*T. vaginalis*-like-2
H				EU215360 (Gerhold)	*T. vaginalis* (U86613) (Felleisen) (98%, 8e-172)	*T. vaginalis*-like-3
J				EU215365 (Gerhold)	*T. vaginalis* (U86613) (Felleisen) (98%, 2e-168)	*T. vaginalis*-like-4
	VI			FN433478 (Grabensteiner)	*T. vaginalis* (FJ813603) (97%, 6e-138)	*T. vaginalis*-like-5
				KF214774 (Kelly-Clark)	Not yet released in GenBank	
				KF993707 (this study)	*T. vaginalis* (FJ813603) (97%, 2e-152)	
G				EU215359 (Gerhold)	*T. vaginalis* (U86613) (Felleisen) (92%, 1e-145)	*Trichomonas* sp.-3
K*				EU215367 (Gerhold)	*T. stableri* (KC215389) (100%, 7e-167)	*T. stableri*-1
				JX089392 (Ecco)*	*T. stableri* (KC215389) (100%, 1e-48)	
				KC215389 (Girard)*	*T. stableri* (KC215390) (99%, 0.0)	
				KC215390 (Girard)*	*T. stableri* (KC215389) (99%, 0.0)	
F				EU215358 (Gerhold)	*T. stableri* (KC215389) (94%, 4e-104)	*T. stableri*-2
				JX089388 (Ecco)*	*Simplicimonas* sp. (HQ334182) (99%, 8e-100)	*Simplicimonas* sp.
				EU290650 (Anderson)	*Histomonas* sp. (HQ334185) (83%, 4e-45)	*Trichomonadida* sp.

Eighty three isolates from oropharyngeal trichomonads of wild birds have been genotyped at their ITS1/5.8S rRNA/ITS2 sequence in this study (table 13). Five different genotypes were detected, although in later investigations two other new genotypes were found in European turtle doves. Three of them were novel identifications in Spain, present in European turtle doves, goshawks and Egyptian vultures: *T. canistomae*-like-1, *T. canistomae*-like-2 and *T. vaginalis*-like-5, respectively. Sequences from new genotypes or hosts were submitted to GenBank database (table 14). Their phylogenetic analysis indicated that they shared a higher similarity with strains of other trichomonad parasites (*T. canistomae* and *T. vaginalis*), than with *T. gallinae* (figure 16).

Table 13. Number of isolates that were genotyped for the ITS1/5.8S rRNA/ITS2 locus according to host species. ITS-OBT-X-n. OBT: Oropharyngeal Bird Trichomonad. X: organism with closest BLAST homology. Nd: not determined.

Genetic group	Species	n	Lesion
ITS-OBT-*T. gallinae*-1	*Accipiter gentilis*	2	No
	Accipiter nisus	1	No
	Aquila pennata	1	Yes
	Asio otus	1	Yes
	Bubo bubo	2	Yes
	Buteo buteo	1	Yes
	Circus cyaneus	1	No
	Falco tinnunculus	18	Yes
		2	Nd*
	Melopsittacus undulatus	1	Yes
	Pica pica	1	Yes
	Otus scops	1	Yes
	Streptopelia decaocto	2	No
	Strix aluco	1	Yes
	Tyto alba	1	Yes
ITS-OBT-*T. gallinae*-2	*Accipiter gentilis*	7	No
	Aquila pennata	1	Yes
	Asio otus	1	Yes
	Buteo buteo	2	No
	Circus aeruginosus	1	No
	Circus cyaneus	1	No
	Columba palumbus	1	Yes
		2	No
	Falco naumanni	3	No
	Falco peregrinus	1	Yes
	Falco tinnunculus	4	No
	Pernis apivorus	1	No
	Streptopelia decaocto	1	Yes
		9	No
Mixed (ITS-OBT-*T. gallinae*-1 +ITS-OBT-*T. gallinae*-2)	*Accipiter gentilis*	1	No
	Accipiter nisus	1	Yes
	Asio otus	1	Yes
	Columba palumbus	1	No
		1	Yes
	Falco tinnunculus	1	Yes
ITS-OBT-*T. canistomae*-like-1	*Streptopelia turtur*	3	No
ITS-OBT-*T. canistomae*-like-2	*Accipiter gentilis*	2	No
ITS-OBT-*T. vaginalis*-like-5	*Neophron percnopterus*	1	No
	Total	**83**	

Table 14. Sequence accession numbers for the ITS1/5.8S rRNA/ITS2 region that were deposited in GenBank database from this study. ITS-OBT-X-n. OBT: Oropharyngeal Bird Trichomonad. X: organism BLAST homology. *: sequences employed for the phylogenetic analysis.

GenBank acc. n.	Genetic group	Host	Lesion	Isolate code
KF993680	ITS-OBT-Tg-2	*Streptopelia decaocto*	No	P94-13
KF993681	ITS-OBT-Tg-2	*Columba palumbus*	No	P178-13
KF993682	ITS-OBT-Tg-2	*Columba palumbus*	Yes	PM1-12
KF993683	ITS-OBT-Tg-2	*Falco tinnunculus*	No	R42-12
KF993684	ITS-OBT-Tg-2	*Accipiter gentilis*	No	R45-12
KF993685	ITS-OBT-Tg-2	*Falco naumanni*	No	R59-13
KF993686	ITS-OBT-Tg-2	*Aquila pennata*	Yes	R134-12
KF993687	ITS-OBT-Tg-2	*Asio otus*	Yes	R137-12
KF993688	ITS-OBT-Tg-2	*Circus aeruginosus*	No	R221-11
KF993689	ITS-OBT-Tg-2	*Pernis apivorus*	No	R237-13
KF993690	ITS-OBT-Tg-1	*Falco peregrinus*	Yes	RM1-12
KF993691*	ITS-OBT-Tg-2	*Accipiter gentilis*	No	RM3-12
KF993692*	ITS-OBT-Tg-2	*Buteo buteo*	No	RM3-13
KF993693	ITS-OBT-Tg-1	*Pica pica*	Yes	OM1-12
KF993694	ITS-OBT-Tg-1	*Streptopelia decaocto*	No	P95-13
KF993695	ITS-OBT-Tg-1	*Melopsittacus undulatus*	Yes	P112-13
KF993696	ITS-OBT-Tg-1	*Bubo bubo*	Yes	R16-12
KF993697	ITS-OBT-Tg-1	*Falco tinnunculus*	Yes	R127-12
KF993698	ITS-OBT-Tg-1	*Tyto alba*	Yes	R183-13
KF993699	ITS-OBT-Tg-1	*Aquila pennata*	Yes	R213-11
KF993700*	ITS-OBT-Tg-1	*Accipiter gentilis*	No	RM7-13
KF993701*	ITS-OBT-Tg-1	*Buteo buteo*	Yes	RM8-13
KF993702	ITS-OBT-Tg-1	*Circus cyaneus*	No	RM9-12
KF993703	ITS-OBT-Tg-1	*Strix aluco*	Yes	RM12-12
KF993704	ITS-OBT-Tg-1	*Asio otus*	Yes	RM14-12
KF993705*	ITS-OBT-Tcl-1	*Streptopelia turtur*	No	P243-12
KF993706*	ITS-OBT-Tcl-2	*Accipiter gentilis*	No	R30-07
KF993707*	ITS-OBT-Tvl-5	*Neophron percnopterus*	No	R235-13
KJ776739	ITS-OBT-Tg-1	*Accipiter nisus*	No	RM13-12
KJ776740	ITS-OBT-Tg-2	*Circus cyaneus*	No	RM5-13
KJ776741	ITS-OBT-Tg-1	*Falco tinnunculus*	Yes	RM2-12
KJ776742	ITS-OBT-Tg-1	*Otus scops*	Yes	R210-13
KJ776743	ITS-OBT-Tg-2	*Streptopelia decaocto*	Yes	PM2-12

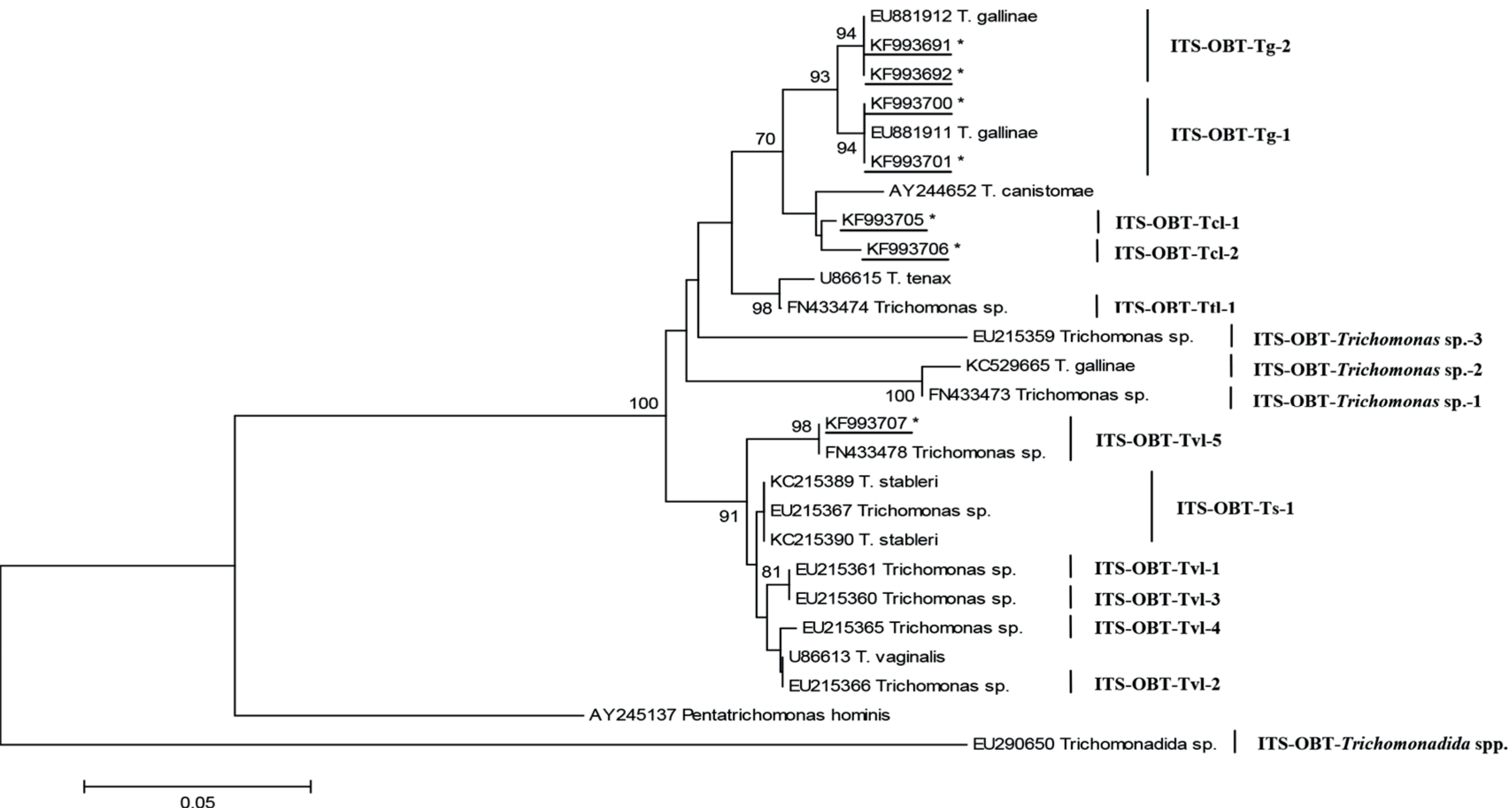


Figure 16.. Neighbor-Joining phylogenetic tree constructed with ITS1/5.8S rRNA/ITS2 sequences of the different genotypes found in this study. Sequences from other authors are included with GenBank acc. n. and organism name. Sequences from this study are indicated with GenBank acc. n., underlined and with an asterisk. Genetic groups are in bold. Final alignment had 256 positions. Distances were calculated with the maximum composite likelihood method. Bootstrap analysis had 2000 replicates. Nodes with values lower than 70 are not shown. Scale indicates the number of base pair substitutions per site.

Genotypes *T. gallinae*-1 and *T. gallinae*-2 were the most frequently identified (92.8%) and widely distributed among the bird species analysed: 19 species were infected with genotype *T. gallinae*-1 or *T. gallinae*-2 and five harboured mixed infections including both genotypes.

T. gallinae-1 was detected mainly in symptomatic birds of prey (order Accipitriformes, Falconiformes and Strigiformes; 60.9%, n= 28, without considering mixed infections, from a total of 46 genotyped birds of prey with gross lesions). Passeriformes (one magpie) and Psittaciformes (one budgerigar) with gross lesions of avian trichomonosis also were infected with this genotype. Overall, 77.8% of the birds infected with genotype *T. gallinae*-1 had gross lesions at the oropharyngeal cavity (28/36 birds infected with *T. gallinae*-1, without mixed infections). In addition, six asymptomatic animals (two goshawks, two Eurasian collared doves, one sparrowhawk and one hen harrier) also presented this genotype.

Birds without symptomatic infections and no gross lesions were infected mainly with genotype *T. gallinae*-2 (68.2%, 30/44). Besides, this genotype was also detected in five animals (a booted eagle, long-eared owl, peregrine falcon, wood pigeon and Eurasian collared dove) with lesions indicative of avian trichomonosis, excluding birds with mixed infections.

In contrast, genotypes *T. canistomae*-like-1, *T. canistomae*-like-2 and *T. vaginalis*-like-5, were associated with particular host species. *T. canistomae*-like-1 with European turtle doves, *T. canistomae*-like-2 with goshawks and *T. vaginalis*-like-5 was present only in Egyptian vultures. This last genotype, *T. vaginalis*-like-5, was later demonstrated to constitute a new species within avian oropharyngeal trichomonad parasites (Martínez-Díaz *et al.*, 2015).

The phylogenetic analysis of avian oropharyngeal trichomonads from wild birds, including sequences from this study and other authors, revealed six different clades for the ITS1/5.8S rRNA/ITS2 region (table 13 and figure 16). One of them was constituted by *T. gallinae* sequences belonging to the *T. gallinae*-1 and *T. gallinae*-2 genetic groups. Three clades were similar to related trichomonad organisms, namely *T.*

canistomae, *T. tenax* and *T. vaginalis*. One clade contained a presumable *T. gallinae* sequence (KC529665) and two *Trichomonas* sp. organisms (EU215359 and FN433473). Another clade was composed by a Trichomonadida sp. sequence (EU290650).

Samples from this study were classified into three of these clades: *T. gallinae*, *T. canistomae*-like and *T. vaginalis*-like clades (figure 16). In the *T. gallinae* clade, sequences from this study (*T. gallinae*-1 and *T. gallinae*-2) showed 100% identity with previous reports of these genetic types by other authors. Several species of birds, including birds of prey and columbiformes were found infected with these genetic types in different studies (Gerhold *et al.*, 2008; Sansano-Maestre *et al.*, 2009; Grabensteiner *et al.*, 2010; Chi *et al.*, 2013). Mixed infections were detected in 7.2% of the birds (6/83) including both genotypes.

For the *T. canistomae*-like isolates, *T. canistomae*-like-1 and *T. canistomae*-like-2, both sequences types differed only in three nucleotide positions (base number 48, 230 and 305) and presented 97% identity with the *T. canistomae* organism (AY244652). They are hitherto new genetic sequence reports.

Finally, samples with the *T. vaginalis*-like-5 genotype were equal to the sequences obtained from parasites found in a bearded vulture (Grabensteiner *et al.*, 2010) and a bald eagle (*Haliaeetus leucocephalus,* Kelly-Clark *et al.*, 2013).

4.1.2. Risk factors for trichomonosis in wild birds

The analysis of risk factors revealed that the presence of gross lesions was associated with the genotype of the parasite, type of host and diet in the case of birds of prey. Besides, the genotype carried by the infected bird was also associated with the type of host.

Genotype and lesions

Most of the birds with gross lesions compatible with avian trichomonosis were infected with genotype *T. gallinae*-1, although *T. gallinae*-2 and mixed infections (*T. gallinae*-1 and *T. gallinae*-2) were found in some of them. Odds ratio calculations indicated that birds infected with genotype *T. gallinae*-1 had higher odds of displaying gross lesions at

the oropharyngeal cavity in comparison with the other genotypes found (OR= 41.9, 95% CI= 13.8-127.5).

Type of host and lesions

The analysis of the type of host species (predator *vs*. prey) indicated that infected predator birds had 4.3 higher odds of having infections with gross lesions than prey species (OR= 4.3, 95% CI= 1.5-12.8).

Diet and lesions

Besides, the diet in the case of birds of prey (non-strict ornithophagous *vs.* strict ornithophagous) was a determinant variable. Non-strict ornithophagous species presented 14.9 higher odds of presenting avian trichomonosis lesions than strict ornithophagous ones (OR= 14.9, 95% CI= 3.7-60.3).

Genotype and type of host

Finally, the genotype of the parasite was associated with the host species, being genotype *T. gallinae*-1 more frequent in birds of prey and genotype *T. gallinae*-2 in prey species (OR= 4, 95% CI= 1.4-12).

In order to detect possible interactions and confounding variables, a stratification of the data and analysis by the Mantel-Haenszel odds ratio (OR_{MH}) method was carried out. After removing the effect of the confounding variables type of host and diet, the influence of the genotype in the development of clinical infections with gross lesions was even higher (OR_{MH}= 35.4, 95% CI= 11.7-107.3 host type stratification; OR_{MH}= 37.6, 95% CI= 10.5-134.4, diet in predator bird stratification).

Stratification of genotype and lesions

Additionally, the genotype was also a determinant factor for the presence of gross lesions when each type of host species was analysed (OR_{prey}= 10.5, 95% CI= 1.2-91.9; $OR_{predator\ bird}$= 59.4, 95% CI= 15.4-228.6). However, genotype and lesions were only associated in non-strict ornithophagous birds ($OR_{non-strict-ornitophagous}$= 195, 95% CI= 116.2-327.2), probably due to the low number of ornithophagous birds with gross lesions (n= 2; $OR_{ornithophagous}$ = 3.3, 95% CI = 0.8-14.6).

Stratification of type of host and lesions

No association was found when the OR_{MH} test was used, the type of host was not a major influence for the development of gross lesions in comparison with the genotype of the parasite (OR_{MH}= 2.5, 95% CI= 0.6-10.1 genotype stratification).

Stratification of diet and lesions

Although a decreased odds was detected after the stratification of genotype for the diet of birds of prey and presence of gross lesions, the association was still significant (OR_{MH}= 8.3, 95% CI= 1.2-58, genotype stratification). However, the effect of the diet was more pronounced in infections with genotype *T. gallinae*-1 ($OR_{genotype\ Tg\text{-}1}$ = 90, 95% CI= 74.9-108; $OR_{genotype\ Tg\text{-}2}$ = 1.5, 95% CI=1.1-2.2).

4.2. Prevalence and MLST characterization of trichomonad parasites in Bonelli´s eagle

This species is an endangered bird of prey with serious conservation problems in the western Mediterranean. The Iberian Peninsula concentrates more than 80% of the population of Europe and different management and conservation programmes have been implemented for improving the present situation.

Cultures were obtained from admissions at different wildlife recovery centers and also from birds of the European Life project NAT/ES/000701: integral recovery of Bonelli's eagle population in Spain. This programme involves the examination of wild nestlings and the reintroduction of animals from captive breeding programmes held at the wildlife veterinary hospital of GREFA (Majadahonda, Madrid, Spain) and French wildlife recovery centers (Ardèche, Vendée, France).

In total, from 2011 to 2015, 98 samples were obtained and cultured from Bonelli's eagles (table 15). The overall prevalence rate of infection was 28.6%. Lesions at the oropharyngeal cavity were detected in 35.7% of the birds, with a wide diversity of presentation types, from grade 1 to grade 3 (figure 17), according to the classification system described in section 3.1.4. (table 4).

Some of the cultures obtained from animals with lesions compatible with avian trichomonosis were negative, even in birds with severe clinical infections of grade 3. A prolonged time from sampling until their incubation in suboptimal temperatures were potential factors for these negative results, although special logistics were implemented during the sampling of nestlings in the field. Consequently, the PCR for the ITS1/5.8S rRNA/ITS2 region was added in order to confirm the diagnosis from a second oropharyngeal swab. These second swabs were obtained following the same methodology as described for the culture of *T. gallinae*, but were stored inside an empty 10 ml polystyrene sterile tube that was kept refrigerated until its arrival to the laboratory, where they were placed at -20 ºC. When a positive ITS PCR result was detected, the PCR of the small subunit of rRNA and later, from the Fe-hydrogenase gene, were also carried out.

Table 15. Number of samples from Bonelli's eagles from 2011 to 2015. n: total number of birds sampled. Lesion: number of birds with gross lesions compatible with avian trichomonosis. Positive: number of birds with a positive culture in TYM medium or positive PCR.

Autonomous community	Province	n	Lesion	Positive	Lesion prevalence	Prevalence
Andalucía		**31**	**17**	**15**	**54.8%**	**48.4%**
	Almería	9	4	3		
	Jaén	6	4	4		
	Granada	9	2	4		
	Huelva	1	1	0		
	Málaga	6	6	4		
Madrid	Madrid	**28**	**5**	**2**	**17.9%**	**7.1%**
Castilla-La Mancha		**5**	**1**	**1**	**20.0%**	**20.0%**
	Albacete	2	1	0		
	Guadalajara	2	0	0		
	Toledo	1	0	1		
Comunidad Valenciana		**33**	**11**	**9**	**33.3%**	**27.3%**
	Alicante	9	6	5		
	Castellón	10	1	2		
	Valencia	14	4	2		
Islas Baleares	Mallorca	**1**	**1**	**1**	**100.0%**	**100.0%**
Total		**98**	**35**	**28**	**35.7%**	**28.6%**

Figure 17. Photographs of oropharyngeal lesions compatible with avian trichomonosis infection in Bonelli's eagles (*Aquila fasciata*). (a-b): lesions of grade 2 (case R3-15 and R12-15). (c): lesions of grade 3 (case 2775-09). See section 4.4, for further case information.

European Life project: Bonelli's eagle nestlings

A special mention should be done for the samples of Bonelli's eagles nestlings of the European Life project that were sampled in the wild with permissions of local administrations.

In total, 37 wild nestlings from Bonelli's eagle were tested for avian trichomonosis infection in 2014 and 2015. The overall infection rate was 48.6% and lesions were present in 62.2% of the birds (n= 23/37 in both cases, table 16). Birds displayed gross lesions at the oropharyngeal cavity that ranged from grade 1 to grade 3. The highest

percentage of affected nestlings had lesions of grade 1 (44.4%) and 2 (33.3%), while severely affected animals that received urgent veterinary care (lesion grade 3) represented the 22.2% .

The MLST analysis of positive birds indicated that genotypes *T. gallinae*-1 and *T. gallina*e-2 of the ITS1/5.8S rRNA/ITS2 region were present in the examined animals. Nestlings with gross lesions were infected with *T. gallinae*-1 genotype, in addition of two mixed infections and four animals with lesions but negative results for culture and PCR. Genotype *T. gallinae*-2 was identified from birds without lesions, with the exception of the two mixed infections already mentioned (tables 16 and 17).

For the small subunit of rRNA, genotypes described by several authors from birds of prey, galliformes and psittaciformes were detected, including one mixed infection. These genotypes were: VI from Grabensteiner *et al.* (2010), EU215373 from Gerhold *et al.* (2008) and KM246609 from Martínez-Díaz *et al.* (2015).

Regarding the single copy gene of Fe-hydrogenase, the genotypes described by Chi *et al.* (2013) as A1, A2 and C4 were found. Isolates of ITS1/5.8S rRNA/ITS2 region *T. gallinae*-1 had Fe-hydrogenase A1, whereas those typified as *T. gallinae*-2 were C4. Two animals presented two genotypes for this genetic marker. Moreover, a new subtype was detected from one of these cases, with 1 SNP for genotype A2.

Table 16. MLST results of oropharyngeal trichomonads isolated from wild nestlings of Bonelli's eagle (*Aquila fasciata*). The analysis involved the ITS1/5.8S rRNA/ITS2 (ITS), the small subunit of rRNA (SSU) and Fe-hydrogenase gene sequences. Genotype nomenclature according to genetic groups of table 12[a], Grabensteiner *et al.* (2010)[b], Chi *et al.* (2013)[c] or with GenBank acc. n. L: lesion grade. Pos: positive. Neg: negative. Nd: not determined.

Year 2014						
Isolate code	**L**	**Culture**	**PCR**	**ITS1/5.8S rRNA/ITS2[a]**	**SSU**	**Fe-hydrogenase**
0273-14	0	Neg	Nd			
0409-14	0	Neg	Nd			
0410-14	0	Neg	Nd			
0411-14	2	Neg	Nd			
0412-14	2	Neg	Nd			
0547-14	1	Neg	Nd			
0734-14	0	Neg	Nd			
0735-14	1	Pos	Pos	*T. gallinae*-1	VI[b]	A1[c]
0974-14	2	Neg	Nd			
0975-14	0	Neg	Nd			
0976-14	0	Pos	Pos	*T. gallinae*-2	EU215373	C4[c]
0977-14	2	Neg	Nd			
1098-14	2	Neg	Nd			
1358-14	1	Pos	Pos	*T. gallinae*-1	VI[b]	A1[c]
3812-14	0	Neg	Nd			
Total: 15	**8**	**3**	**3**			

Year 2015						
Isolate code	**L**	**Culture**	**PCR**	**ITS1/5.8S rRNA/ITS2[a]**	**SSU**	**Fe-hydrogenase**
0439-15	0	Neg	Pos	*T. gallinae*-2	EU215373	C4[c]
0440-15	0	Neg	Nd			
0441-15	0	Neg	Pos	*T. gallinae*-2	Negative	Negative
0442-15	0	Pos	Pos	*T. gallinae*-2	EU215373	C4[c]
0443-15	1	Neg	Pos	*T. gallinae*-1	VI[b]	A1[c]
0444-15	3	Neg	Pos	*T. gallinae*-1	VI[b]	Negative
0445-15	1	Neg	Pos	*T. gallinae*-1	VI[b]	A2[c]
0446-15	3	Neg	Pos	*T. gallinae*-1	VI[b]	A2[c]-1 SNP+C4[c]
0447-15	1	Neg	Pos	*T. gallinae*-1+ *T. gallinae*-2	VI[b] +EU215373	A1[c]+C4[c]
0449-15	1	Neg	Pos	*T. gallinae*-1	VI[b]	A1[c]
0450-15	1	Neg	Neg			
0467-15	2	Neg	Neg			
0884-15	0	Neg	Neg			
0885-15	0	Neg	Pos	*T. gallinae*-2	EU215373	Negative
1108-15	2	Neg	Neg			
1109-15	2	Neg	Pos	*T. gallinae*-1+ *T. gallinae*-2	EU215373	Negative
1110-15	1	Neg	Pos	*T. gallinae*-1	Negative	Negative
1111-15	2	Neg	Nd			
1112-15	3	Neg	Pos	*T. gallinae*-1	VI[b]	A1[c]
1113-15	3	Neg	Neg			
1483-15	0	Neg	Pos	*T. gallinae*-2	Negative	Negative
1484-15	1	Pos	Pos	*T. gallinae*-1	VI[b]	A1[c]
Total: 22	**15**	**2**	**15**			

Table 17. MLST groups of the different genotypes of oropharyngeal trichomonads isolated from wild nestlings of Bonelli's eagle (*Aquila fasciata*). ITS: ITS1/5.8S rRNA/ITS2 sequence. SSU: small subunit of rRNA sequence. Fe: Fe-hydrogenase gene sequence. Genotype nomenclature according to genetic groups of table 12[a], Grabensteiner *et al.* (2010)[b], Chi *et al.* (2013)[c] or with GenBank acc. n. Un: unsuccessful reaction.

ITS[a]	SSU	Fe	Isolate code	Lesion	n
T. gallinae-1	VI[b]	A1[c]	0735-14, 1358-14, 0443-15, 0449-15, 1112-15, 1484-15	Yes	6
		A2[c]	0445-15	Yes	1
		A2[c]-1 SNP+C4[c]	0446-15	Yes	1
		Un	0444-15	Yes	1
	Un		1110-15	Yes	1
T. gallinae-2	EU215373	C4[c]	0976-14, 0439-15, 0442-15	No	3
		Un	0885-15	No	1
	Un		0441-15, 1483-15	No	2
T. gallinae-1+ *T. gallinae*-2	VI[b]+EU215373	A1[c]+C4[c]	0447-15	Yes	1
	EU215373	Un	1109-15	Yes	1
				Total	**18**

4.3. Prevalence and MLST characterization of trichomonad parasites in European turtle doves

Isolates from European turtle doves would be presented separately due to the diversity of oropharyngeal trichomonads found in this vulnerable columbiforme. Despite that the maximum level of infection (100%) was detected, a small sample size was obtained (n= 8). The collection of a higher number of samples was especially difficult due to its lower abundance in comparison with other species of columbiformes, the cynegetic pressure that exists in Spain and the migratory character of the species, present only during the breeding season.

Noteworthy, European turtle doves had the highest diversity of oropharyngeal trichomonads, with three genotypes (*Trichomonas* sp.-1, *T. canistomae*-like-1 and *T. tenax*-like-2) of the ITS1/5.8S rRNA/ITS2 region detected from only seven individuals (table 18 and figure 18). Moreover, these genotypes were described for the first time in Spain and some of them were descriptions not reported previously by other authors (*T. canistomae*-like-1 and *T. tenax*-like-2). Therefore, a MLST analysis was completed from these isolates, including the small rRNA subunit and Fe-

hydrogenase gene (table 18, figures 19 and 20). The results indicated three new genotypes, one of the small rRNA subunit and two of the Fe-hydrogenase gene, with mixed infections also for both loci.

For the small rRNA subunit, the phylogenetic analysis of the sequences from European turtle doves revealed that two isolates (P13-14 and P14-14) belonged to the genotype 18S-I described by Grabensteiner *et al.* (2010) in racing pigeons from Austria, also reported in a grey-capped Emerald dove (*Chalcophaps indica*) from Australasia (GenBank acc. n. JQ030997, unpublished). Besides, a novel genotype (novel-1) was found in three birds, that did not clustered with any *T. gallinae*, *T. canistomae*, *T. tenax* or *Trichomonas* sp. sequences (figure 19).

Further diversity was detected at the Fe-hydrogenase locus, with two novel genotypes (novel-1 and 2) that did not match with any other sequences available in the NCBI public database. Genotype novel-1 presented 98% identity with sequence JF681142, isolated from a Zebra dove (*Geopelia striata*) in the Seychelles islands (Lawson *et al.*, 2011). BLAST analysis indicated that the genotype novel-2 had 96% identity with sequence KP900040, genotype C5 (Sansano-Maestre *et al.*, 2016) isolated from a rock pigeon.

Table 18. MLST results of oropharyngeal trichomonads isolated from European turtle doves (*Streptopelia turtur*) in this study. The analysis involved the ITS1/5.8S rRNA/ITS2 (ITS), the small rRNA subunit (SSU) and Fe-hydrogenase gene sequences. Genotype nomenclature according to genetic groups of table 12[a] and Grabensteiner *et al.* (2010)[b]. Novel genotypes are indicated with a number (novel-n).

<table>
<tr><th>Code</th><th>Lesion</th><th>ITS genotype</th><th>SSU genotype</th><th>Fe-hydrogenase genotype</th></tr>
<tr><td>P12-14</td><td rowspan="7">No</td><td>III[b]- Trichomonas sp.[a]</td><td>Mixed</td><td>Novel-1</td></tr>
<tr><td>P196-13</td><td rowspan="3">T. canistomae-like-1[a]</td><td rowspan="3">Novel-1</td><td rowspan="3">Novel-2</td></tr>
<tr><td>P243-12</td></tr>
<tr><td>P262-12</td></tr>
<tr><td>P13-14</td><td rowspan="3">T. tenax-like-2[a]</td><td rowspan="2">I[b]</td><td>Mixed</td></tr>
<tr><td>P14-14</td><td>Mixed</td></tr>
<tr><td>P15-14</td><td>Mixed</td><td>Mixed</td></tr>
</table>

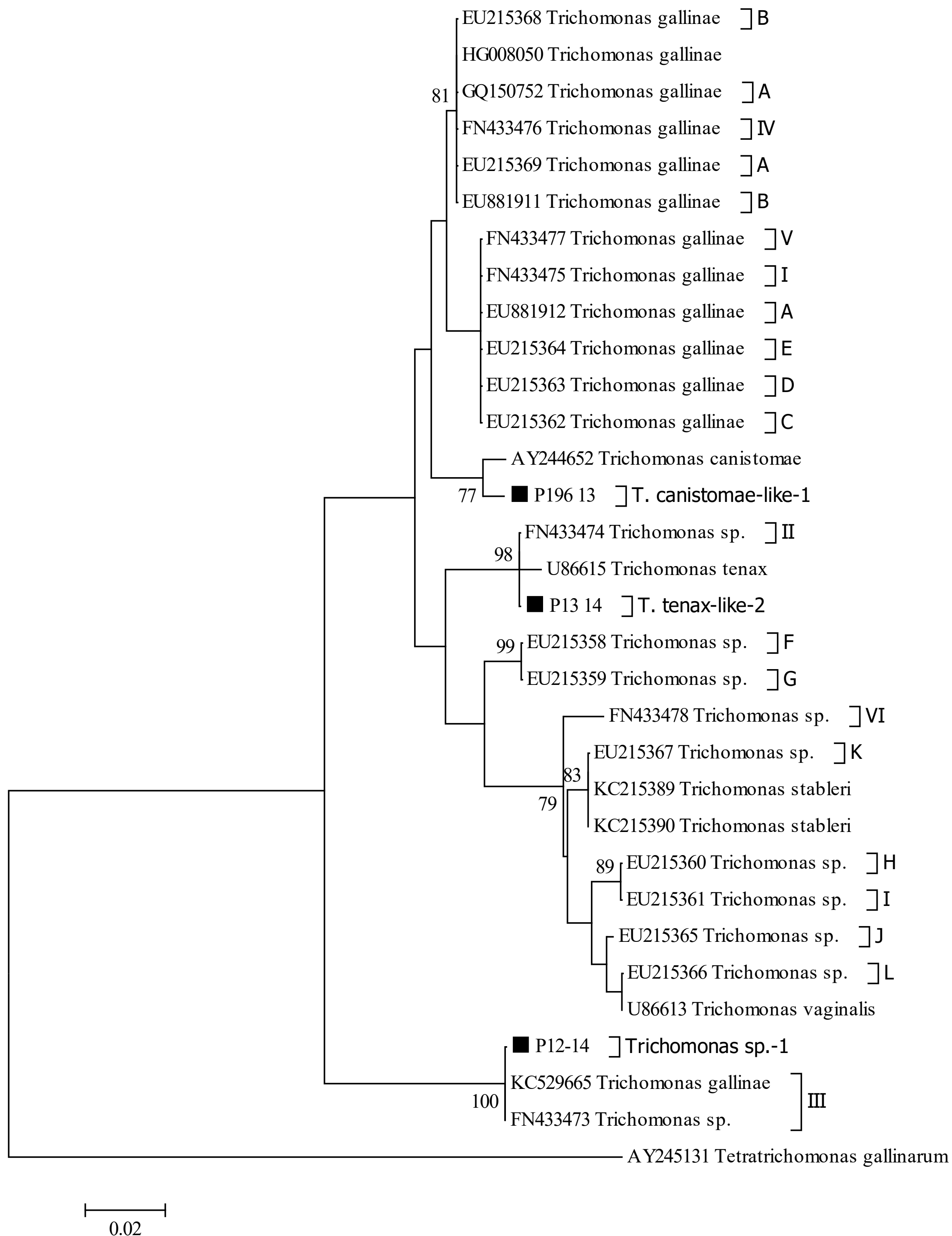


Figure 18. Neighbor-Joining phylogenetic tree constructed with ITS1/5.8S rRNA/ITS2 sequences of isolates from European turtle doves (*Streptopelia turtur*). Sequences from other authors are included with GenBank acc. n. and organism description. Sequences from this study are indicated with symbol ■ and laboratory isolate code. Final alignment had 184 positions. Distances were calculated with Tamura 3-parameter method and rate variation among sites was modeled with a gamma distribution (shape parameter = 5). Bootstrap analysis had 2000 replicates. Nodes with values lower than 70 are not shown. Scale indicates number of base pair substitutions per site.

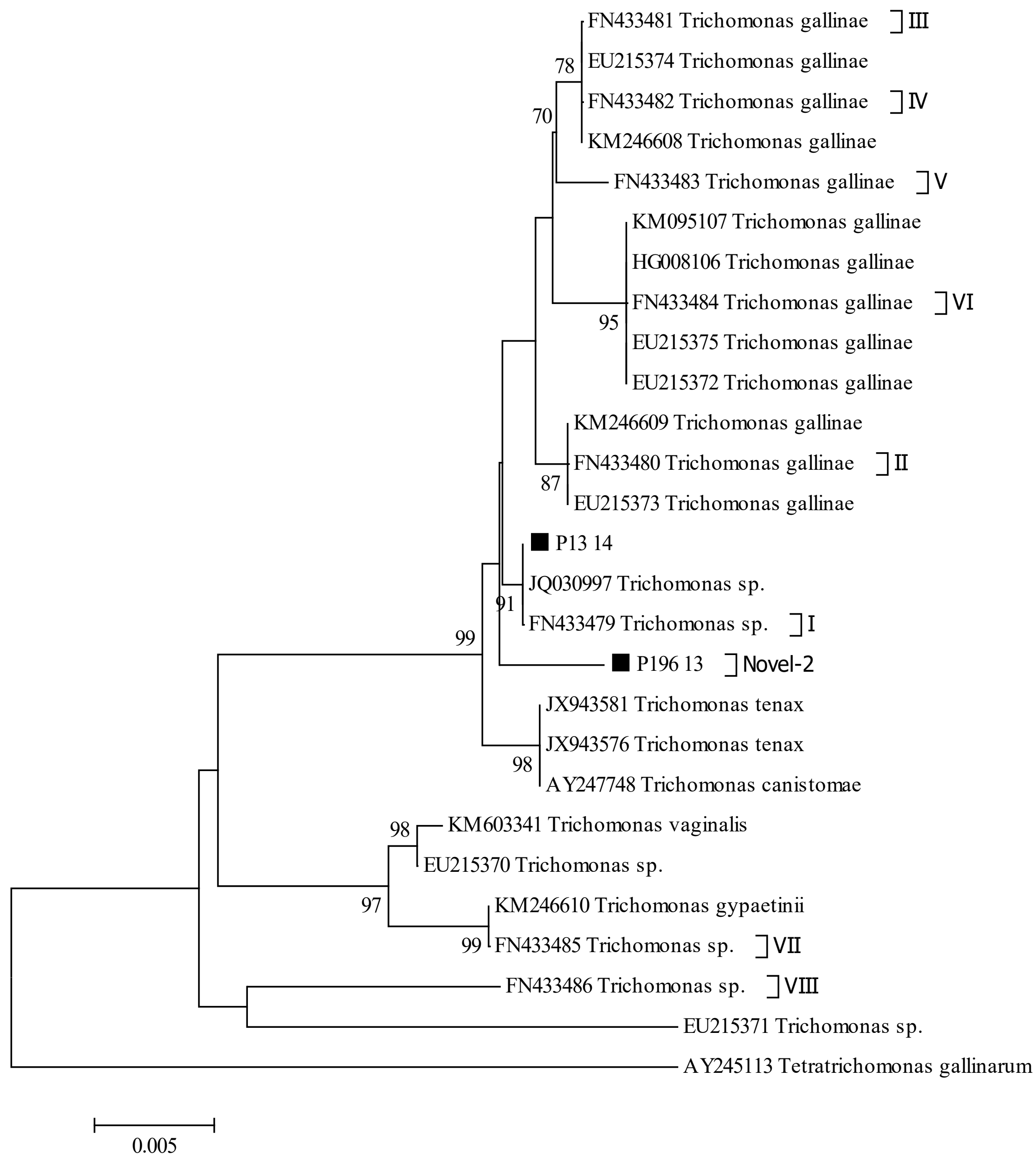


Figure 19. Neighbor-Joining phylogenetic tree constructed with small subunit of rRNA sequences of isolates from European turtle doves (*Streptopelia turtur*). Sequences from other authors are included with GenBank acc. n. and organism description. Sequences from this study are indicated with symbol ■ and laboratory isolate code. Final alignment had 934 positions. Distances were calculated with Kimura 2-parameter method and rate variation among sites was modeled with a gamma distribution (shape parameter = 5). Bootstrap analysis had 2000 replicates. Nodes with values lower than 70 are not shown. Scale indicates number of base pair substitutions per site.

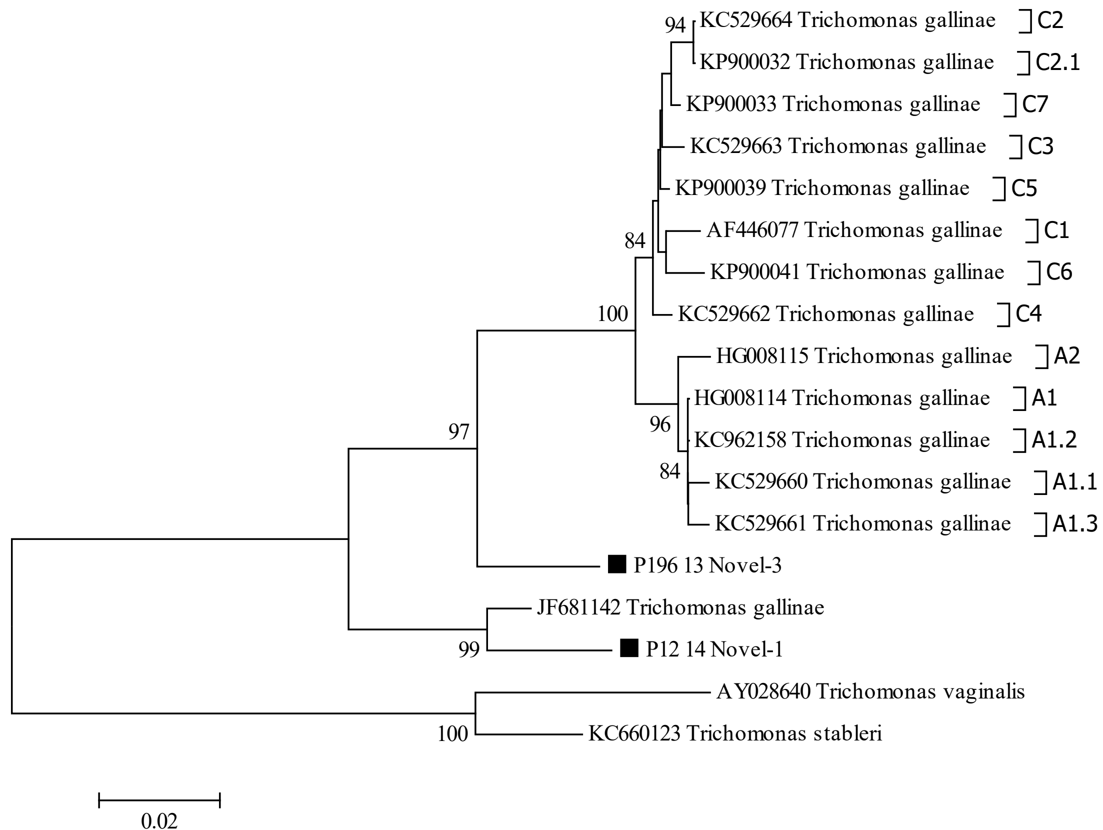


Figure 20. Neighbor-Joining phylogenetic tree constructed with Fe-hydrogenase sequences of isolates from European turtle doves (*Streptopelia turtur*). Sequences from other authors are included with GenBank accession number and organism description. Sequences from this study are indicated with symbol ■ and the laboratory isolate code. Final alignment had 591 positions. Distances were calculated with Kimura 2-parameter method. Bootstrap analysis had 2000 replicates. Nodes with values lower than 70 are not shown. Scale indicates number of base pair substitutions per site.

4.4. Proposed classification of oropharyngeal lesions compatible with avian trichomonosis

A total of 77 clinical cases were evaluated for the categorization of their lesions, 65 from birds of prey and 12 from columbiformes. The established classification system divided gross lesions in three different grades, according to their degree of severity based on several criteria (see table 4, section 3.1.3 of material and methods).

Lesions of grade 1 corresponded to 1.3% of the cases (n= 1), grade 2 with 20.8% (n= 16) and grade 3 with 77.9% (n= 60, figure 21). Birds of prey were found with lesions of grade 1, 2 and 3, whereas columbiformes had grades 2 and 3.

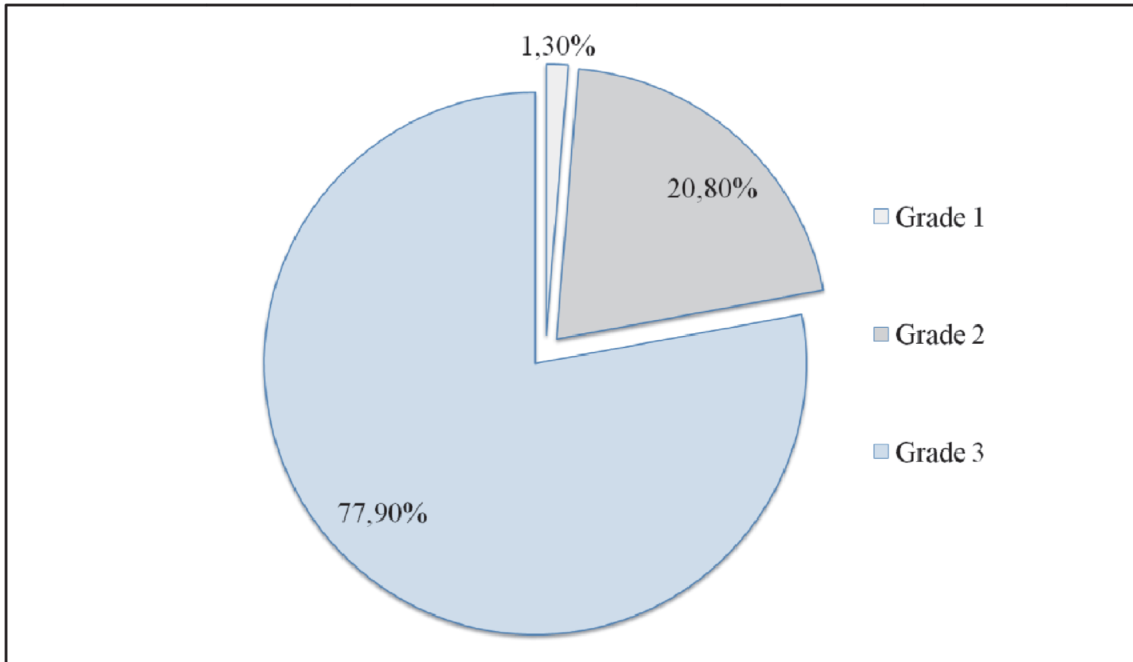


Figure 21. Percentage of number of cases according to their lesion grade classification type.

Urgent treatment that included surgery and supportive general care (rehydration and electrolytic balance normalization) as well as antiprotozoal medication was required in lesions of grade 3. The prognosis of the birds was poor, as 77% (n= 47) of the birds died as a result of the lesions caused by the protozoan (tables 19 and 20).

Lesions of grade 2 had a favourable prognosis, since a lower value of fatalities were recorded (6.3%, n= 1). Antiprotozoal medication should be administered as soon as possible as lesions at this stage already had a multifocal distribution, indicative of a rapid spread and multiplication rate of trophozoites through the mucosal layer of the oropharyngeal cavity.

Finally, the case with lesions of grade 1 had an optimal prognosis and recovered quickly after treatment. However, it is essential to remark that alternative diagnosis for lesions should be considered while diagnostic tests are performed as the gross findings are unspecific. In fact, the cases of birds with lesions of grade 3 and negative result of *Trichomonas* spp. culture were from goshawks that harboured capillarid nematodes on the oesophagus and stomach (n= 3).

Table 19. Classification of gross lesions compatible with *T. gallinae* infections. ID: case identification code. OC: oropharyngeal cavity. D: distribution type F-focal, M-multifocal. L: length in cm. T: *Trichomonas* spp. infection. Pos: positive. Neg: negative. LGC: lesion grade classification.

Species	ID	Anatomical location	Fibrinonecrotic lesion type	D	L	T	Outcome	LGC
Accipiter gentilis (goshawk)	1739-06	Choanal slit-left margin	Nodular	F	< 5	Pos	Death	3
	R158-12	OC-lateral areas of lower and upper jaw	Membranous, (capillarid nematodes in stomach)	M	≤ 2	Neg	Carcass	3
	R3-13	OC-lateral areas of lower and upper jaw	Membranous, (capillarid nematodes in stomach)	M	≤ 2	Neg	Carcass	3
	R6-13	OC-lateral areas of lower and upper jaw	Membranous, (capillarid nematodes in stomach)	M	≤ 2	Neg	Carcass	3
Accipiter nisus (Eurasian sparrowhawk)	1310-10	OC-lower jaw and upper jaw	Nodules	M	≤ 5	Pos	Death	3
	0029-13	OC-hard palate and conjunctivitis on right eye	Nodules	M	≤ 5	Pos	Death	3
Aquila fasciata (Bonelli's eagle)	2775-09	Choanal slit-right margin and tongue-right area at the base	Membranous	M	≤ 0.5	Pos	Recovery	3
	0169-10	Choanal slit-left margin	Membranous	M	≤ 0.3	Pos	Recovery	2
	0504-10	Tongue-left area at the base	Nodular	F	3	Pos	Recovery	3
	3012-11	OC-left margin of lower jaw	Membranous	F	< 2	Pos	Recovery	2
	0444-15	OC-left margin from choanal slit to lower jaw	Membranous	F	< 5	Pos	Recovery	3
	0446-15	OC-right margin from choanal slit to lower jaw and tongue-base	Nodules	M	≥ 5	Pos	Recovery	3
	0732-15	Infundibular cleft-left margin of choanal slit	Membranous	F	< 0.5	Pos	Recovery	2
	0734-15	Infundibular cleft-right margin	Membranous	F	0.5	Pos	Recovery	2
	1112-15	OC-right area of lower jaw	Nodular	F	< 5	Pos	Recovery	3
	1113-15	OC-right lateral and caudal area of lower jaw	Nodular	F	3	Pos	Recovery	3
	1382-15	Infundibular cleft-left margin	Membranous	F	< 0.5	Pos	Recovery	2
	1383-15	Choanal slit-left margin	Membranous	M	≤ 0.5	Pos	Recovery	2
	1606-15	Tongue-right area of the base	Nodular	F	< 0.5	Pos	Recovery	2
	R2-15	Tongue-at the tip	Membranous	M	≤ 1	Neg	Recovery	2
	R3-15	Infundibular cleft-both margins	Membranous	M	≤ 1	Pos	Recovery	2

Table 19. (Cont.)

Species	ID	Anatomical location	Fibrinonecrotic lesion type	D	L	T	Outcome	LGC
Aquila fasciata (Bonelli's eagle)	R8-15	Infundibular cleft-right margin	Membranous	F	< 1	Neg	Recovery	2
	R12-15	Infundibular cleft-both margins	Membranous	M	≤ 1	Neg	Recovery	2
	0482-16	OC-right area of lower jaw	Nodular	F	< 0.5	Pos	Recovery	1
	VR10-16	Choanal slit-margins	Membranous	M	≤ 0.5	Neg	Recovery	2
Aquila pennata (booted eagle)	R172-11	OC-lateral and caudal areas of lower and upper jaw	Membranous	M	≤ 5	Pos	Death	3
	R213-11	Thoracic oesophagus-proventricular junction	Nodular	F	5	Pos	Death	3
Asio otus (long-eared owl)	R137-12	OC-lateral areas of upper jaw, infundibular cleft-caudal area and larynx	Membranous	M	≤ 0.5	Pos	Recovery	2
Bubo bubo (eagle owl)	0094-09	OC-lower and upper jaw	Nodules	M	≤ 5	Pos	Carcass	3
	R16-12	Infundibular cleft-right margin	Nodular	F	< 5	Pos	Recovery	3
	R17-12	Infundibular cleft-both margins	Nodules	M	≤ 5	Pos	Recovery	3
	9298-13	OC-upper jaw	Nodular	F	> 5	Pos	Carcass	3
Buteo buteo (common buzzard)	0621-06	OC-lower right jaw and tongue-base	Nodules	M	≤ 4	Pos	Recovery	3
	0041-10	OC-lower right jaw and tongue-right area of the base	Nodules	M	≤ 4	Pos	Recovery	3
	3378-11	OC-lower jaw and tongue-base	Nodules	M	≤ 5	Pos	Death	3
Circus aeruginosus (marsh harrier)	1360-05	OC-choanal slit-both margins, lower jaw and tongue	Nodules	M	≤ 2	Pos	Recovery	3
Columba livia (rock pigeon)	0708-08	OC-lower jaw and upper jaw	Nodular	F	< 5	Pos	Death	3
	P10-11	OC-lower jaw and upper jaw and liver	Nodules	M	≤ 5	Pos	Death	3
	P25-11	OC-lower jaw and upper jaw	Nodular	F	< 5	Pos	Death	3
	P27-11	OC-lower jaw and upper jaw	Nodular	F	< 5	Pos	Death	3
Columba palumbus (wood pigeon)	P349-12	OC-lower jaw and upper jaw and conjunctivitis on right eye	Nodules	M	≤ 2	Pos	Death	3
	1562-13	OC-lower jaw and upper jaw	Nodules	M	≤ 2	Pos	Death	3
	2201-14	Crop and oesophagus	Nodules	M	≤ 5	Pos	Death	3
	2862-16	Infundibular cleft-caudal area	Nodular	F	< 2	Pos	Death	2

Table 19. (Cont.)

Species	ID	Anatomical location	Fibrinonecrotic lesion type	D	L	T	Outcome	LGC
Falco peregrinus (peregrine falcon)	0727-08	OC-lower and upper jaw and conjunctivitis on left eye	Nodules	M	≤ 5	Pos	Death	3
	0189-10	OC-lower and upper jaw and conjunctivitis on left eye	Membranous	M	≤ 2	Pos	Recovery	3
	1706-14	OC-lower and upper jaw and tongue	Membranous and nodules	M	≤ 4	Pos	Recovery	3
Falco tinnunculus (common kestrel)	0207-11	OC-lower jaw and upper jaw	Nodules	M	≤ 4	Pos	Death	3
	R126-11	OC-lower and upper jaw	Nodular	M	≤ 5	Pos	Death	3
	R166-11	OC-lower and upper jaw, crop and oesophagus	Nodules	M	≤ 3	Pos	Death	3
	R176-11	OC-lower and upper jaw	Nodules	M	≤ 3	Pos	Death	3
	R94-12	OC-lower and upper jaw	Nodules	M	≤ 5	Pos	Death	3
	R98-12	OC-lower, upper jaw and crop	Nodules	M	≤ 5	Pos	Death	3
	2866-13	OC-lower jaw and tongue	Nodules	M	≤ 5	Pos	Death	3
	R186-13	OC-lower jaw	Nodular	F	< 5	Pos	Death	3
	R191-13	OC-lower and upper jaw	Nodules	M	≤ 3	Pos	Death	3
	R198-13	OC-lower jaw and crop	Nodular	F	< 5	Pos	Death	3
	1203-14	OC-lower jaw and crop	Nodular	F	< 5	Pos	Death	3
	1216-14	OC-lower jaw	Nodular	F	< 5	Pos	Death	3
	3742-15	OC-lower and upper jaw and tongue	Membranous and nodules	M	≤ 4	Pos	Death	3
	VR4-15	OC-right area of hard palate	Nodular	F	> 5	Pos	Death	3
	VR5-15	OC-right area of hard palate and crop	Nodules	M	≤ 5	Pos	Death	3
	VR6-15	OC-lower jaw and crop	Nodular	F	< 5	Pos	Death	3
Milvus milvus (red kite)	1550-99	Larynx	Nodular	F	< 1	Pos	Recovery	2
Streptopelia decaocto (eurasian collared dove)	VP21-15	OC-lower jaw and upper jaw	Nodular	F	< 5	Pos	Death	3
	0794-16	OC-lower jaw and upper jaw	Nodular	F	< 5	Pos	Death	3
	0815-16	OC-lower jaw and upper jaw, infundibular cleft-margins and caudal area and tongue	Nodules	M	≤ 2	Pos	Death	3
	1464-16	OC-lower right jaw	Nodular	F	< 2	Pos	Death	3

Table 19. (Cont.)

Species	ID	Anatomical location	Fibrinonecrotic lesion type	D	L	T	Outcome	LGC
Strix aluco (tawny owl)	4137-11	OC-right area of hard palate	Nodular	F	> 5	Pos	Death	3
	0060-12	Infundibular cleft-right margin and tongue-right area of the base	Nodules	M	≤ 1	Pos	Recovery	2
	0063-12	Infundibular cleft-both margins and choanal slit-left area	Nodular	F	> 5	Pos	Death	3
	1411-12	OC-hard palate	Nodular	F	> 5	Pos	Death	3
	3244-12	Choanal slit-both margins and infundibular cleft	Nodular	F	< 5	Pos	Death	3
	R18-12	OC-upper jaw	Nodular	F	> 5	Pos	Carcass	3
	5147-15	Lower right eyelid	Membranous	F	< 5	Pos	Death	3
Tyto alba (barn owl)	0050-12	OC-hard palate and skull base	Nodular	F	< 5	Pos	Death	3
	R183-13	OC-upper jaw	Nodular	F	< 5	Pos	Death	3

Table 20. Number of cases according to their clinical evolution and lesion grade classification type. N: number.

Lesion grade classification	Outcome	N of cases	Total n of cases for each lesion grade	% of the total for each lesion grade	% from the total n cases
1	Recovery	1	1	100.0	1.3
	Death	0		0.0	0.0
2	Recovery	15	16	93.75	19.5
	Death	1		6.25	1.3
3	Recovery	13	60	21.7	16.9
	Death	47		78.3	61.0
Total		**77**			**100.0**

In reference to the distribution of gross lesions, 53.3% (n= 41) of the birds had multiple anatomical locations, whilst 46.8% presented a unique lesion (n= 36).

The most commonly affected area was the oropharyngeal cavity, with the hard palate including the upper jaw, choanal slit and infundibular cleft being the areas where the higher number of lesions were located (n= 30, figure 22 and table 21). Secondly, the lower jaw region accounted for 13. Thirty two cases had both regions affected. The tongue and crop were also involved in certain number of cases, 12 and 6, respectively. Next, the eye orbits, considering animals which presented conjunctivis defined as external swelling of the eye lids, with ocular discharge, was determined in 5 cases. Finally, in a lower number of cases, birds presented lesions on the oesophagus (n= 3), larynx (n= 2) and liver (n= 1). Nevertheless, lesions on internal organs were under reported as the necropsy of the birds was performed only in some cases. For the present classification of gross lesions further diagnostic analysis results, such as endoscopy or radiography, were not included.

If we consider the lesions grouped by orders of birds, species from the order Accipitriformes had lesions located at the oropharyngeal cavity, tongue, eye orbits and sinuses, larynx and oesophagus (table 21). In Falconiformes they extended to the oropharyngeal cavity, crop, tongue, eye orbits, crop and oesophagus. Nocturnal birds of prey (order Strigiformes) presented lesions in oropharyngeal cavity as well, tongue, eye orbits and larynx. Finally, in the case of prey species, order Columbiformes, lesions were located at the oropharyngeal cavity, tongue, eyes, crop, liver and oesophagus.

Table 21. Information of bird species and the anatomical location/s affected with gross lesions compatible with avian trichomonosis. OC-UJ: oropharyngeal cavity-upper jaw, the choanal slit and infundibular cleft locations are also included. OC-LJ: oropharyngeal cavity-lower jaw.

Anatomical location	Order	Species
OC-UJ	Accipitriformes	*Accipiter gentilis* (goshawk)
		Accipiter nisus (Eurasian sparrowhawk)
		Aquila fasciata (Bonelli's eagle)
		Aquila pennata (booted eagle)
		Circus aeruginosus (marsh harrier)
	Columbiformes	*Columba livia* (rock pigeon)
		Columba palumbus (wood pigeon)
		Streptopelia decaocto (Eurasian collared dove)
	Falconiformes	*Falco peregrinus* (peregrine falcon)
		Falco tinnunculus (common kestrel)
	Strigiformes	*Asio otus* (long-eared owl)
		Bubo bubo (eagle owl)
		Strix aluco (tawny owl)
		Tyto alba (barn owl)
OC-LJ	Accipitriformes	*Accipiter gentilis* (goshawk)
		Accipiter nisus (Eurasian sparrowhawk)
		Aquila fasciata (Bonelli's eagle)
		Aquila pennata (booted eagle)
		Buteo buteo (common buzzard)
		Circus aeruginosus (marsh harrier)
	Columbiformes	*Columba livia* (rock pigeon)
		Columba palumbus (wood pigeon)
		Streptopelia decaocto (Eurasian collared dove)
	Falconiformes	*Falco peregrinus* (peregrine falcon)
		Falco tinnunculus (common kestrel)
	Strigiformes	*Bubo bubo* (eagle owl)
Tongue	Accipitriformes	*Aquila fasciata* (Bonelli's eagle)
		Buteo buteo (common buzzard)
		Circus aeruginosus (marsh harrier)
	Columbiformes	*Streptopelia decaocto* (Eurasian collared dove)
	Falconiformes	*Falco peregrinus* (peregrine falcon)
		Falco tinnunculus (common kestrel)
	Strigiformes	*Strix aluco* (tawny owl)
Eye orbits	Accipitriformes	*Accipiter nisus* (Eurasian sparrowhawk)
	Columbiformes	*Columba palumbus* (wood pigeon)
	Falconiformes	*Falco peregrinus* (peregrine falcon)
	Strigiformes	*Strix aluco* (tawny owl)
Crop	Columbiformes	*Columba palumbus* (wood pigeon)
	Falconiformes	*Falco tinnunculus* (common kestrel)
Larynx	Accipitriformes	*Milvus milvus* (red kite)
	Strigiformes	*Asio otus* (long-eared owl)
Liver	Columbiformes	*Columba livia* (rock pigeon)
Oesophagus	Accipitriformes	*Aquila pennata* (booted eagle)
	Columbiformes	*Columba palumbus* (wood pigeon)
	Falconiformes	*Falco tinnunculus* (common kestrel)

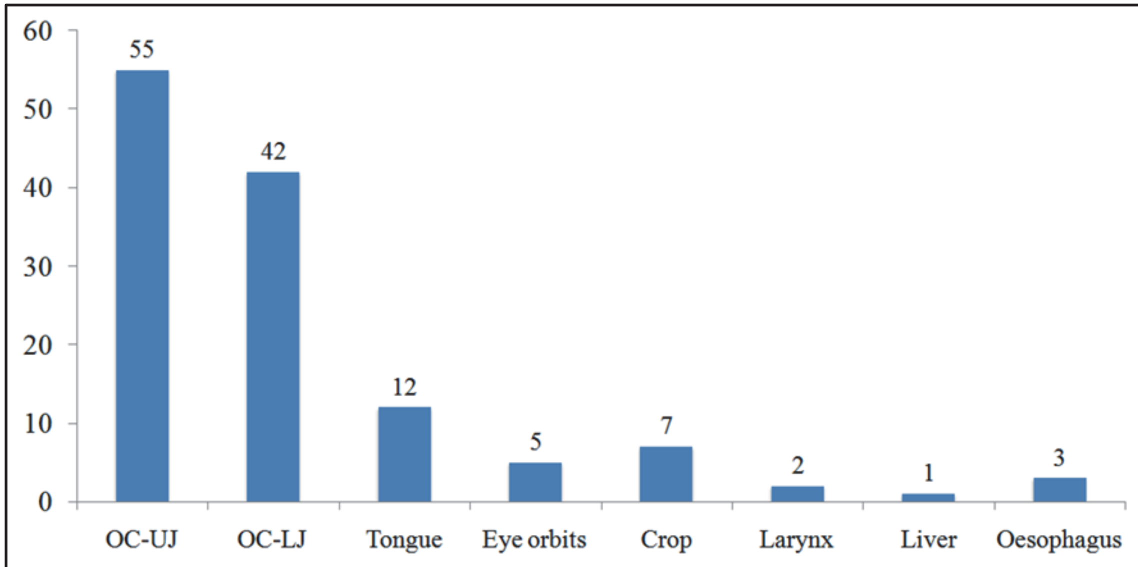


Figure 22. Histogram with the number of cases which included the corresponding anatomical location on their gross lesion distribution. OC-UJ: oropharyngeal cavity-upper jaw, the choanal slit and infundibular cleft locations are also included. OC-LJ: oropharyngeal cavity-lower jaw.

In reference to the dimensions of lesions, the largest (≥ 5 cm in length) but also the smallest ones (< 0.5 cm) were detected in birds from orders Accipitriformes, Falconiformes and Strigiformes (table 22), although most of Falconiformes and Strigiformes had lesions larger than two cm in length. Columbiformes had lesions between 0.6 to less than five cm in length.

Table 22. Information of bird species in relation to size of gross lesions compatible with avian trichomonosis.

Lesion size	Order	Species	n
≤ 0.5 cm	Accipitriformes	*Aquila fasciata* (Bonelli's eagle)	9
	Strigiformes	*Asio otus* (long-eared owl)	1
0.6-2 cm	Accipitriformes	*Accipiter gentilis* (goshawk)	3
		Aquila fasciata (Bonelli's eagle)	5
		Circus aeruginosus (marsh harrier)	1
		Milvus milvus (red kite)	1
	Columbiformes	*Columba palumbus* (wood pigeon)	3
		Streptopelia decaocto (Eurasian collared dove)	2
	Falconiformes	*Falco peregrinus* (peregrine falcon)	1
	Strigiformes	*Strix aluco* (tawny owl)	1
> 2-5 cm	Accipitriformes	*Accipiter gentilis* (goshawk)	1
		Accipiter nisus (Eurasian sparrowhawk)	2
		Aquila fasciata (Bonelli's eagle)	4
		Aquila pennata (booted eagle)	2
		Buteo buteo (common buzzard)	3
	Columbiformes	*Columba livia* (rock pigeon)	4
		Columba palumbus (wood pigeon)	1
		Streptopelia decaocto (Eurasian collared dove)	2
	Falconiformes	*Falco peregrinus* (peregrine falcon)	2
		Falco tinnunculus (common kestrel)	15
	Strigiformes	*Bubo bubo* (eagle owl)	3
		Strix aluco (tawny owl)	2
		Tyto alba (barn owl)	2
> 5 cm	Accipitriformes	*Aquila fasciata* (Bonelli's eagle)	1
	Falconiformes	*Falco tinnunculus* (common kestrel)	1
	Strigiformes	*Bubo bubo* (eagle owl)	1
		Strix aluco (tawny owl)	4
		Total	**77**

4.4.1. Lesions in columbiformes

In columbiformes, one lesion of grade 2 and 11 of grade 3 were evaluated from Eurasian collared doves, rock pigeons and wood pigeons. All birds died shortly after admission.

Eurasian collared doves

Four Eurasian collared doves were found with lesions of grade 3 at the oropharyngeal cavity that consisted of typical caseonecrotic growths from 0.5 to more than two cm in length (figure 23). Hard palate, lower jaw and/or tongue were the affected areas. All birds perished due to the infection.

Figure 23. Lesions at the oropharyngeal cavity of Eurasian collared doves (*Streptopelia decaocto*, cases VP21-15 and 0815-16).

Rock pigeons

Four rock pigeons were found with caseonecrotic nodules of large size (> 2 or 3 cm in length). Necrotic foci on the liver were reported also in one juvenile (figure 24).

Figure 24. From left to right: at the top gross lesions in a rock pigeon (*Columba livia*, case P10-11) and at the bottom detail of the liver (ventral area of the dorsal surface, visceral surface and cross section).

Wood pigeons

Lesions of grade 2 and 3 were detected in four wood pigeons. Lesions extended through the upper and lower regions of the oropharyngeal cavity or were located on the rims of the infundibular cleft. Crop and oesophagus were affected in one case, while unilateral conjunctivitis was reported from another animal in association with extensive lesions at the oropharynx (figure 25).

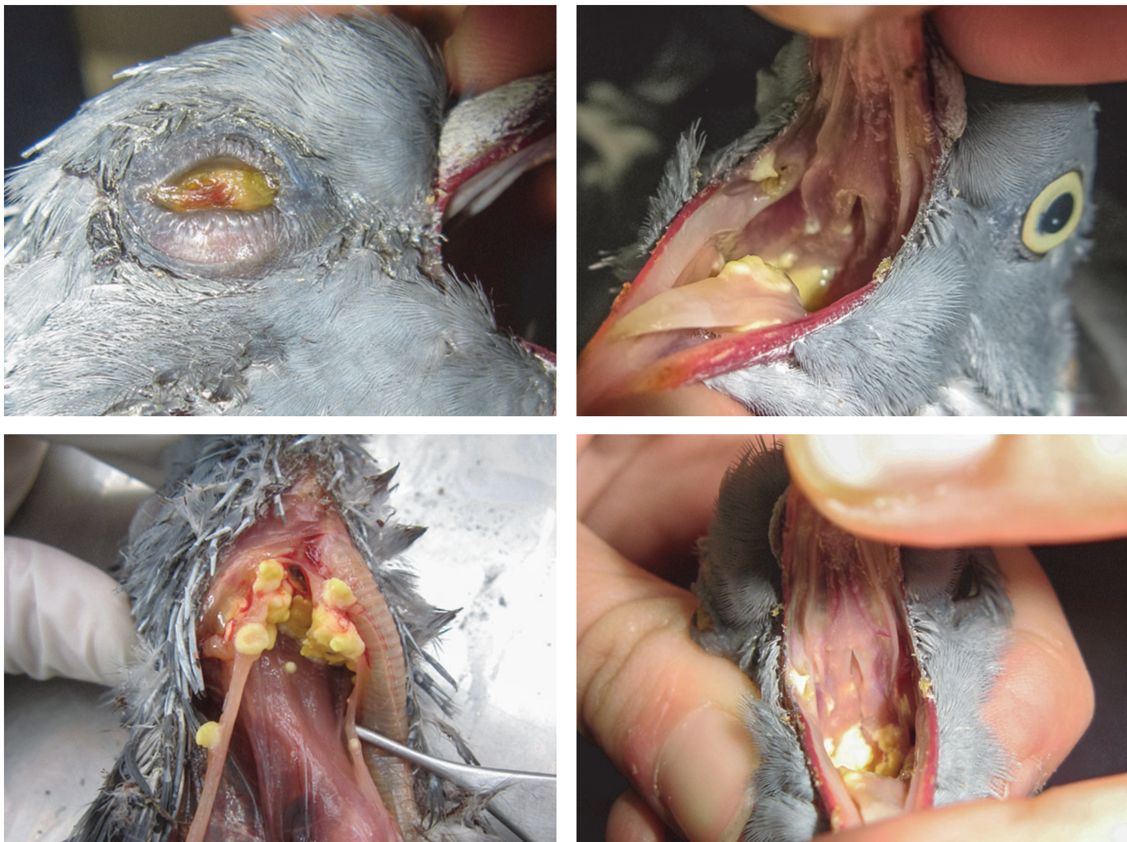


Figure 25. Photographs of the wood pigeon (*Columba palumbus)* case P349-12. From left to right: at the top appearance of the right eye and oropharyngeal cavity and at the bottom lesions in the oesophagus and detail of oropharyngeal cavity.

4.4.2. Lesions in accipitriformes

In this order of birds, lesions were evaluated from seven host species: Bonelli's eagles, booted eagles, common buzzards, Eurasian sparrowhawks, goshawks, marsh harriers and red kites. The three gradations of gross lesions were detected.

Bonelli's eagles

In Bonelli's eagles, lesions of grade 1, 2 and 3 were detected (figure 26). All birds (n= 20) survived the infection after being submitted for proper veterinary examination and treatment.

Lesions of grade 1 consisted of fibrinonecrotic material or small caseonecrotic growths. In the first case, the hard palate was affected and a negative result was obtained for *Trichomonas* spp. infection. Antiprotozoal treatment was administered due to the sanitary protocols implemented for these birds and the lesion disappeared in few days. The second case was a small caseonecrotic nodule that was surgically excised.

Lesions of grade 2 were found in 11 birds, 8 with a positive *T. gallinae* culture. These birds had mainly fibrinonecrotic material at the hard palate, with one case on the lower jaw. Besides, a caseonecrotic nodule was observed at the base of the tongue of one bird. Clinical cases of lesions of grade 2 and negative diagnosis of *Trichomonas* spp. infection were found with fibrinonecrotic material at the tip of the tongue or at the infundibular cleft margins. All cases recovered successfully after administration of antiprotozoal medication.

Grade 3 was determined on birds in which the parasite was identified (n= 6). The oropharyngeal cavity, from the hard palate to the lower jaw and tongue were the areas with gross lesions. Treatment was successful in all cases and birds were reintroduced into their habitat.

Booted eagles

Two fatal cases of grade 3 were evaluated. A large caseonecrotic nodule on the oesophagus or fibrinonecrotic material occupying 30% of the oropharyngeal mucosa, were the gross findings on these birds.

Common buzzards

Three common buzzards had lesions of grade 3 located at the base of the tongue and lower jaw that consisted of caseonecrotic growths. Two birds were reintroduced after proper treatment and one of them did not recover from the infection.

Eurasian sparrowhawks

Data from two fatalities were collected. Large caseonecrotic nodular lesions (> 3 cm in length) on the oropharyngeal cavity were detected at necropsy. Besides, unilateral conjunctivitis was found in one animal with a necrotic nodule located on the hard palate.

Goshawks

Four cases of lesions of grade 3 were examined in this species. One animal positive to *Trichomonas* spp. had a large caseonecrotic nodule (between 3 and 4.5 cm) on the left margin of the choanal slit (case 1739-06). The three other cases corresponded to infections with capillarid nematodes that accumulated fibrinonecrotic material at the oropharynx. These birds died shortly after admission and were necropsied (indicated as carcass in table 19).

Marsh harriers

One case with lesions at the oropharyngeal cavity was studied. Multifocally distributed fibrinonecrotic lesions of approximately 2 cm in length had a brownish superficial colouration. The animal recovered well after treatment.

Red kites

A unique lesion present at the proximal laryngeal area was found in one animal. The caseonecrotic nodule of small dimensions (< 1 cm) was surgically removed and the animal quickly recovered.

Figure 26. From left to right: detail of oesophageal and oropharyngeal lesions in Bonelli's eagles (*Aquila fasciata*, cases 0482-16 and 1113-15), booted eagle (*Aquila pennata*, case R172-11), marsh harrier (*Circus aeruginosus*, case 1360-05), red kite (*Milvus milvus*, case 1550-99) and goshawk (*Accipiter gentilis*, case R158-12).

4.4.3. Lesions in falconiformes

Two species of falconiformes were included in the study, common kestrels and peregrine falcons. Lesions of grade 3 were the unique category type.

Common kestrels

Sixteen common kestrels with lesions of grade 3 and positive *T. gallinae* cultures were evaluated. Focal or multifocally distributed caseonecrotic growths that ranged from 0.2 to more than 5 cm in length at the oropharyngeal cavity were identified (figure 27). The parasite-induced lesions caused the death of the birds in all cases. Maximum mortality was observed in these cases (100%).

Peregrine falcons

Three admissions with a positive *T. gallinae* culture were included. Lower and upper jaw were the areas affected at the oropharynx. The tongue was also involved in one case. Additionally, two birds had unilateral conjunctivitis and concomitant caseonecrotic masses at the oropharyngeal cavity, one of them did not survive (recovery rate 66.7%).

Figure 27. From left to right: detail of oesophageal and oropharyngeal lesions in common kestrels (*Falco tinnunculus,* cases R166-11, R198-13 and 3742-15) and in a peregrine falcon (*Falco peregrinus*, case 1706-14).

4.4.4. Lesions in strigiformes

Gross lesions from four species of owls were analysed: barn owls, eagle owls, long-eared owls and tawny owls. Grade 2 and 3 were the phenotypes found.

Barn owls

Two clinical cases of grade 3 were evaluated, both with single caseonecrotic nodules at the oropharyngeal cavity of large size (> 3 cm, figure 28). One of the animals died.

Eagle owls

Four animals with lesions of grade 3 had late stage, unique necrotic nodules of more than 2 cm in length involving the hard palate of the oropharynx (figure 28). Recovery rate was 50% for these cases.

Long-eared owls

One case of grade 2 lesions was examined (figure 28). Several white areas of fibrinonecrotic material were attached to the caudal margins of the infundibular cleft and posterior oropharynx. The animal was positive to *T. gallinae* culture and recovered succesfully after appropriate treatment.

Tawny owls

Seven clinical cases were studied. One bird with lesions of grade 2 had two caseonecrotic nodular lesions of one cm in length approximately. The animal achieved a complete recovery and was reintroduced in the nature after adequate treatment. Five animals with lesions of grade 3 were unable to survive. All birds had unique, focally distributed lesions of large size (> 3 cm) located on the hard palate. Finally, unilateral conjunctivitis was reported in one case. Final recovery rate was 14.28%.

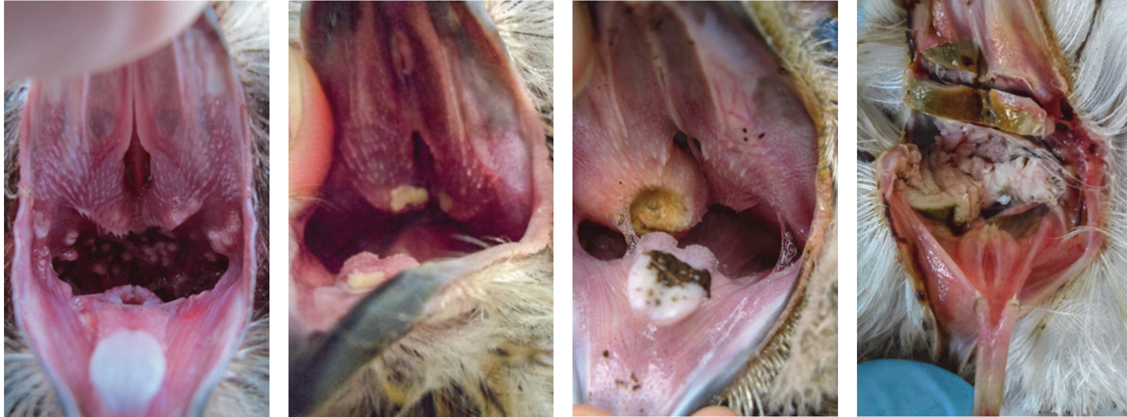


Figure 28. From left to right: lesions at the oropharyngeal cavity of a long-eared owl (*Asio otus,* case R137-12), tawny owl (*Strix aluco*, case 0060-12), eagle owl (*Bubo bubo,* case R16-12) and barn owl (*Tyto alba*, case 0050-12).

4.5. Characterization of new variants in avian oropharyngeal trichomonads

4.5.1. Analysis of mixed infections

Those parasitic strains with mixed infections detected by the ITS1/5.8S rRNA/ITS2 genotype were selected for further genetic characterization. Clonal cultures established through micromanipulation were developed in order to isolate the different genotypes present. In addition, parasites recovered from infections originated by unique genotypes were included for comparison and internal quality control.

A total of 118 clones were obtained from the selected parasites (table 23). Growth efficacy of the single-celled organisms varied considerably, with values ranging from 23 to 90%, according to the isolate. This fluctuation could be explained by the different adaptation of strains to *in vitro* growth conditions. Indeed, differences on growth rate were detected between genotype *T. gallinae*-1 and *T. gallinae*-2 in TYM medium by Sansano-Maestre (2009), with a lower growth rate in genotype *T. gallinae*-1.

For clonal cultures, isolates of genotype *T. gallinae*-1 obtained slightly lower efficacy (60-90%) than those with *T. gallinae*-2 genotype (80-90%). Isolates with mixed infections (*T. gallinae*-1 and *T. gallinae*-2) demonstrated even lower values (40-70%). Moreover, one isolate of genotype *T. canistomae*-like-1 had the lowest one, with only 23% of the cells successfully growing. A difficult subculture and adaptation to the growth medium was also observed in this isolate during previous multiplication of the parasite at the laboratory.

Table 23. Number of clonal cultures obtained from each isolate. Code: isolate code, the first letter refers to prey species (P) or raptor (R) and the last two digits to the sampling year. P: number of passage or subculture. ITS: genotype of the ITS1/5.8S rRNA/ITS2 region. L: gross lesions at the oropharyngeal cavity. MICRO P: passage used for micromanipulation. N clones: number of clonal cultures obtained. N cells: number of single-cell isolations for clonal culture obtention. Efficacy: percentage of clones that grew successfully.

Code	P	ITS	Host	L	Micro P	N clones	N cells	Efficacy
P349-12	2	*T. gallinae*-1+ *T. gallinae*-2	*Columba palumbus*	Yes	5	16	40	40%
P95-13	2	*T. gallinae*-1	*Streptopelia decaocto*	No	7	6	10	60%
P178-13	1	*T. gallinae*-2	*Columba palumbus*	No	6	8	10	80%
P196-13	0	*T. canistomae*-like-1	*Streptopelia turtur*	No	6	7	30	23%
R16-12	4	*T. gallinae*-1	*Bubo bubo*	Yes	10	7	10	70%
R17-12	0	*T. gallinae*-1	*Bubo bubo*	Yes	3	25	30	83%
R24-12	7	*T. gallinae*-2	*Falco tinnunculus*	No	13	9	10	90%
R44-12	3	*T. gallinae*-1+ *T. gallinae*-2	*Accipiter gentilis*	No	6	18	30	60%
R186-13	3	*T. gallinae*-1+ *T. gallinae*-2	*Falco tinnunculus*	Yes	7	7	10	70%
R193-13	1	*T. gallinae*-1	*Falco tinnunculus*	Yes	6	9	10	90%
R198-13	6	*T. gallinae*-1	*Falco tinnunculus*	Yes	9	6	10	60%
Total						**118**	**200**	**59%**

For the ITS1/5.8S rRNA/ITS2 region, clonal cultures obtained from isolates P95-13, P196-13, R16-12, R17-12, R24-12, R193-13 and R198-13, showed a unique genotype that was identical to the genotype of the original isolate. These genotypes were *T. gallinae*-1, *T. gallinae*-2 and *T. canistomae*-like-1. Besides, clones from isolates P349-12, R44-12 and R186-13 that were typified as mixed infections, revealed one single genotype (*T. gallinae*-1 or *T. gallinae*-2). Finally, a single nucleotide polymorphism was detected in all clones from isolate P178-13 (*T. gallinae*-2-1 SNP).

Table 24. Number of sequences obtained from the clonal cultures of the ITS1/5.8S rRNA/ITS2 region. Code: isolate code, the first letter refers to prey species (P) or raptor (R) and the last two digits to the sampling year. L: gross lesions at the oropharyngeal cavity. ITS: number of clones that were genotyped at the ITS1/5.8S rRNA/ITS2 region/total number of clones. ITS I/C: genotype of the ITS1/5.8S rRNA/ITS2 region of the isolate/clonal cultures.

Code	Host	L	ITS	ITS I/C
P349-12	*Columba palumbus*	Yes	1/16	*T. gallinae*-1+*T. gallinae*-2/ *T. gallinae*-1
P95-13	*Streptopelia decaocto*	No	6/6	*T. gallinae*-1/*T. gallinae*-1
P178-13	*Columba palumbus*	No	8/8	*T. gallinae*-2/*T. gallinae*-2-1 SNP
P196-13	*Streptopelia turtur*	No	2/7	*T. canistomae*-like-1/ *T. canistomae*-like-1
R16-12	*Bubo bubo*	Yes	7/7	*T. gallinae*-1/*T. gallinae*-1
R17-12	*Bubo bubo*	Yes	7/25	*T. gallinae*-1/*T. gallinae*-1
R24-12	*Falco tinnunculus*	No	9/9	*T. gallinae*-2/*T. gallinae*-2
R44-12	*Accipiter gentilis*	No	4/18	*T. gallinae*-1+*T. gallinae*-2/ *T. gallinae*-2
R186-13	*Falco tinnunculus*	Yes	7/7	*T. gallinae*-1+*T. gallinae*-2/ *T. gallinae*-2
R193-13	*Falco tinnunculus*	Yes	9/9	*T. gallinae*-1/*T. gallinae*-1
R198-13	*Falco tinnunculus*	Yes	6/6	*T. gallinae*-1/*T. gallinae*-1
Total			**65/118 (55%)**	

4.5.2. MLST analysis of new variants

T. gallinae isolates were chosen according to their ITS1/5.8S rRNA/ITS2 cryptic sequence type, as it has been previously cited (see table 7 of material and methods, section 3.4.1).

The MLST analysis indentified three new genotypes in clones from the new variants (7895-06 C2 and P196-13 C20), one for the small subunit of rRNA and two for the Fe-hydrogenase gene (table 25).

Clones P178-13 C7 and R17-12 C1 were typified as *T. gallinae* organisms for the three genetic loci with 100% BLAST identity to other reference sequences from other authors. However, clones 7895-06 C2 and P196-13 C20 displayed higher identity with other trichomonad parasites, *T. tenax* and *T. canistomae*, respectively. Their ITS1/5.8S rRNA/ITS2 region indicated 99% and 97% identity with these organisms, correspondingly. Clone 7895-06 C2 also had 99% BLAST identity at the ITS1/5.8S rRNA/ITS2 sequence with *Trichomonas* sp. organisms detected in European turtle

doves, wood pigeons and stock doves (*Columba oenas*) from the UK (Lennon *et al.*, 2013). For the small subunit of rRNA, a *Trichomonas* sp. sequence from an Australasian grey-capped Emerald dove (*Chalcophaps indica*) had the highest similarity (99%) and a *T. gallinae* isolate from a Eurasian collared dove of Austria was the first match considering an organism identified to the taxonomic species level. Finally, regarding the Fe-hydrogenase gene, 97% sequence identity was found with a *T. gallinae* strain from a zebra dove (*Geopelia striata*) from Seychelles islands.

In the analysis of clone P196-13 C20, the ITS1/5.8S rRNA/ITS2 locus revealed 99% identity with a *Trichomonas* sp. from a goshawk obtained in this study (R30-07) and, as it has been mentioned before, 97% with a *T. canistomae* strain of a dog. The other ribosomal marker (small subunit of rRNA) was a novel sequence with 99% identity with a *T. gallinae* strain from a common buzzard sampled in this study (RM11-12). Fe-hydrogenase gene revealed that a novel sequence was present in this region and indicated the lowest identity (95%) with a *T. gallinae* sequence from a rock pigeon of Canada.

Table 25. Information of the MLST clonal culture analysis for the study of new genetic variants. BLAST results are listed with the first match and further relevant identifications for comparison. L: presence of gross lesions. Acc. n.: GenBank acc. n. BP: sequence length in base pairs. I %: percentage of BLAST identity. Genotype nomenclature according to genetic groups of table 12[1], Grabensteiner *et al.*, 2010[2]; Chi *et al.*, 2013[3]. ITS: ITS1/5.8S/ITS2, SSU: small subunit of rRNA and Fe: Fe-hydrogenase gene. Ref: author reference of the sequence, a: Sansano-Maestre *et al.*, 2009, b: Martínez-Díaz *et al.*, 2015, c: McBurney *et al.*, 2015, d: Kunca *et al.*, 2015, e: Girard *et al.*, 2014, f: Peters and Raidal, unpublished, g: Jiang *et al.*, 2016, h: López-Escamilla *et al.*, 2013, i: Grabensteiner *et al.*, 2010, j: Lawson *et al.*, 2011, k: Martínez-Herrero *et al.*, 2014, l: Kutisova *et al.*, 2005.

Clone	Host	L	Acc. n. / Genotype	PCR	Bp	BLAST			
						I %	Acc. n.[ref]	Organism	Host
P178-13 C7	*Columba palumbus* (wood pigeon)	No	KX524372 / *T. gallinae*-2- 1 SNP[1]	ITS	244	99	EU881912[a]	*Trichomonas gallinae*	*Columba livia* (rock pigeon)
			KX514373	SSU	1407	100	KM246609[b]	*Trichomonas gallinae*	*Buteo buteo* (common buzzard)
			KX514374 / C4[3]	Fe	820	100	KJ184172[c]	*Trichomonas gallinae*	*Columba livia* (rock pigeon)
R17-12 C1	*Bubo bubo* (eagle owl)	Yes	KX514378 / *T. gallinae*-1[1]	ITS	260	100	EU881913[a]	*Trichomonas gallinae*	*Tyto alba* (barn owl)
			KX514379 / VI[2]	SSU	1396	100	KM095107[d]	*Trichomonas gallinae*	*Accipiter nisus* (Eurasian sparrowhawk)
			KX514380 / A1[3]	Fe	909	100	KC244201[e]	*Trichomonas gallinae*	*Patagioenas fasciata monilis* (band-tailed pigeon)
7895-06 C2	*Columba livia* (rock pigeon)	Yes	FN433474 / II[2] / *T. tenax*-like-1[1]	ITS	296	99	JQ755287[f]	*Trichomonas* sp.	Australasian columbiforme
						99	KJ721786[g]	*Trichomonas* sp.	*Columba livia* (rock pigeon)
						99	KF164607[h]	*Trichomonas tenax*	*Homo sapiens* (man)
						95	KC215388[e]	*Trichomonas gallinae*	*Patagioenas fasciata monilis* (band-tailed pigeon)
			FN433479 / I[2]	SSU	1128	100	JQ030997[f]	*Trichomonas* sp.	*Chalcophaps indica* (grey-capped Emerald dove)
						99	FN433481[i]	*Trichomonas gallinae*	*Streptopelia decaocto* (Eurasian collared dove)
			KX514371 / Novel-3	Fe	842	97	JF681142[j]	*Trichomonas gallinae*	*Geopelia striata* (zebra dove)
P196-13 C20	*Streptopelia turtur* (European turtle dove)	No	KX514375 / *T. canistomae*-like-1[1]	ITS	256	100	KF993705[k]	*Trichomonas* sp.	*Streptopelia turtur* (European turtle dove)
						99	KF993706[k]	*Trichomonas* sp.	*Accipiter gentilis* (goshawk)
						97	AY244652[l]	*Trichomonas canistomae*	*Canis familiaris* (dog)
						97	KC215388[e]	*Trichomonas gallinae*	*Patagioenas fasciata monilis* (band-tailed pigeon)
			KX514376 / Novel-1	SSU	1407	99	KM246609[b]	*Trichomonas gallinae*	*Buteo buteo* (common buzzard)
			KX524377 / Novel-2	Fe	853	95	KJ184172[c]	*Trichomonas gallinae*	*Columba livia* (rock pigeon)

4.5.3. Morphometric study of new variants

4.5.3.1. Optical microscopy study

After the analysis of Pearson's correlation coeficients, the criteria of cell perimeter and cell surface were eliminated from the analysis due to their high correlation ($r \geq 0.7$; *p*-value < 0.01). The measurements of individual flagellar length (flagella 1 to 4) were substituted by the sum of them because of their high correlation too, using total flagellar length as valid criteria.

The morphometrical study by optical microscopy revealed statistical differences between the clones (figure 29, tables 26 and 27). Average cell size dimensions were 8-11 µm length and 4.3-7.2 µm width (table 26). The smaller cell size was obtained by clones P196-13 C20 and R17-12 C1, with mean values of 8.3 x 4.4 µm and 8.4 x 4.3 µm, respectively. Clone 7895-06 C2, that was ceded by the research group of Professor Dr. Michael Hess (Veterinary Medicine University, Vetmeduni, Vienna, Austria), had intermediate results (9.2 x 7.2 µm), while clone P178-13-C7 revealed the highest values (11 x 6.5 µm).

Mean values for individual flagellar length were 10.0-17.9 µm, with significant differences between the clones, that grouped P178-13 C7 and P196-13 C20 with the longest value, clone 7895-06 C2 with intermediate results and clone R17-12 C1 with the shortest length.

The study of the axostyle showed average values that ranged 15.2-18.4 µm in length, with an axostyle projection between 5.9-8.2 µm length. Significant differences were detected in clone P196-13 C20 which showed the longest axostyle projection (mean value of 8.2 µm length).

Finally, statistical analysis determined that significant differences were found among the clones (MANOVA Pillai's Trace test, *p*-value < 0.01). Clone P196-13 C20 showed different morphological traits, with a smaller cell body size and the longest axostyle projection, this later criteria allowing the differentiation from clone R17-12 C1, also small-sized.

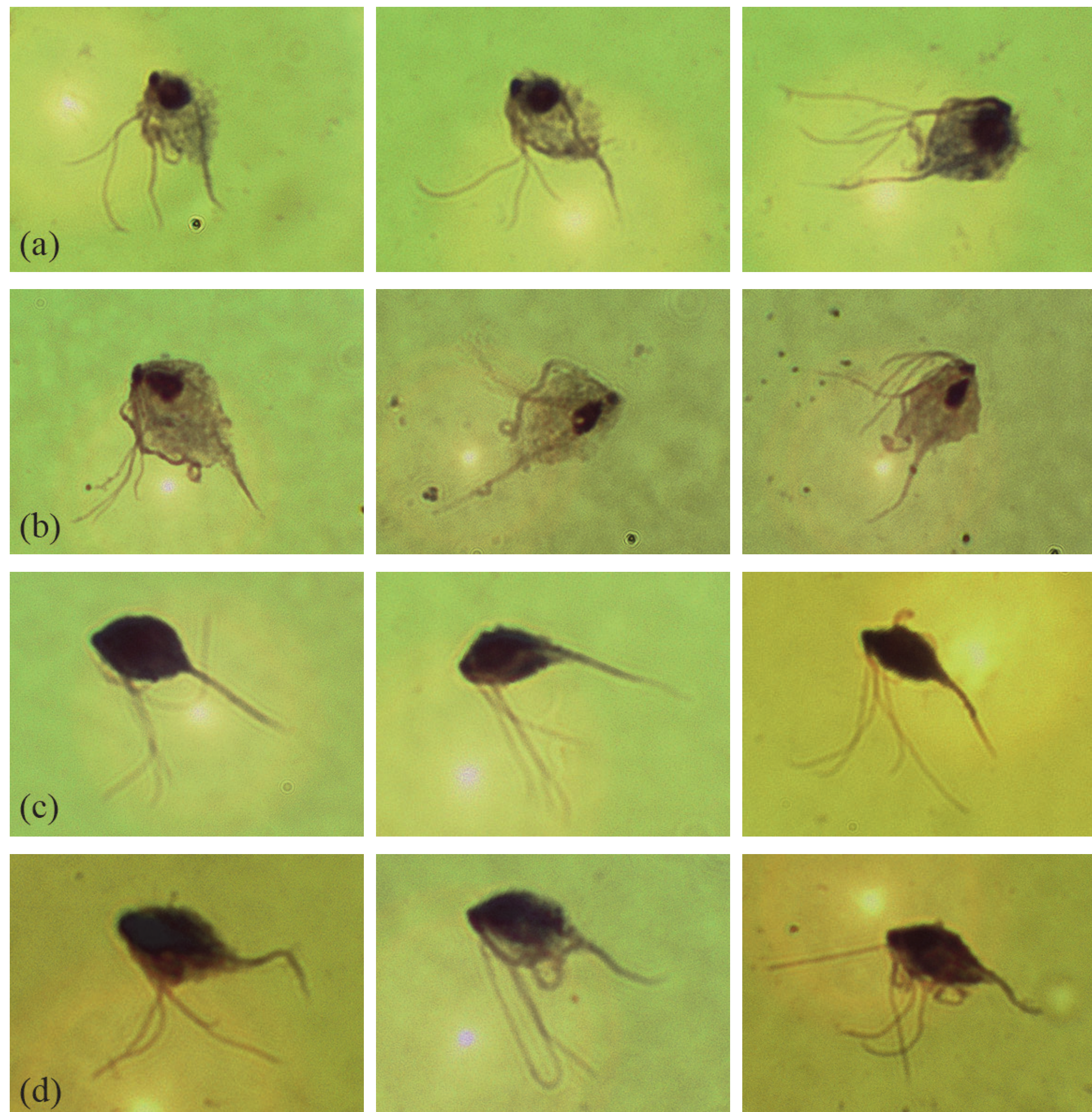


Figure 29. Images of optical microscopy from trophozoites of the clonal cultures selected for morphometric comparison. Diff-Quick staining. (a): clone 7895-06 C2. (b): clone P178-13 C7. (c): clone P196-13 C20. (d): clone R17-12 C1.

Table 26. Morphometric results of *Trichomonas* spp. trophozoites from clonal cultures. Values are expressed in micrometers. The four free flagella are ordered from longest to shortest (flagellum 1 to 4).

Parameter	Mean ± standard deviation (range)			
	Clone 7895-06 C2	**Clone P178-13 C7**	**Clone P196-13 C20**	**Clone R17-12 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)

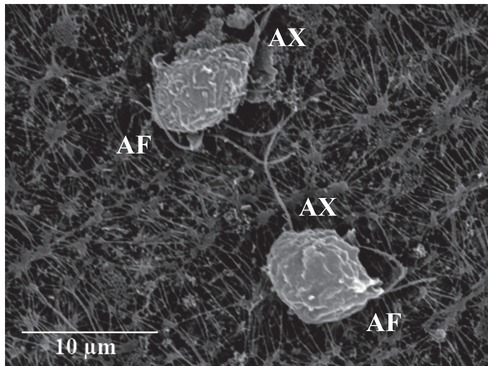
Table 27. Statistical results from the different morphometric criteria considered in this study. Scheffé post hoc tests are indicated with their *p*-value. Mean values correspond with original measurements (not transformed data) in µm. *Indicates significant differences (*p*-value < 0.01).

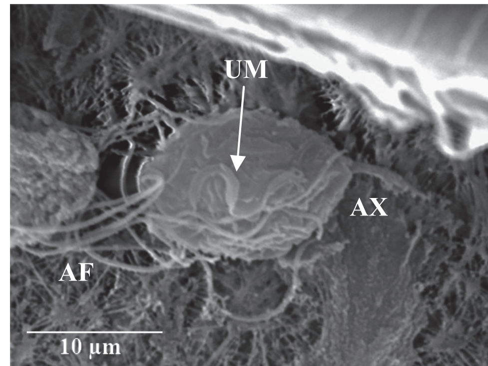
Transformed variable	Homogeneous group/s	*p* - value	Clone	Mean value
Cell length	1	0.000*	7895-06 C2	9.2
	2	0.000*	P178-13 C7	11
	3	0.089	P196-13 C20	8.3
			R17-12 C1	8
Cell width	1	0.004*	P196-13 C20	4.4
			R17-12 C1	4.3
	2	0.844	7895-06 C2	7.2
			P178-13 C7	6.5
Total flagellar length	1	0.000*	7895-06 C2	54.5
	2	0.000*	R17-12 C1	48.2
	3	0.084	P178-13 C7	59.7
			P196-13 C20	61
Axostyle projection	1	0.549	7895-06 C2	5.9
			P178-13 C7	6.4
			R17-12 C1	6.5
	2	0.000*	P196-13 C20	8.2

4.5.3.2. Structural analysis by scanning electron microscopy

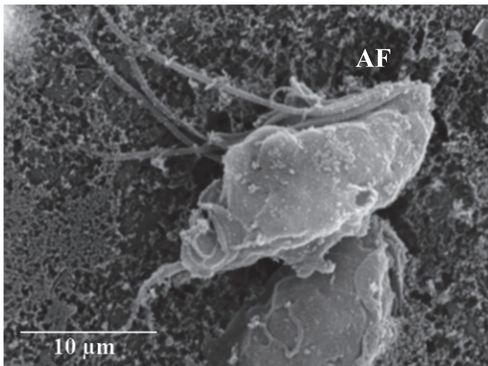
The comparative study by scanning electron microscopy allowed the assessment of superficial structures on trophozoites. In all cases, the plasmatic membrane had a rough appearance, with small curvatures and indentations. External shape was pyriform or rounded. No alternative or new structures were detected on the visual inspection of the images (figure 30, next page).

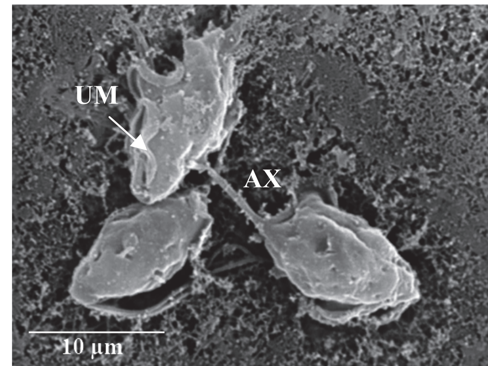
A: clone 7895-06 C2



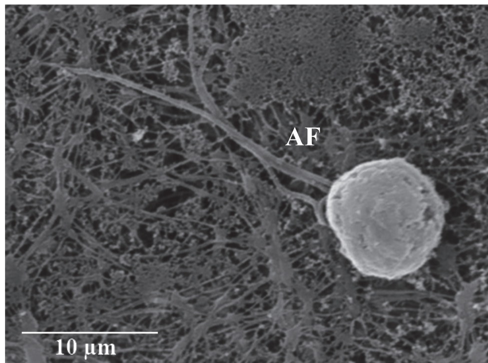


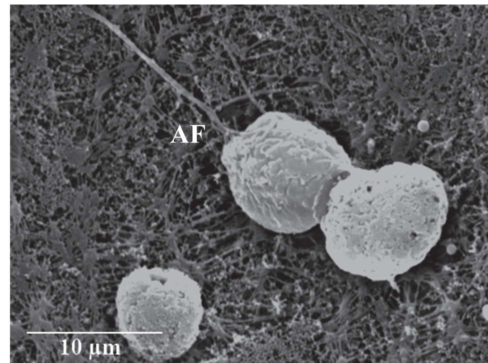
B: clone P178-13 C7



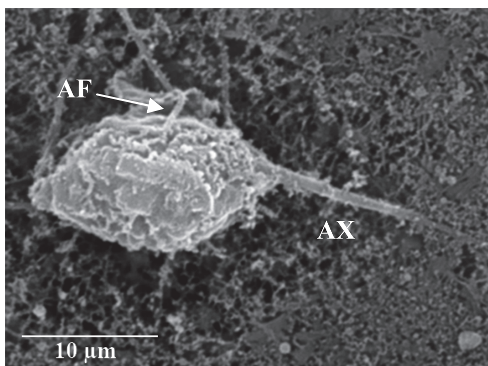


C: clone P196-13 C20





D: clone R17-12 C1



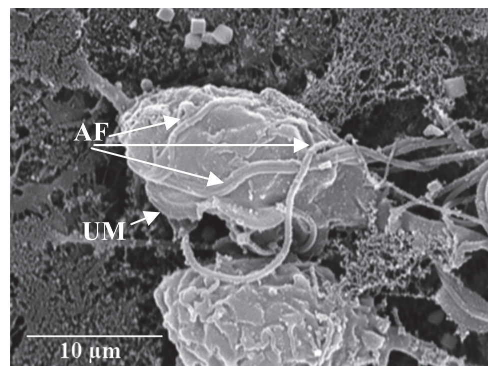


Figure 30. Scanning electron microscopy images from the selected clonal cultures of avian oropharyngeal trichomonads. The scale bar is denoted at the bottom of each photograph. AF: anterior flagella. AX: axostyle. UM: undulating membrane.

4.6. Virulence assays of clonal cultures of *T. gallinae* in LMH cells

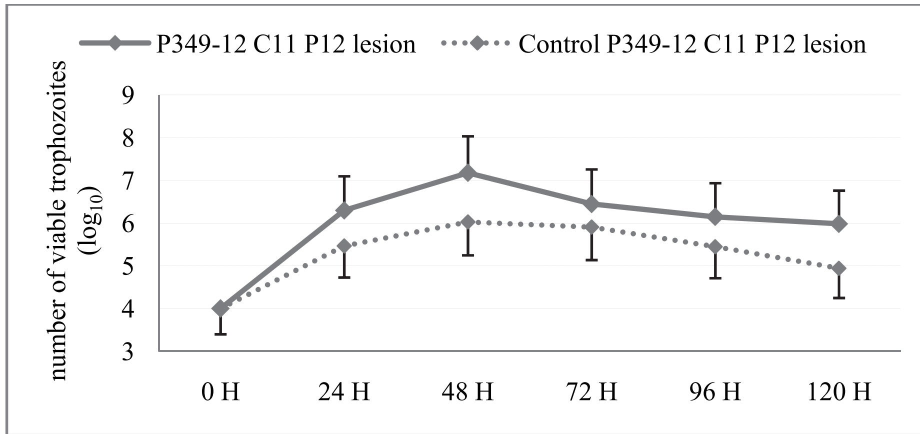
4.6.1. Trophozoite growth in LMH cells

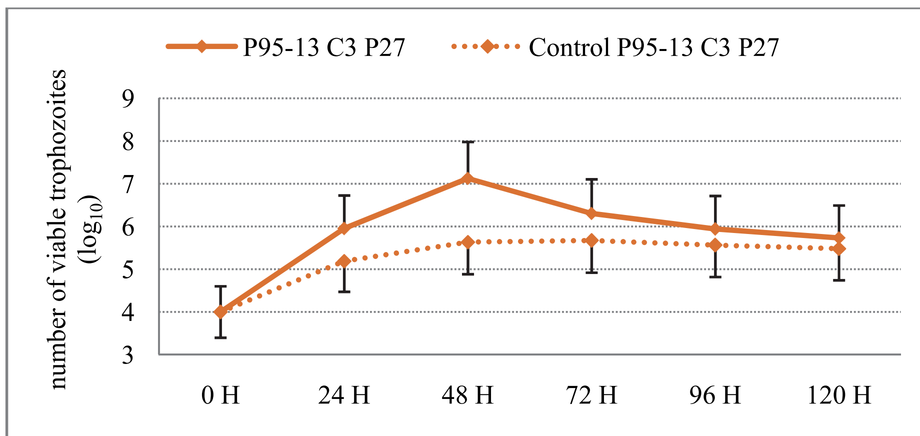
A total of $1 \cdot 10^4$ trophozoites from axenic clonal cultures of *T. gallinae* were added to LMH cell cultures. The growth of trichomonads was observed in all the flask containing cells and trophozoites (table 28, figure 31). The maximum growth was observed at two days post-infection for the majority of the clones (6/7) and at the third day post-infection for one clone (1/7). Trophozoites growth was also recorded in all flasks maintained as positive controls (figure 31).

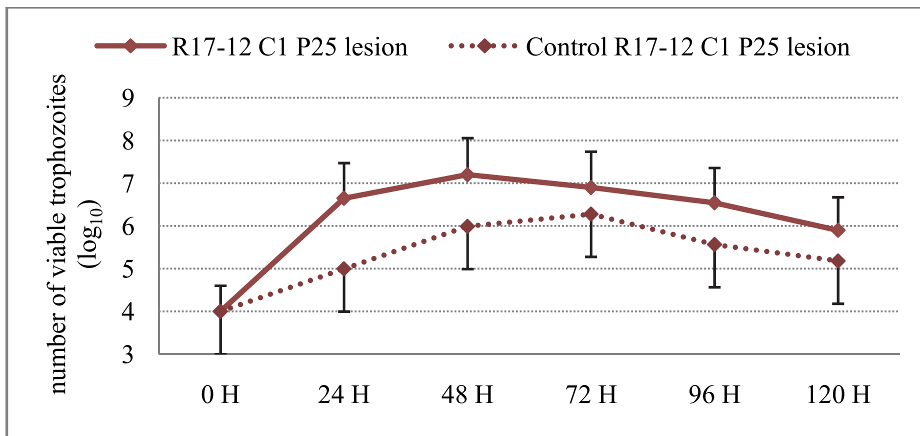
Table 28. Total number of live trichomonads from infected LMH cell cultures and positive controls. Standard deviation is preceded by the ± sign. Code: clonal culture identification code (first letter for type of host P-columbiforme, R-bird of prey; two or three numbers for laboratory internal code; next two numbers of sampling year; C and number for clonal culture, and P and two numbers for passage). H: hours post-infection. L: clonal cultures from birds with gross lesions. ITS: ITS1/5.8S rRNA/ITS2 region genotype. *: maximum growth.

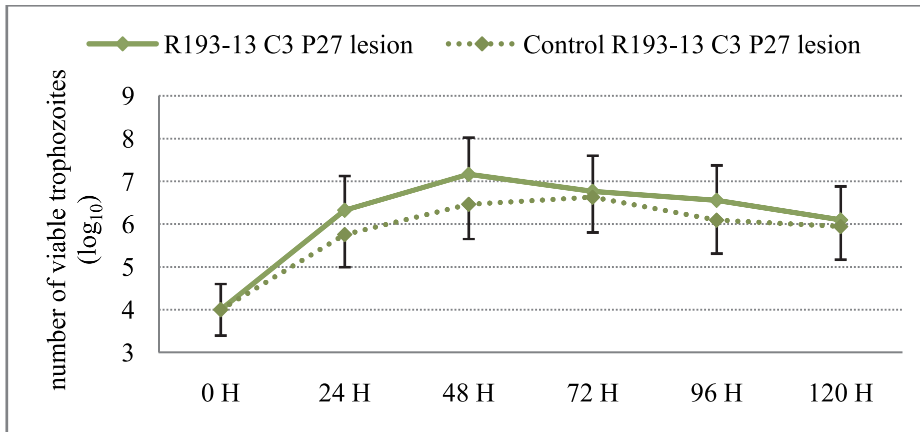
ITS	Code	24H	48H	72H	96H	120H
***T. gallinae*-1**	P95-13 C3 P27	898,333 ± 556,251	13,387,500* ± 1,515,545	2,024,167 ± 399,189	875,000 ± 63,097	542,500 ± 87,500
	Positive control	154,583 ± 118,798	431,667 ± 44,041	472,500* ± 35,000	367,500 ± 63,097	303,333 ± 375,469
	P349-12 C11 P12 L	1,983,333 ± 50,518	14,991,667* ± 353,627	2,800,000 ± 231,503	1,400,000 ± 87,500	962,500 ± 175,000
	Positive control	291,667 ± 53,463	1,061,667* ± 26,732	805,000 ± 35,000	280,000 ± 46,301	87,500 ± 17,500
	R17-12 C1 P25 L	4,450,833 ± 58,6272	15,822,917* ± 1,277,270	7,962,500 ± 918,750	3,488,333 ± 78,912	793,333 ± 82,702
	Positive control	99,167 ± 10,104	980,000 ± 311,087	1,895,833* ± 102,541	367,500 ± 63,097	151,667 ± 96,383
	R193-13 C3 P27 L	2,114,583 ± 165,634	14,685,417* ± 886,230	5,862,500 ± 660,610	3,616,667 ± 50,518	1,254,167 ± 267,317
	Positive control	571,667 ± 119,120	2,910,833 ± 78,912	4,275,833* ± 772,845	1,248,333 ± 145,717	880,833 ± 119,120
***T. gallinae*-2**	P178-13 C7 P27	1,522,500 ± 355,211	12,384,167* ± 6,295,291	5,322,917 ± 1,051,215	2,893,333 ± 359,212	1,960,000 ± 30,311
	Positive control	198,333 ± 36,429	1,090,833 ± 148,836	1,621,667* ± 26,732	554,167 ± 61,458	140,000 ± 60,622
	R24-12 C10 P19	2,216,667 ± 281,273	10,179,166* ± 281,273	3,762,500 ± 350,000	1,604,167 ± 267,317	145,833 ± 50,518
	Positive control	262,500 ± 60,622	945,000 ± 46,301	1,487,500* ± 63,097	1,213,333 ± 66,254	589,167 ± 66,254
	R44-12 C5 P14	1,575,000 ± 87,500	6,620,833 ± 1,265,981	11,725,000* ± 175,000	5,920,833 ± 182,146	1,137,500 ± 231,503
	Positive control	87,500 ± 35,000	198,333* ± 26,732	64,167 ± 10,104	23,333 ± 10,104	17,500 ± 0

A: clonal cultures of ITS1/5.8S rRNA/ITS2 genotype *T. gallinae*-1.









B: clonal cultures of ITS1/5.8S rRNA/ITS2 genotype *T. gallinae-2*.

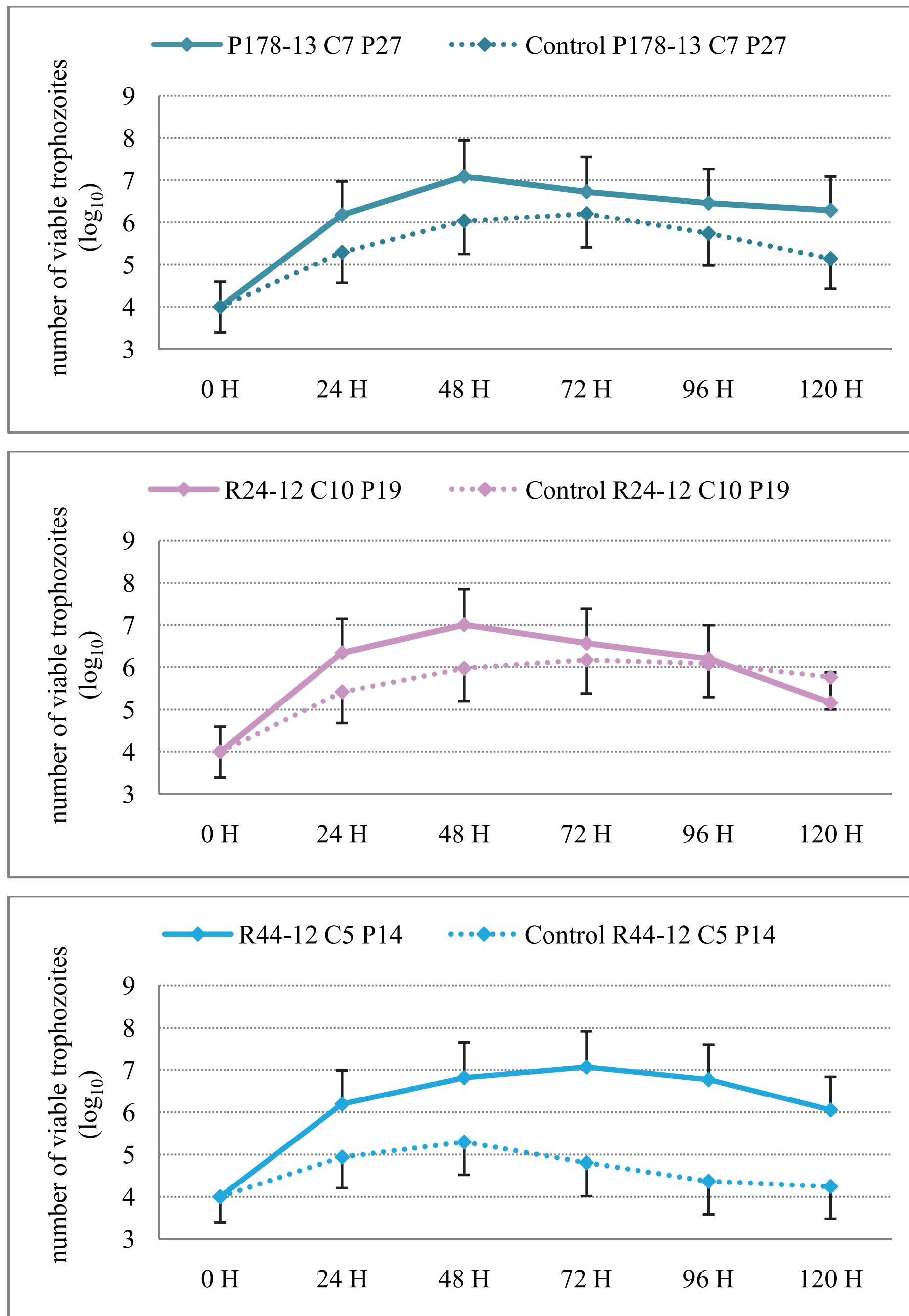


Figure 31. Graphics with the number of live *T. gallinae* trophozoites from clonal cultures on infected LMH cells. Error bars indicate standard deviation. Control flasks containing only trophozoites without LMH cells are also indicated in discontinuous lines. A: results from clonal cultures of ITS1/5.8S rRNA/ITS2 genotype *T. gallinae*-1. B: results from clonal cultures of ITS1/5.8S rRNA/ITS2 genotype *T. gallinae*-2.

In all cases, a complete destruction of the LMH cell monolayer was observed, although differences in the time required for the complete cell removal were detected. On the first hours post-infection, motile trichomonad cells were seen easily on the media, some of them swimming across the flask and others already attached to the LMH cell monolayer. On day two post-infection, trophozoites had increased their numbers and were easily visualized in clusters attached to LMH cells. Areas without cells were abundant on the LMH cell culture, which grew in size until a complete detachment was assessed on day three post-infection.

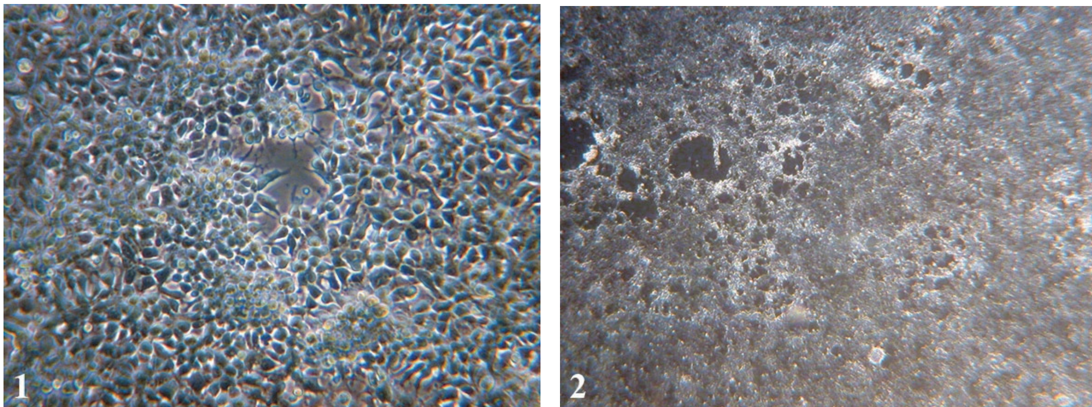
Statistical analysis was performed to analyze if there were differences in the number of viable trophozoites on flasks with LMH cells in comparison with positive controls (those that contained only trophozoites) for each different clonal culture. The Wilcoxon signed rank test was employed as the data did not fit with the normal distribution. All clones, with the exception of R24-12 C10 P19, obtained values that were statistically significant (p-value < 0.05).

4.6.2. Cytopathogenic score in LMH cells

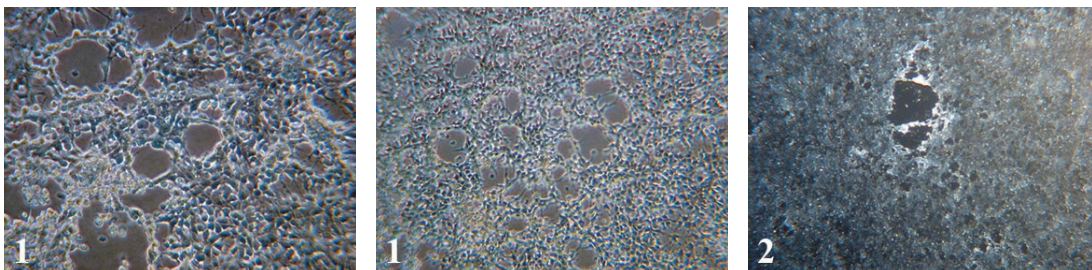
The maximum cytopathogenic score (4) was observed on day three post-infection in all clonal cultures (figure 32). Consequently, differences on the degree of pathogenicity were detected on day one and two post-infection. At 24 hours post-infection, clones P178-13 C7 and R24-12 C10, with genotype *T. gallinae*-2 for the ITS1/5.8S rRNA/ITS2 region, obtained the lowest values of cytophatogenicity (score 1). Score 2 was observed in clones R193-13 C3 and R44-12 C5, from both ITS1/5.8S rRNA/ITS2 genotypes (*T. gallinae*-1 and *T. gallinae*-2, respectively). In contrast, three of the four clones of genotype *T. gallinae*-1 (P95-13 C3, P349-12 C11 and R17-12 C1), produced the maximum cytopathogenic scores (3 and 4) at 24 hours post-infection.

These differences continued at 48 hours post-infection, as all the clones of genotype *T. gallinae*-1 achieved the maximum degree of cytopathogenicity (score 4), while clones of genotype *T. gallinae*-2 varied from score 2 (clone R44-12 C5), to 3 (clone P178-13 C7) or 4 (clone R24-12 C10).

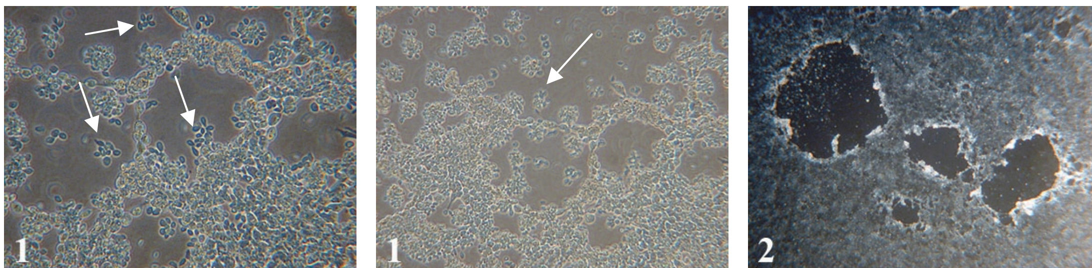
A: ≤ 25% of monolayer destruction (cytopathogenic effect score 1).



B: 25-50% of monolayer destruction (cytopathogenic effect score 2).



C: 50-75% of monolayer destruction (cytopathogenic effect score 3).



D: more than 75% of the cells destroyed (cytopathogenic effect score 4).

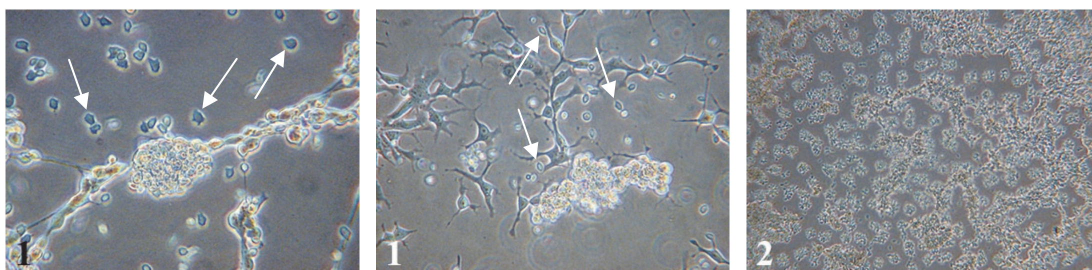


Figure 32. Images of gradual LMH cell monolayer damage originated by the growth of *T. gallinae* trophozoites from clonal cultures. Cytophatogenic effect (CPE) score was determined as proposed by Amin *et al.* (2012a). A: ≤ 25% of monolayer destruction (CPE 1), B: 25-50% (CPE 2), C: 50-75% (CPE 3) and D: more than 75% of the cells destroyed (CPE 4). 1: higher magnification. 2: lower magnification. Arrows point *T. gallinae* trophozoites.

Significant statistical differences were detected on the average CPE score of clones on day one and two post-infection according to their ITS1/5.8S rRNA/ITS2 genotype (Chi-square test of association; $\chi^2_{day\ 1}$= 56.00, d.f.= 3, p-value < 0.01; $\chi^2_{day\ 2}$= 39.74, d.f.= 2, p-value < 0.01).

Table 29. Average cytopathogenic effect (CPE) scores detected on LMH cells infected with clonal cultures of *T. gallinae* during the first three days post-infection. ITS: ITS1/5.8S rRNA/ITS2. Genotype nomenclature according to genetic groups of table 12[a]. *: statistical significant results (Chi-square test, p-value < 0.01).

Genotype		CPE score		
ITS[a]	Clone	Days post-infection		
		1*	2*	3
T. gallinae-1	R193-13 C3 P27 lesion	2	4	4
	P95-13 C3 P27	3	4	4
	P349-12 C11 P12 lesion	4	4	4
	R17-12 C1 P25 lesion	4	4	4
T. gallinae-2	R44-12 C5 P14	2	2	4
	R24-12 C10 P19	1	4	4
	P178-13 C7 P27	1	3	4

4.7. Comparative analysis of membrane proteins in two *T. gallinae* clonal cultures with different genotype by RP-LC-MS/MS

4.7.1. SDS preliminary evaluation

Different cell yields were obtained for each biological replicate of axenized *T. gallinae* clones grown in TYM medium without antibiotics and antifungals. After their subcellular fractionation and acetone precipitation, the fraction containing the cell and organelle membranes was quantified for protein concentration by the Bradford method (RC DC protein assay, Bio-Rad Laboratories, Hercules, California, USA, table 30).

Table 30. Protein quantification from biological replicates of clone P178-13 C7 P11 and R17-12 C1 P9.

Biological replicate	Protein concentration (µg/µl)
P178 - R1	1.46
P178 - R2	2.12
P178 - R3	2.06
P178 - R4	1.34
P178 - R5	3.72
R17- R1	2.25
R17- R2	0.95
R17- R3	1.84

Protein fractions were loaded in a 12% SDS-PAGE gel as a quality control prior to the RP-LC-MS/MS analysis. The electrophoresis confirmed the differences in protein concentrations determined by the Bradford method, and also in the protein profiles obtained from the clones (figure 33). Three biological replicates were selected from each clone for further proteomic analysis in accordance to their higher protein concentration. For clone P178-13 C7, samples from lane 4, 5 and 7 were employed, and for clone R17-12 C1 samples from lane 8, 9 and 10.

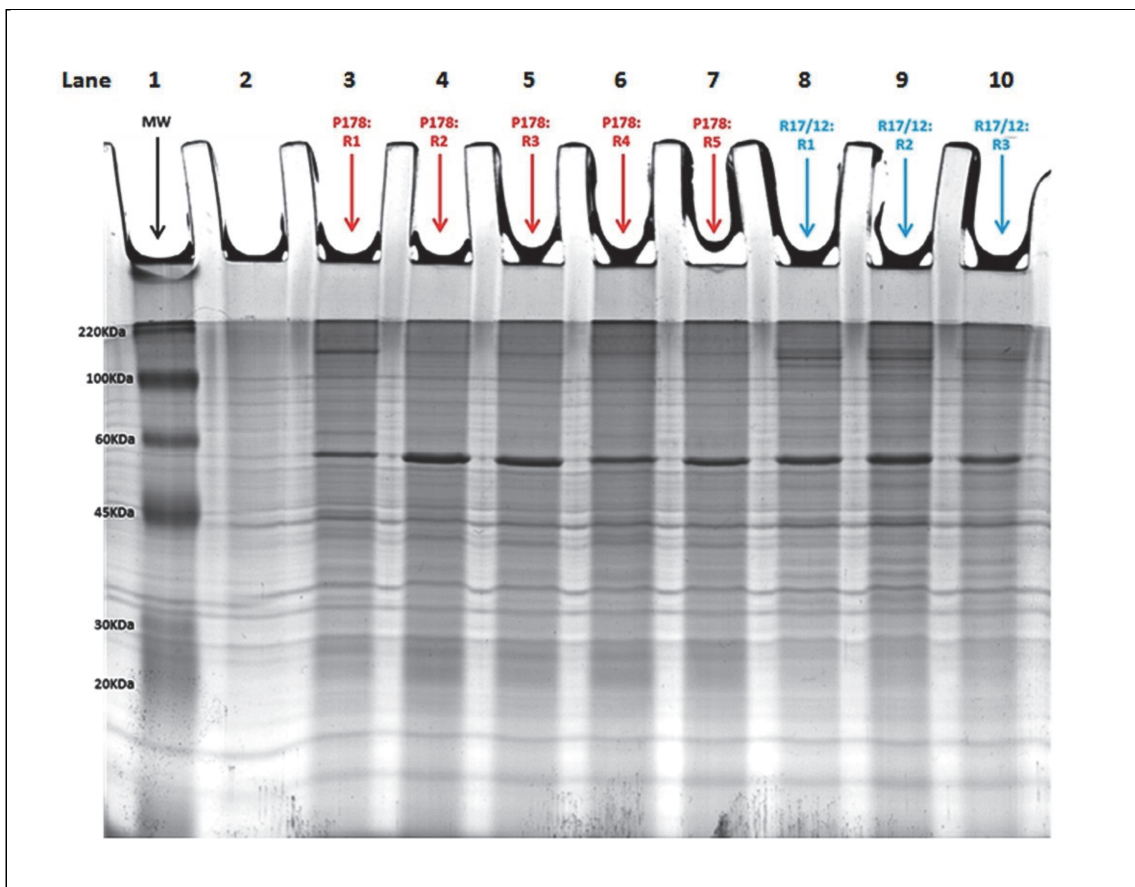


Figure 33. Image of the 12% SDS-PAGE gel from the biological replicates of membrane and organelle membrane protein fraction of *T. gallinae* clonal cultures P178-13 C7 P11 and R17-12 C1 P9. Coomassie blue staining.

4.7.2. LC-MS/MS analysis

The results of mass spectrometry study also revealed substantial differences in the peak profiles replicates of clone P178-13 C7 in comparison with replicates of clone R17-12 C1 (appendix I). Similar chromatogram profiles were obtained between each biological and technical replicate originated from the same clonal culture.

4.7.3. Quantification of relative protein abundance and predicted functions

Common proteins, i.e. identified in both clonal cultures (P178-13 C7 and R17-12 C1) in every biological and technical replicates, containing two or more peptides and a *p*-value < 0.01 were quantified. Those with ≥ 3 fold change difference in abundance between both clones were considered differentially expressed. As a result, 302 proteins were detected with differential expression, 170 more abundant in clone P178-13 and 132 in clone R17-12 C1 (appendices II and III, figures 34 and 35).

Most of the identified proteins had no information available regarding the subcellular location (73% of the proteins in clone P178-13 C7 and 62% in clone R17-12 C1, figure 35). The largest group of identified proteins corresponded with plasmatic membrane, with 8% of proteins in clone P178-13 C7 and 13% in clone R17-12 C1. Peptides from organelle membranes were found in 6% and 4% of the samples from clone P178-13 C7 and R17-12 C1, respectively. Cytoplasmic proteins were detected in 3% of the proteins from clone P178-13 C7 and 11% in clone R17-12 C1. Besides, cytoskeletal associated proteins accounted for 5% of the proteins in clone P178-13 C7 and 9% in clone R17-12 C1. Finally, a small percentage of nuclear proteins were present in clone P178-13 C7 (5%) and clone R17-12 C1 (1%).

Considering the category assigned to the predicted function, there were also a high percentage of proteins (35%) with unknown function in both clones (figure 35). The group of proteins involved in cellular metabolism comprised 40% of the identified proteins in clone P178-13 C7, whilst in clone R17-12 C1 included 33% of them. Proteins of the cytoskeleton were more abundant in clone R17-12 C1 and included 6% and 8% of the identified proteins (clones P178-13 C7 and R17-12 C1, respectively). Proteins acting as constituents of cellular components were more numerous in clone R17-12 C1 (10%) than in clone P178-13 C7 (5%). The lower amounts of those corresponded to proteins involved in translation (4% in both clones), DNA replication (3% and 4% in clones P178-13 C7 and R17-12 C1, respectively), signal transduction (3% in clone P178-13 C7 and 1% in clone R17-12 C1), cellular adhesion (2% in both clones) and exocytosis (2% and 3% in clones P178-13 C7 and R17-12 C1, respectively).

Among the proteins that differed in abundance between the clones, BspA-like proteins (*Bacteroides* sp. surface A-like) were identified, including cytoskeletal proteins like one alpha-actinin (more abundant in clone P178-13 C7) and dyneins (four more abundant in clone R17-12 C1). Also, other possible proteins related with the parasite virulence were identified in both clones. For instance, several peptidases and proteins involved in transmembrane trafficking and vesicle-mediated transport. Besides, other proteins that were found more abundant in clone R17-12 C1 were two malate dehydrogenases (fold change 3.46 and 6.54), a Ras family protein (rat sarcome small guanosine triphosphatases, fold change 3.8), a GP63-like protein (fold change 7.78) and a immuno-dominant variable surface antigen-like (fold change 18.96; appendices II and III).

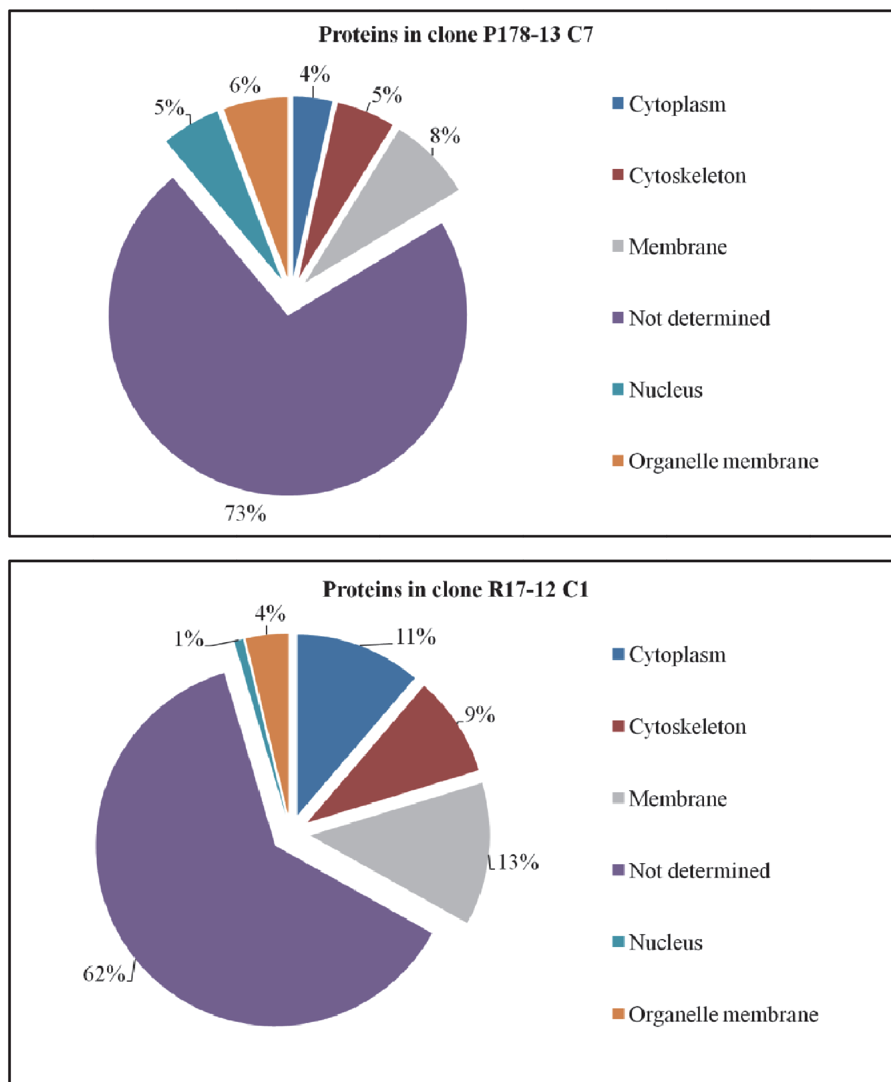


Figure 34. Graph with the percentage of relatively quantified proteins, according to their subcellular location, that were more abundant in clone P178-13 C7 and clone R17-12 C1, respectively.

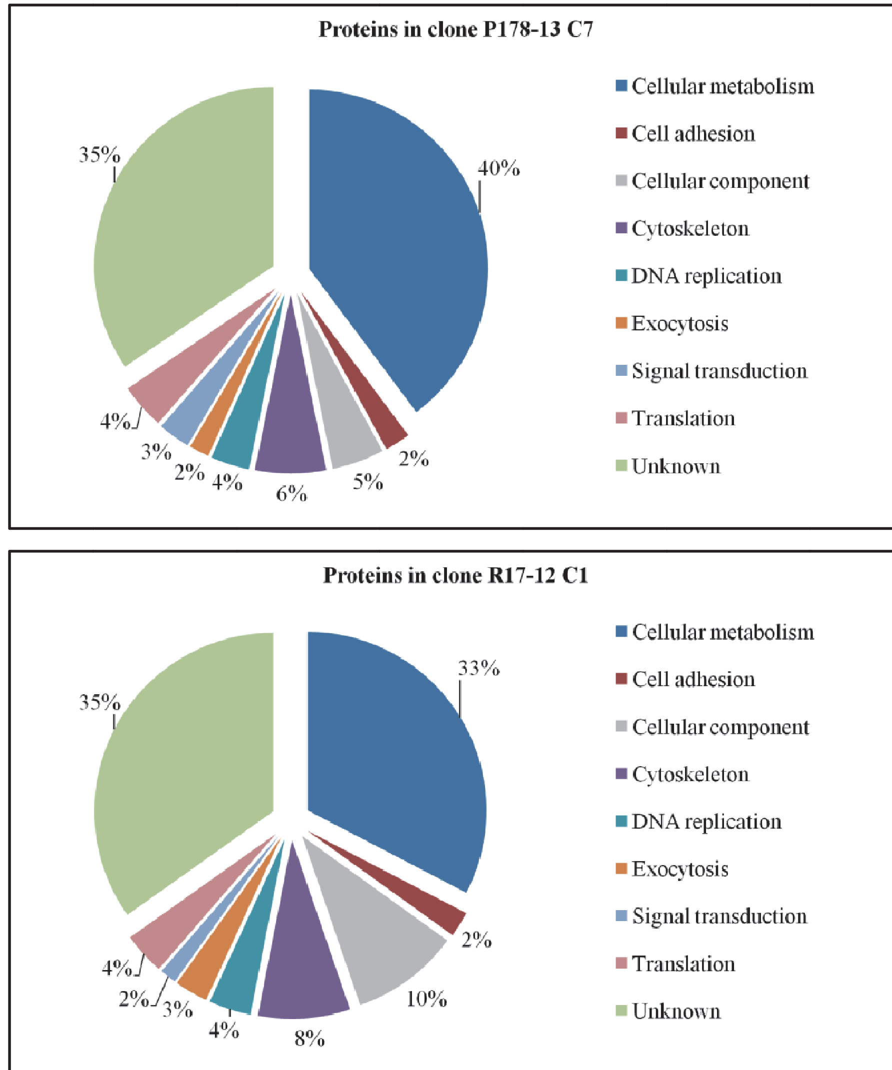


Figure 35. Graph with the percentage of relatively quantified proteins, according to their subcellular location, that were more abundant in clone P178-13 C7 and clone R17-12 C1, respectively.

5. DISCUSSION

5.1. Presence of oropharyngeal trichomonads in wild birds

The latest emergence of an epidemic avian trichomonosis in wild finches raised the concerns about the negative impacts of this disease for wildlife and warranted the need of further investigations. The high levels of population decline detected in greenfinches (35%) and chaffinches (21%) could be influencing other species, such as birds of prey, with unpredicted consequences (Robinson *et al.*, 2010). Indeed, Chi *et al.* (2013) mentioned that a significant population decline has been observed in sparrowhawks of the UK since 2006, the same year of the first report of the finch epidemic. However, not only birds of prey would be affected, other columbiformes and passeriformes will be at risk as well. Furthermore, domestic birds reared in farms with a low biosecurity control, where wild birds such as columbiformes or passeriformes gain access to water bowls and feeding pens, could get infected easily.

The fact that samples are collected from wild birds implies that these may be non homogeneous. The desirable condition of a random selection of individuals was not fulfilled in this case due to the biological characteristics of the avian species considered and the different possibilities for the capture and handling of the birds. Consequently, the values of prevalence should not be interpreted as a precise indicator of the population in the nature. Furthermore, most studies that involve wild species imply accepting this restriction. Nevertheless, valuable information was obtained in spite of this condition.

Additionally, the genetic characterization of avian oropharyngeal trichomonads has been an interesting field of research as cryptic genotypes and even new species, *T. stableri* as an example, have been recently described (Girard *et al.*, 2013).

At the present study, trichomonad isolates from the oropharyngeal cavity of wild birds were recovered by culture. This method was selected, as it has been proved to be the gold standard for the diagnosis of infection caused by *T. gallinae* (Cover *et al.*, 1994; Bunbury *et al.*, 2005).

TYM medium was successfully used for the cultivation of *T. gallinae* by Diamond (1954), Sansano-Maestre *et al.* (2009) and Amin *et al.* (2010). This rich liquid medium favoured the multiplication of the parasite, with an average incubation time for the detection of growth of 48 hours. However, some isolates needed a prolonged period, of 7-10 days in order to detect active trophozoite movement. This feature was detected mainly in cultures from necrophagous birds of prey that also demonstrated a smaller cell size during the examination at optical microscopy, and later, by morphometric analysis.

The highest percentage of infection was found in columbiformes, the primary host of the parasite. European turtle doves (100%, 3/3), rock pigeons (79.4%, 27/34), wood pigeons (70%, 7/10) and Eurasian collared doves (57.8%, 167/289) were the species with the maximum values. These results are in accordance with other studies, such as the one carried out by Sansano-Maestre *et al.* (2009), where 41% of wild rock pigeons of the Valencian Community (Spain), were positive to culture. Also, Lennon *et al.* (2013) found 86% of European turtle doves, 86% of Eurasian collared doves and 47% of wood pigeons infected with the parasite in the UK. Later, Stockdale *et al.* (2015) reported 100% positive European turtle doves from 25 wild animals sampled next to farms from the same country. Columbiformes are known to act as the reservoir host of the parasite; consequently, we have detected high levels of infected individuals in our study. In addition, low numbers of birds with lesions were obtained in Eurasian collared doves (1%), the species of columbiformes with the highest number of birds sampled (289). The high infection rates and low abundance of gross lesions found in Eurasian collared doves reflect the enzootic character of the infection in this species, acting as reservoir host of the parasite. Previous studies from rock pigeons of the Valencian Community (Spain), demonstrated the same findings, with only one rock pigeon (0.37%) with macroscopical lesions of avian trichomonosis from a total of 612 birds examined (Sansano-Maestre *et al.,* 2009).

Similarly, in the group of birds of prey, goshawks, a species with an ornitophagous diet, had the highest values of prevalence of infection (74.5%, 41/55) and lesions were not detected. Cooper and Petty (1988) reported the impact of avian trichomonosis in an upland goshawk population of Great Britain. Mortality associated with this infection was up to 39%, affecting nestlings and fledglings. The infection route of raptors was by consumption of infected columbiformes, the main prey for goshawks. Negative consequences on the expansion and increase of the raptor population were suspected and avian trichomonosis was designated as the most important mortality factor for young birds. However, in our study area, birds with lesions were not detected. In the case of chicks, we sampled 39 animals directly at the nest in the region of Murcia. Our findings may reflect a host-parasite adaptation or the presence of non pathogenic strains of the parasite. Krone *et al.* (2005) found the same pattern of infection and gross lesions in goshawk nestlings from Germany. They reported a higher infection rate (65.1%) with low numbers of nestlings presenting avian trichomonosis (4.5%) from birds breeding close to urban areas. These birds fed mainly on columbiformes and the infection rate increased with age in chicks.

Other authors have found similar infection rates, but a higher presence of lesions. Wieliczko *et al.* (2003) studied goshawk nestlings from Wroclaw (Poland) and also found that the prevalence increased with age. Indeed, all birds tested positive at 35-40 days of age. However, a higher rate of gross lesions was recorded (22.2%). The genotype of the parasite may be influencing this difference in the percentage of nestlings affected by avian trichomonosis, but no sequence data was reported in this study. In our case, 86.7% (39/45) of goshawk nestlings with 30-32 days were infected, while only 20% (2/10) of adults and juveniles were positive to culture.

It seems that the age of the bird is a determinant factor for infection in young animals. Urban and Mannan (2014) also found differences in the prevalence rate according to the age of the bird, but included measurements of the oral pH in Cooper's hawks. Authors reported that older animals, adults and fledglings of 55-120 days old, were not infected, whilst 16% of 14-28 day old nestlings harboured the protozoan. Additionally, higher levels of acidity at oral pH of older animals demonstrated to reduce the risk for the establishment of the infection. A physiological explanation for this change of pH

conditions at the oropharyngeal cavity of the birds as they matured was suggested. Obviously, the immune response of the bird, including passive maternal immunity, in addition to the physiological changes according to the age, would be also principal host determinants of the susceptibility to infection.

Kunca *et al.* (2015) reported differences in prevalence according to the nest location of sparrowhawks, another ornitophagous species. Higher levels of infection were detected in birds breeding in urban areas compared with birds in rural areas (32.9% *vs.* 12.2%). Columbiformes were identified at their feeding places. During the first year of study, 2012, few nestlings displayed gross lesions at the oropharyngeal cavity. However, all infected nestlings of 2013 had avian trichomonosis. According to the authors, it was probably due to the expansion of the finch trichomonosis epidemic from the UK and Fennoscandia through central Europe, as the same genotype was detected. During our study, outbreaks were not detected in the sampling area for any of the avian species included.

A special consideration would be done with lesser kestrels from the captive breeding center of DEMA (Defensa y Estudio del Medio Ambiente, Almendralejo, Badajoz, Spain). These animals were the main colony for the reintroduction of this species in Bulgaria, integrants of the European project LIFE11 NAT/BG/000360: "lesser kestrel recovery". Surprisingly, 7.8% of the birds were found positive to culture (11 of 141) without gross lesions. The parasite was probably present on wild chicks without lesions and clinical symptoms that were rehabilitated without testing for the parasite and introduced into the colony. A proper treatment with carnidazole at 50 mg/kg PO, which was repeated after one week, was administered.

Remarkably, the present study added new genotypic data from strains isolated from sixteen wild avian host species without previous reports: common buzzards, Egyptian vultures, Eurasian eagle-owls, Eurasian scops owls, European honey-buzzards, European turtle doves, goshawks, hen harriers, lesser kestrels, long-eared owls, magpies, marsh harriers, peregrine falcons, sparrowhawks, tawny owls and wood pigeons. Overall, these results indicate that many wild bird species are susceptible hosts for the parasite in the nature, some of them being first descriptions. These findings

reveal the importance of investigating the epidemiology of avian trichomonosis to understand the disease, including the potential consequences of this emerging agent for domestic species of birds.

Two multicopy ribosomal genetic markers, the ITS1/5.8S rRNA/ITS2 and small subunit of rRNA, and a single-copy gene, the Fe-hydrogenase, were the selected loci for the study. Their validity for the phylogenetic classification of trichomonad parasites has been proved in several studies (Felleisen, 1997; Kleina *et al.*, 2004; Gaspar da Silva *et al.*, 2007; Gerhold *et al.*, 2008; Grabensteiner *et al.*, 2010; Lawson *et al.*, 2011; Chi *et al.*, 2013; Ganas *et al.*, 2014; McBurney *et al.*, 2015).

For the ITS1/5.8S rRNA/ITS2 sequence, a remarkable diversity was detected in Spain with seven genotypes of the parasite, three of them being new reports (*T. canistomae*-like-1, *T. canistomae*-like-2 and *T. tenax*-like-2) and also mixed infections (Martínez-Herrero *et al.*, 2014). Our results are in contrast with the findings of Sansano-Maestre *et al.* (2009), who found only two genotypes (A and B) widely distributed among columbiformes and birds of prey in the Valencian Community (Spain). Nevertheless, those genotypes were also identified in this study and again, were the most prevalent in raptors and columbiformes. As several different genotypes have been described around the world, a new nomenclature system was used in order to try to group the different variants and codes used by other authors. *T. gallinae*-1 corresponds with genotype B of Sansano-Maestre *et al.*, (2009) and *T. gallinae*-2 with genotype A of Sansano-Maestre *et al.*, (2009) (Martínez-Herrero *et al.*, 2014). Chi *et al.* (2013) also identified those genotypes as the most prevalent among wild columbiformes and raptors from the UK. In the Canadian Maritime provinces, McBurney *et al.* (2015) found the same genetic types in columbiformes and finches. Genotype *T. gallinae*-1 was detected more frequently in birds of prey, while genotype *T. gallinae*-2 was predominant in columbiformes, results in agreement with the findings of Sansano-Maestre *et al.* (2009) and Chi *et al.* (2013).

Regarding the presence of gross lesions compatible with avian trichomonosis, genotype *T. gallinae*-1 was detected in 75.7% (28/37) of these animals, 13.5% (5/37) with genotype *T. gallinae*-2 and 10.8% (4/37) with mixed infections (*T. gallinae*-1+*T.*

gallinae-2). These findings confirm the results of Sansano-Maestre *et al.* (2009), who found a significantly higher presence of genotype *T. gallinae*-1 in birds with gross lesions of avian trichomonosis. McBurney *et al.* (2015) also found similar results. However, both authors noted that this genotype was isolated from healthy birds as well. Animals with lesions harbouring genotype *T. gallinae*-2 have been also identified in two racing pigeons of Austria (genotypes II and III of Grabensteiner *et al.*, 2010) and a small proportion (9.3%, 4/43) of wild columbiformes from the UK by Chi *et al.* (2013). Mixed infections of both genotypes were detected in a lower percentage of birds (6%, 5/83) and other authors also reported this finding (Sansano-Maestre, 2009; Grabensteiner *et al.*, 2010).

The three new genotypes detected (*T. canistomae*-like-1, *T. canistomae*-like-2 and *T. tenax*-like-2) were related to particular avian host species. *T. canistomae*-like-1 and *T. tenax*-like-2 appeared on European turtle doves from Valencia province (Spain) and *T. canistomae*-like-2 on goshawk nestlings from the region of Murcia (Spain). Also, *T. vaginalis*-like-5 was associated with particular host species, Egyptian and cinereous vultures from different Spanish locations. *T. canistomae*-like-1 and *T. canistomae*-like-2 were described for the first time. In contrast, *T. vaginalis*-like-5 was previously identified by two authors. Grabensteiner *et al.* (2010) reported a *Trichomonas* sp. from a bearded vulture without clinical signs from the Czech Republic. Later, Kelly-Clark *et al.* (2013) found the same strain in a bald eagle without clinical signs in Canada. This strain led later to the description of a new trichomonad species, *T. gypaetinii*, by Martínez-Díaz *et al.* (2015). The strain was found in necrophagous birds of prey in Spain, Egyptian and cinereous vultures, and it was not associated with clinical signs of disease.

Genotype *T. gallinae*-1 and the non-strict ornithophagous diet of birds of prey were identified as major risk factors for the presence of gross lesions. The influence of genotype on the clinical presentation of the birds was previously reported by Sansano-Maestre *et al.* (2009). This finding also agrees with the results of Chi *et al.* (2013) and McBurney *et al.* (2015). The higher susceptibility related to the feeding habits of birds of prey indicates that the number of re-infections is a major factor for the development of lesions and may reflect the generation of local, not persistent, protective immunity.

Similarly, *T. foetus* does not generate and an adequate immune response in bulls, which become chronic carriers of the organism (Cobo *et al.*, 2011). A comparable situation occurs in the human pathogen *T. vaginalis*, where the local immune response of vaginal epithelial cells in infected women is not sufficient for the clearance of the infection (Chapwanya *et al.*, 2016).

Oropharyngeal trichomonad parasites in Bonelli's eagle

The European project LIFE12 NAT/ES/000701: "integral recovery of Bonelli's eagle population in Spain" and other national conservation projects permitted the analysis of 98 samples of adults and chicks of this emblematic species. Overall, 28.6% (28/98) of the birds were found positive to culture, with a higher prevalence in nestlings sampled in the field (48.6%, 18/37).

Real *et al.* (2000) found lower values of prevalence (36%) in chicks from Catalonia, Spain. Nestling mortality attributable to this parasitic disease was of 22%, a considerable amount that pointed out the necessity to monitor the infection. Other studies have reported similar values of infection in nestlings from southern Portugal (50%) and the Valencian Community (52.4%) in Spain (Höfle *et al.*, 2000; Sansano-Maestre *et al.*, 2009). The dietary inclusion of infected columbiformes was the cause of the results found in Portugal, which consisted of more than one third of their diet. In the Valencian Community, the same scenario was present. Moreover, due to conflicts with predation of birds of prey on racing pigeons, barrier lofts with pigeons were established to mitigate this problem (Sansano-Maestre *et al.*, 2009; Law 10/2002, of 12th of December, BOE of 10.01.2003, Spain).

Regarding the presence of lesions, 23 nestlings were found with lesions at the oropharyngeal cavity compatible with early to late stage avian trichomonosis (grade 1 to grade 3). Birds with lesions and a positive result to *T. gallinae* infection were mainly of grade 1 (n= 8), while grade 2 and 3 accounted for few animals (n= 1, n= 3, respectively). However, one animal with lesions of grade 1, two of grade 2 and one of grade 3, had negative culture and PCR. As it has been noted before, lesions of grade 1 and 2 could be originated by another agent, as there are many differential diagnosis for these types of lesion classification grades. For the animal with lesions of grade 3, maybe

the oropharyngeal swab did not recover any trophozoite from the peripheral areas of the lesion, being not present at the superficial mucosa but in deeper layers of the tissue.

Positive Bonelli's eagle nestlings were found infected with the ITS1/5.8S rRNA/ITS2 genotypes *T. gallinae*-1 or *T. gallinae*-2, with also two mixed infections of both genotypes. The same genotypes were found previously by Sansano-Maestre *et al.* (2009) in this host species from eastern Spain. The association of genotype *T. gallinae*-1 with gross lesions was also observed, being this genotype isolated from all the birds with lesions and from one without lesions.

The addition of the small subunit of rRNA and Fe-hydrogenase gene sequence analysis, indicated that genotypes isolated from birds of prey, galliformes and psittaciformes were also present in this species and an association between genotype ITS1/5.8S rRNA/ITS2, the small subunit of rRNA and Fe-hydrogenase subtype was found. Actually, genotype *T. gallinae*-1 of the ITS1/5.8S rRNA/ITS2 corresponded to genotype VI (Grabensteiner *et al.*, 2010) of small subunit of rRNA and Fe-hydrogenase subtype A (A1 or A2; Chi *et al.*, 2013); while genotype *T. gallinae*-2 to sequence type EU215373 (Gerhold *et al.*, 2008) and Fe-hydrogenase subtype C4 (Chi *et al.*, 2013). Similar findings were previously reported by Chi *et al.* (2013) in wild birds of the UK. In addition, a new subtype of the Fe-hydrogenase gene was detected, with one SNP.

The results of this study demonstrate the importance of surveillance and monitoring of trichomonosis in this endangered raptor species, due to the existence of pathogenic strains among the population and its negative impact on nestling survival.

Oropharyngeal trichomonad parasites in European turtle doves

A total of seven genotypes, plus mixed infections for the small subunit of rRNA and Fe-hydrogenase loci, were detected from European turtle doves after MLST analysis. Five of them were new reports: two of the ITS1/5.8S rRNA/ITS2 region (*T. canistomae*-like-1 and *T. tenax*-like-2), one of the small subunit of rRNA (novel-1) and two of the Fe-hydrogenase gene (novel-1 and novel-2). Nomenclature guidelines for new genotypes of

the small subunit of rRNA and Fe-hydrogenase gene have not been established yet, therefore they were named with the word "novel" plus numbers.

For the ITS1/5.8S rRNA/ITS2 fragment, genotype *Trichomonas* sp.-1 has been previously found in racing pigeons of Austria (sequence type III of Grabensteiner *et al.*, 2010) and *T. canistomae*-like-1 had maximum identity with the *T. canistomae*-like-2 trichomonad organism identified from goshawks in this study. Finally, genotype *T. tenax*-like-1 had 99% sequence identity (1 base pair different) with a *T. tenax*-like strain found by Jiang *et al.* (2016) in domestic pigeons used for human consumption from Chinese farms.

The small subunit of rRNA revealed genotype I, described in racing pigeons by Grabensteiner *et al.* (2010), two mixed infections and the novel genotype 1. This latter genetic type had 99% identity with other *T. gallinae* strains from columbiformes and birds of prey (Gerhold *et al.*, 2008; Martínez-Díaz *et al.*, 2015).

The Fe-hydrogenase marker revealed two novel types (novel-1 and 2) and mixed infections. For the genotype coded as novel-1, only 98% BLAST identity was achieved with a *T. gallinae* sequence obtained from a columbiforme (Lawson *et al.*, 2011). Genotype novel-2 obtained a 96% sequence similarity with the C5 subtype of *T. gallinae* found in a Bonelli's eagle and rock pigeons (Sansano-Maestre *et al.*, 2016).

Overall, the phylogenetic analysis supported a clear distinction of *T. gallinae* organisms from other trichomonad species for the small subunit of rRNA and Fe-hydrogenase genes. The ITS1/5.8S rRNA/ITS2 phylogenetic tree showed that one *T. gallinae* sequence clustered also with a *Trichomonas* sp. organism. Actually, this finding will presumably correspond with a different criteria used by authors, as the mentioned sequence (KC529665, Chi *et al.*, 2013) differed only in two nucleotides (99% BLAST identity) from the genotype described as *Trichomonas* sp. (FN433473, Grabensteiner *et al.*, 2010), but they classified it as *T. gallinae*.

The sequences of oropharyngeal trichomonads from European turtle doves indicated that higher similarity was found with *T. canistomae*, *T. tenax* and *Trichomonas* sp.

organisms than with *T. gallinae* for the ITS1/5.8S rRNA/ITS2 region and the small subunit of rRNA. For the Fe-hydrogenase, a lower percentage of identity was obtained, due to the lower amount of sequences available in public databases. The absence of data of this last target from other trichomonad species was also remarkable.

Lennon *et al.* (2013) detected ITS1/5.8S rRNA/ITS2 genotypes *T. gallinae*-1, *T. gallinae*-2 and a *T. tenax*-like strain in European turtle doves without clinical signs from the UK. Another study carried out with this avian species found clinical signs of trichomonosis in adult birds from the same country (Stockdale *et al.*, 2014). The genotypic characterization of the isolates indicated the presence of *T. gallinae*-1, *T. gallinae*-2 and two *Trichomonas* sp. genotypes at the ITS1/5.8S rRNA/ITS2 region. Birds with clinical signs were infected with genotype *T. gallinae*-1. *Trichomonas* sp. genotypes were similar to *T. tenax* and a strain isolated from a dog. These results are in accordance with the *T. tenax*-like and *T. canistomae*-like genotypes found in the present study.

To conclude, potentially new species of oropharyngeal trichomonads were detected in this host species. None of them were associated with gross lesions in the birds. However, considering the low amount of samples, this condition would be only presumptive information. Further investigations would be required to unravel the genetic diversity of the parasite in this migrant columbiforme. International concerns have been declared for the conservation of this species, as a substantial decline of 73% from 1980 to 2010 has been documented in Europe (Stockdale *et al.*, 2014). Indeed, the species is catalogued as vulnerable at the IUCN red list of threatened species (IUCN, 2015). Moreover, their migration to Africa and South Asia could be a risk for the introduction of different genotypes of the parasite in Europe and vice versa.

5.2. Proposed classification of lesions compatible with avian trichomonosis

Order Columbiformes

Lesions in reservoir hosts of the parasite have been described at the oropharyngeal cavity, upper gastrointestinal tract (crop, oesophagus) and internal organs (liver, lungs, pancreas, etc.). We have detected lesions in these locations as well, mainly at the oropharyngeal cavity but also at the crop, oesophagus, orbital sinuses and liver.

Eurasian collared doves, a species that has recently increased its population size and geographical range in western Europe and America, was found with gross lesions of avian trichomonosis as described for other columbiform hosts, such as rock pigeons (Stabler and Engley, 1946; Pérez-Mesa *et al.*, 1961) or wood pigeons (Höfle *et al.*, 2004; Lennon *et al.*, 2013). This finding confirms that this species is also susceptible to the mortality caused by pathogenic strains of the parasite, as Stimmelmayr *et al.* (2012) reported from an outbreak in the Caribbean.

Orders Accipitriformes and Falconiformes

The subset of birds examined from orders Accipitriformes and Falconiformes included anatomical locations of lesions already described by several authors in diurnal birds of prey (Samour, 2000; Real *et al.*, 2000; Samour and Naldo, 2003; Krone *et al.*, 2005; Labrut *et al.*, 2012).

Lesions of grade 3 were the unique category evaluated in Falconiformes and treatment was required as soon as possible to recover the birds. Samour and Naldo (2003) described two types of clinical presentations with lesions on this group of birds: a nodular and membranous type. The first one was applied to caseous growths of necrotic masses that protruded through the surrounding tissue, while the second one corresponded to fibrinonecrotic material with the shape of diffuse plaques. In this study, we have also observed these two types of presentations.

Additionally, lesions were evaluated from species without previous reports in scientific literature. For instance, booted eagles, common buzzards, a marsh harrier and a red kite were positive to culture and had gross lesions of grades 2 and 3. These hosts are species not routinely reported with avian trichomonosis, presumably, due to their diet without the inclusion of infected preys.

Order Strigiformes

From the evaluated cases, all birds displayed gross lesions that involved the upper region of the oropharyngeal cavity (hard palate, choanal slit, infundibular cleft and/or tissues of the skull basis).

The same anatomical regions have been described to be affected with severe, extensive lesions in other nocturnal birds of prey (Jessup, 1980; Pokras *et al.*, 1993; Sansano-Maestre, 2009; Ecco *et al.*, 2012). In 1980, Jessup reported three cases of avian trichomonosis infections in great horned owls (*Bubo virginianus*) that reached the soft tissues and bones of the skull base. The parasite was sensitive only to higher dosages of dimetridazole. In this study, we have observed the same trend for the parasite locations in species belonging to the same order of birds.

The three carcasses in which a complete necropsy was performed did not show further internal lesions of avian trichomonosis. It could be possible that the absence of crop in nocturnal birds of prey implies that the parasite will primarily start its multiplication at the oropharyngeal cavity, extending to the hard palate and bones of the skull, instead of other organs. Certainly, the specific tissue pH would be another factor that influences the areas were the flagellated thrives (Urban and Mannan, 2014).

Lesions of grade 1 and 2 were found in 17 cases, with four of them being negative for *Trichomonas* sp. infection by culture. A proper diagnosis of the etiology of the process, as it has been mentioned, is essential here. In fact, three cases of goshawks with lesions of fibrinonecrotic material extended through the mucosa of the upper and lower regions of the oropharyngeal cavity (grade 3) were caused by capillarid nematodes, as it was revealed during the necropsy of the carcasses.

The importance of a correct diagnosis of lesions present at the oropharyngeal cavity was equally highlighted by Samour and Naldo (2003) in falconiformes. Moreover, these authors disclosed the diagnostic challenge of atypical infections of the parasite present in other internal locations: nasal cavity, infraorbital sinuses, oesophagus and/or tracheobronchial syrinx. Similarly, Anderson *et al.* (2010) indicated the necessity of an early detection to avoid the transmission of the parasite among finch admissions in wildlife recovery centers, as non classical signs of avian trichomonosis infection were found in these birds.

The classification system applied included an indication for the outcome of the bird. Namely, grade 2 required treatment to be administered as soon as possible as lesions

already have a multifocal distribution, while grade 3 implied an urgent veterinary evaluation due to extensive lesions with a poor general body condition and a high risk of death.

Finally, we would like to remark the importance of the evaluation of lesions in birds, as some relevant publications did not detail this information. The presence and description of lesions for this emergent protozoal disease is particularly valuable with the current scenario where new species, also present in co-infections with *T. gallinae,* and new hosts have been reported. This classification system relies on a visual inspection of the birds to determine the presence, extent and distribution of the lesions, providing a standard categorization that will help comparing data and unifying the criteria for the description of different types of lesions.

5.3. Characterization of new variants in avian oropharyngeal trichomonads

Clonal generation of cultures can be performed by two basic methodologies, serial dilution or micromanipulation. In the present study, micromanipulation was selected as it has been previously used for the obtention of *T. gallinae* clones with good results by Hess *et al.* (2006) and Grabensteiner *et al.* (2010). Moreover, a complete visual monitoring of the process is achieved by this technique and single cells are assured to be isolated for their clonal development.

The efficacy of single trichomonad cell growth and clonal culture establishment varied with each isolate (23-90%). This fluctuation could be explained by the different adaptation of strains to *in vitro* growth conditions. Indeed, differences on growth rate were detected between genotype *T. gallinae*-1 and *T. gallinae*-2 in TYM medium by Sansano-Maestre (2009), with genotype *T. gallinae*-1 showing a lower multiplication rate. For clonal cultures, isolates of genotype *T. gallinae*-1 obtained slightly lower efficacy (60-90%) than those of *T. gallinae*-2 genotype (80-90%). Isolates with mixed infections (*T. gallinae*-1 and *T. gallinae*-2) demonstrated even lower values (40-70%). Moreover, one isolate of genotype *T. canistomae*-like-1 had the lowest ones with only 23% of the cells successfully established. A difficult subculture and adaptation to the growth medium were also observed in this isolate during previous (isolates) and posterior (clones) culture of the parasite at the laboratory.

The genetic characterization of the clonal cultures revealed that the same genotype was detected in all the clonal cultures derived from the isolate, as it was expected. In the case of mixed infections, clones displayed only one of the genotypes that was present in the original isolate. These results are in contrast with the study of Granbensteiner *et al.* (2010), where two genotypes were detected in clonal cultures from isolates of clinically affected birds.

Our results could reflect the intrinsic limitations of the technique, as single trophozoites are selected randomly from a suspension. A small number of cells are used for the establishment of clonal cultures in comparison with the quantity of trophozoites present in the original isolate. Besides, the *in vitro* subcultivation at the laboratory could cause that particular genotypes prevail in the sample, due to their better adaptation to the culture medium.

All clonal cultures from the wood pigeon isolate P178-13 had one SNP at the ITS1/5.8S rRNA/ITS2 region (*T. gallinae*-2-1 SNP), a transition of two pyrimidines, a cytosine instead of a thymine. A similar genotypic variation has been detected in other protozoa parasites after *in vitro* culture. For instance, *Plasmodium falciparum* demonstrated modifications in the number of SNPs and microsatellite markers in short and long term cultures (Yeda *et al.*, 2016). Moreover, this type of DNA polymorphisms (SNPs) has also been detected in infected wild birds. Chi *et al.* (2013) found three different SNPs (A1.1, A1.2 and A1.3) of the subtype A1 from the Fe-hydrogenase locus in isolates from one sparrowhawk and two wood pigeons in the UK.

The MLST analysis of the four clonal cultures selected to study new genetic variants revealed three novel genotypes, one of the small subunit of rRNA (novel-1) and two of the Fe-hydrogenase gene (novel-2 and 3). The low BLAST identity observed on the new Fe-hydrogenase genotypes (97% and 95% homology for clone 7895-C2 and P196-13 C20, respectively) indicates that a higher subtyping capacity is obtained with this single-copy gene, in comparison with the ITS1/5.8S rRNA/ITS2 region, as it has been demonstrated by other authors (Lawson *et al.*, 2011; Chi *et al.*, 2013; Ganas *et al.*, 2014). In addition, the recent incorporation of this genetic marker to the study of avian oropharyngeal trichomonads implies that a lower number of sequences are available on

public databases in comparison with the ITS1/5.8S rRNA/ITS2 and small subunit of rRNA, and therefore, also explains these results.

Clone 7895-06 C2, obtained from an isolate recovered from a racing pigeon in Austria, presented a higher sequence homology with a *T. tenax* strain than with *T. gallinae* at the ITS1/5.8S rRNA/ITS2 fragment. Besides, the small subunit of rRNA revealed an identical nucleotide sequence with a *Trichomonas* sp. strain isolated from an Australian grey-capped Emerald dove (*Chalcophaps indica*). These results indicate that a new species of avian oropharyngeal trichomonad could be present. In fact, *T. tenax*-like strains have been described in columbiformes of Austria, the UK and more recently in China (Grabensteiner *et al.*, 2010; Lennon *et al.*, 2013; Jiang *et al.*, 2016).

The clone P196-13 C20, from an European turtle dove, also obtained higher BLAST identity with other trichomonad parasites than with *T. gallinae* at the ITS1/5.8SrRNA/ITS2 sequence. In this case, a *T. canistomae* isolate obtained the maximum score. The small subunit of rRNA had 99% identity with a *T. gallinae* strain from a common buzzard. *T. canistomae*-like strains were also described in goshawks from Spain (Martínez-Herrero *et al.*, 2014). The genetic evidence indicates that possible new species are present also in this particular host species, although more isolates need to be characterized in order to confirm the host distribution of these organisms.

The Diff-Quick modified stain provided an adequate flagellar and intracellular structure delimitation. The fast application was also an advantage against other methodologies. Martínez-Díaz *et al.* (2015) reported good results with this staining in *T. gallinae* and *T. gypaetinii* isolates.

Optical microscopy revealed significant statistical differences in clonal cell measurements. The possible new species of clone P196-13 C20 was clearly distinguished from *T. gallinae* clones (P178-13 C7 and R17-12 C1) and *T. tenax*-like clone 7895-06 C2 by its longer axostyle projection (average of 8.2 µm *vs*. 5.9-6.5 µm for the other clones). This criterion was also the distinctive trait observed in the recently described new species *T. gypaetinii*, although smaller cell dimensions were reported in comparison with *T. gallinae* (Martínez-Díaz *et al.*, 2015). In our case, clone P196-13

C20 had smaller trophozoite size values in comparison with the *T. gallinae* clone P178-13 C7, with statistical significance, but was not distinguishable from the *T. gallinae* clone R17-12 C1, which was smaller than other *T. gallinae* isolates or clones.

Smaller cell dimensions were detected in trophozoites of clone R17-12 C1 of ITS1/5.8S rRNA/ITS2 genotype *T. gallinae*-1 (length range of 6-10 µm and width range of 3-6 µm) while other authors reported mean values of 7-11 µm or 10.6 x 6.7 µm for clones and isolates of the same genotypic profile (Mehlhorn *et al.*, 2009; Martínez-Díaz *et al.*, 2015, respectively). We obtained a higher homogeneity in trophozoites cell dimensions, as our standard deviation of cell length and width was of 1 and 0.7 µm, in contrast with the larger values of 1.9 and 1.8 µm, respectively, reported by Martínez-Díaz *et al.* (2015). A more homogeneous sample would be expected from the use of clones instead of isolates. The clone with the largest trophozoite dimensions, P178-13 C7, obtained similar results to three isolates of the same ITS1/5.8SrRNA/ITS2 region (*T. gallinae*-2) from the study of Martínez-Díaz *et al.* (2015).

Girard *et al.* (2013) did not report such a wide range of measurements in *T. gallinae* or *T. stableri*, although they described two morphotypes of *T. stableri*, a slender and a rounded form. During our study, the use of clonal cultures could have selected one particular morphotype, if they were present in the original isolate, and slight variations would be expected in the morphology of the cells in comparison with parental cultures (isolates). It is also notable the characteristic trophozoite plasticity of trichomonads, as it has been observed by several authors with the use of either clones or isolates (Stabler, 1941; Petrin *et al.*, 1998; Tasca and De Carli, 2003; Mehlhorn *et al.*, 2009; Martínez-Díaz *et al.*, 2015).

Another factor that could be influencing the shape of the trophozoites is their adaptation to *in vitro* growth conditions. The smaller cell size observed in clones with ITS1/5.8S rRNA/ITS2 genotypes *T. gallinae*-1 (R17-12 C1) and *T. canistomae*-like (P196-13 C20) may reflect a poor adaptation of the parasite to the growth medium. A comparison of different culture media with the same clonal cultures would be desirable in order to test this hypothesis.

Scanning electron microscopy (SEM) showed superficial irregularities at the external membrane of all the clonal cultures. This fact has also been reported by other authors and would be attributable to the required preliminary protocol used for SEM sample preparation (Tasca and De Carli, 2003). This procedure involved the cell dehydration in acetones of increasing gradation and several centrifugation steps that will damage the plasmatic membrane of the protozoa.

Trophozoites had the classical pyriform or rounded shape, as it is described for *T. gallinae*, *T. stableri* and *T. gypaetinii* (Tasca and De Carli, 2003; Mehlhorn *et al.,* 2009; Girard *et al.*, 2013; Martínez-Díaz *et al.,* 2015). However, no differences in the structures were observed among the clones. These results are in accordance with the genetic and species identification of the parasite, as only one species, *T. gallinae*, was recognized during decades. Nevertheless, the use of molecular biology techniques for the genetic characterization of the strains and their morphological study revealed that novel species were present (*T. stableri* and *T. gypaetinii*; Girard *et al.*, 2013; Martínez-Díaz *et al.*, 2015). The genetic study of isolates has revealed the existence of cryptic species and variants in other parasitic protozoans. As examples, we can mention the genetic assemblages present in *Giardia duodenalis*, the species described in the last decade within the genus *Cryptosporidium*, the species of *E. histolytica* and *T. gallinarum*, the new species in the genus *Tritrichomonas* (*T. blagburni*) and the cryptic genotypes of *E. tenella* in chickens (Andrews *et al.*, 1998; Clark, 2000; Cepicka *et al.*, 2005; Frey and Müller, 2012; Walden *et al.*, 2013; Clark *et al.*, 2016; Kváč *et al.*, 2016).

These results indicate that a notable genetic and morphological diversity is still present in isolates from wild birds and further characterization of isolates from new variants is needed to describe the different strains and possible novel species.

5.4. Virulence assays of *T. gallinae* clonal cultures in LMH cells

In vitro virulence analysis was performed using chicken hepatocytes, the LMH cell line, as substrate. This cell line derived from a hepatocellular carcinoma of a domestic chicken.

The use of cells to assess the pathogenic potential of clinical isolates of *T. gallinae* was demonstrated as a powerful tool by different authors. Honigberg *et al.* (1964) used trypsin-dispersed chicken liver cells to test one virulent *T. gallinae* strain (Jones' Barn) and a non-virulent one (Lahore), finding significant different outcomes. Kulda (1967) tested *T. gallinae* in kidney cells of monkeys and reported cell death due to the multiplication of protozoans. In recent years, Amin *et al.* (2012a and b) validated the use of LMH cells for the *in vitro* evaluation of the cytophatogenic capacity of *T. gallinae* and *T. gallinarum* clones. The authors reported that LMH cells were more suitable for this purpose than quail fibroblasts (cell line QT35), which were also tested in the study. The liver is one of the registered target organs in *T. gallinae* infections, where hepatotrophic strains have been described, such as the Eiberg and Jones' Barn strains.

The use of an *in vitro* methodology to categorize the pathogenic capacity of isolates offers obvious advantages in comparison with animal trials. Firstly, more convenient and reproducible experiments can be achieved, as new variables from individual animals are eliminated. For example, intrinsic factors, such as the different immune response developed by the animals will add further variation to experimental infections if it is not properly evaluated. Besides, a validated methodology would be easily reproduced by other laboratories and could be incorporated as a routine analysis in the characterization of avian trichomonads.

The tested clonal cultures revealed differences in the cytophatogenic score used to evaluate the damage on the LMH cell monolayer. These differences were detected at 24 and 48 hours post-infection, as at 72 hours post-infection a complete monolayer destruction was observed independently of the clonal culture considered. Clones with ITS1/5.8S rRNA/ITS2 genotype *T. gallinae*-2 had lower pathogenic scores that clones of genotype *T. gallinae*-1 with statistical significante (Chi-square test, p-value < 0.01). These findings are in accordance with the clinical evaluation of the birds, as two from the three clonal cultures derived from birds with lesions revealed complete monolayer destruction (CPE score 4) at 24 hours post-infection corresponding with isolates from lesions of grade 3 (P349-12, R17-12), while the third one, which was obtained from an animal with lesions of grade 2 (R193-13), reached this value at 48 hours post-infection.

Amin *et al.* (2012a) also described variations in the degree of monolayer destruction by *T. gallinae* clones of different ITS1/5.8S rRNA/ITS2 genotype. These authors reported that 50% of the cell monolayer was destroyed by one clonal culture originated from a racing pigeon with gross lesions of avian trichomonosis (clone 8855-06 C3, corresponding with genotype *T. gallinae*-2). A complete detachment was observed in clones from a budgerigar with clinical signs of disease (clone 5895-06 C1, genotype *T. gallinae*-1) and another racing pigeon with lesions (clone 231-07 C1 of genotype *Trichomonas* sp-1). Our results also corroborate their findings as the four clones tested of genotype *T. gallinae*-1 obtained the highest cytopathogenic scores with complete monolayer detachment, whilst the three *T. gallinae*-2 clones had lower scores during the first two days of incubation, although a complete detachment was detected in one clone of this group at 48 hours post-infection.

In order to evaluate the interaction with LMH cells of the clonal cultures that have been employed, a lower amount of trophozoites would be desirable as initial inoculum. This modification would probably slow down the multiplication of the parasite and the cytopathogenic process would be more easily inspected, with a better differentiation among the clones.

In conclusion, a substantial increase in trophozoite growth was observed on flasks with LMH cells in comparison with their controls. In fact, maximum growth was reached one day before than controls in 71.4% (5/7) of the clones. Consequently, the presence of cells favor the growth of the parasite and this *in vitro* methodology provides a convenient system to evaluate the virulence of clinical isolates in order to use them for further applications. Although a strong pathogenicity was detected in all the clonal cultures tested on day five post-infection, significant differences were found on day one and two post-infection that were related to the ITS1/5.8S rRNA/ITS2 genotype of the clones.

5.5. Analysis of membrane proteins in two *T. gallinae* clonal cultures with different genotype by RP-LC-MS/MS

The plasmatic and organelle membrane fraction from two *T. gallinae* clones of the most commonly distributed ITS1/5.8S rRNA/ITS2 genotypes, *T. gallinae*-1 and *T.*

gallinae-2, was analyzed for the identification and relative quantification of proteins (Sansano-Maestre *et al.*, 2009; Chi *et al.*, 2013; Martínez-Herrero *et al.*, 2014). This fraction was selected due to the potential role of membrane proteins in the process of trophozoite adhesion to the host cells, an essential phase for the establishment of infection demonstrated in other extracellular protozoa, such as *Giardia duodenalis* and *E. histolytica* (Hernández-Sánchez *et al.*, 2008; Hernández *et al.*, 2014; Emmanuel *et al.*, 2015).

Proteins involved in diverse processes, comprising cellular adhesion and metabolism, cytoskeleton, organelle membranes, DNA replication, exocytosis, signal transduction and translation were identified in both clones with significant differential expression.

The subcellular compartment of these proteins was expected to be mainly from the plasmatic and organelle membranes. However, the majority of proteins were not assigned to any particular location (73% in clone P178-13 and 62% in clone R17-12). In fact, the targeted proteome, which comprised plasmatic and organelle membrane proteins, accounted for 17% and 14% of the relatively quantified proteins of clone R17-12 and P178-13, respectively.

The presence of proteins from other locations has been reported in similar proteomic studies. For instance, de Miguel *et al.* (2010) also found cytoskeletal proteins in surface proteome experiments of *T. vaginalis* and their presence was related to their close association with the trophozoite plasmatic membrane. Moreover, moonlighting proteins, i. e. proteins with diverse functions that could be present in different cell locations, may contribute to the observed results (Huberts *et al.*, 2010; Khan *et al.*, 2014). Indeed, aldolases have been identified as moonlighting proteins in *Plasmodium vivax* with glycolytic and host-cell invasion functions (Bosch *et al.*, 2007). Two types of aldolases were also differentially expressed with more abundance in clone P178-13 (see appendix II).

Proteins with relevant predicted biological functions that could be considered as virulence factors or involved in the parasite pathogenesis will be discussed below.

Proteins grouped by function that differed in abundance between the clones

Extracellular vesicles: exosomes and microvesicles

Extracellular vesicles have been identified as virulence factors and modulators of the host's immune response in several protozoa (*Leishmania* spp., *Plasmodium falciparum*, *Toxoplasma gondii*, *Trypanosoma brucei, Trypanosoma cruzi*, *T. vaginalis*; Cronemberger-Andrade *et al.*, 2014; Mantel and Marti, 2014; Atayde *et al.*, 2015; Mugnier *et al.*, 2016; Szempruch *et al.*, 2016a and b). In *T. vaginalis*, highly antigenic exosomes attach to the host epithelium and modulate the immune response of the cells, inducing cytokine expression. Noteworthy, cellular invasion was also exosome-dependent as microvesicles from adherent strains increased the adhesion of low adherent ones (Twu *et al.*, 2013). The significant presence of proteins with exocytic function, three in clone P178-13 and four in clone R17-12, reveals that this virulence factor is likely to be also present in *T. gallinae*.

Peptidases

Several peptidases differed in abundance between both clones, although one of these proteins was an intracellular peptidase for clone R17-12. In reference to clone P178-13, two clan CA family C1 cathepsin L-like and two of family C19 ubiquitin hydrolase-like cysteine peptidases, one clan MH family M20 T-like metallopeptidase and one hypothetical protein with peptidase activity were found in higher abundance. For clone R17-12, one clan CA family C2 calpain-like and two family 19 ubiquitin hydrolase-like cysteine peptidases, one clan SB family S8 subtilisin-like serine peptidase were more abundantly detected.

Peptidases are essential metabolic cell proteins that have been identified as potential virulence factors and vaccine target molecules for pathogenic trichomonads as *T. vaginalis* and *T. gallinae* (Carlton *et al.*, 2007; Amin *et al.*, 2012b; Carvajal-Gamez *et al.*, 2014; Hernández *et al.*, 2014).

In *T. gallinae*, cysteine peptidases were identified in the parasite's secreted proteins on LMH cell cultures (Amin *et al.*, 2012b). Two clan CA family C1 cathepsin L-like cysteine peptidases were detected in parasite-free filtrates and four different genetic sequences were recognized. The authors demonstrated their activity in the process of

cell culture monolayer destruction. Thus, the virulence of particular strains seems to be determined by the delivery of these molecules to the extracellular environment. In the present study, several peptidases were found differentially expressed in both clones. From the two clan CA family C1 cysteine peptidases identified in clone P178-13, one of them corresponded to the *T. gallinae* peptidase CP4 described by Amin *et al.* (2012b). Therefore, the cytopathogenic character of the parasite was confirmed as these molecules were present in both clonal cultures of the present study, indicating their essential role in the regulation of host invasion and parasite proliferation.

BspA-like proteins

Bacteroides sp. surface A-like proteins (BspA) are known as virulence factors of mucosal bacteria involved in adherence to the host's cells. They have been characterized from human oral pathogenic bacteria (*Tannerella forsythia, Treponema denticola*) and streptococci (*Streptococcus agalactiae*) and seem to influence the host's immune response, their adhesion to oral tissues and coaggregation phenomena (Ikegami *et al.*, 2004; Inagaki *et al.*, 2005; Rego *et al.*, 2016). Coaggregation occurs when bacteria attach with one another through surface interactions and is believed to promote biofilm formation (Rickard *et al.*, 2003). Recently, BspA-like proteins have been identified in pathogenic protozoans, such as *T. vaginalis, E. histolytica* and *E. dispar* (Hirt *et al.*, 2007; de Miguel *et al.*, 2010; Noël *et al.*, 2010; Hirt, 2013; Kusdian and Gould, 2014). Moreover, it has been suggested that these proteins conduct important roles in *T. vaginalis*. Mucosal tissue invasion, host's innate immune response, parasite's immune evasion and chronification of the infections are some examples (Nöel *et al.*, 2010).

In this study, three BspA-like proteins were detected in *T. gallinae*, one of them more abundantly expressed in clone P178-13 (29.82 fold change) and two with a higher quantification in clone R17-12 (fold changes of 4.68 and 7.83).

Proteins more abundant in clone P178-13

Cytoskeletal proteins

One putative alpha-actinin more expressed in clone P178-13 (6.23 fold change) obtained a match with a *Histomonas meleagridis* sequence. This cytoskeletal protein has been recognized as an immunogenic component in *T. vaginalis* and *H. meleagridis*

(Addis *et al.*, 1999; Leberl *et al.*, 2010). Indeed, in *T. vaginalis* alpha-actinins are active molecules during the infection, involved in the conversion to the amoeboid stage and effective parasitic motility through the host mucosa. Furthermore, they have been associated with the cytopathogenicity of the strain (Fiori *et al.*, 1999; Kusdian *et al.*, 2013).

Proteins more abundant in clone R17-12

Cytoskeletal proteins

We have detected four dynein proteins with higher abundance in clone R17-12, that was isolated from a bird with lesions of avian trichomonosis and genotype *T. gallinae*-1 for the ITS1/5.8S rRNA/ITS2 region. These proteins are involved in a wide variety of cytoskeletal functions, like as examples: actin and microtubule organization, cell morphogenesis or flagellar activity. In addition, clone R17-12 had the erythrocyte membrane-associated giant antigen 332 protein more abundantly expressed (10.23 fold change) than clone P178-13. This protein is aslso related to microtubule organization. Other investigations with the pathogen *T. vaginalis* have indicated that cytoskeletal proteins such as actin, were differentially over expressed in virulent strains (Cuervo *et al*., 2008). It is also well known that the morphological changes underwent by *T. vaginalis*, from the trophozoite stage to the amoeboid form found attached to the host cells and the parasite migration through the tissue are based on actin reorganization (Kusdian *et al.*, 2013). Therefore, our results could be indirect markers of trophozoite virulence.

Malate dehydrogenase proteins

Two malate dehydrogenase proteins were significantly more expressed in clone R17-12 (fold changes of 3.46 and 6.54). In *T. vaginalis*, this enzyme has been detected more abundantly in a highly virulent fresh clinical isolate, in comparison with its long term culture (Cuervo *et al*., 2008). Furthermore, other authors have detected its binding to fibronectin in amoeboid forms of *T. vaginalis*, with a possible role in the adherence mechanism of the parasite (Huang *et al*., 2012).

Membrane proteins: immuno-dominant variable surface antigen-like, GP63-like and Ras protein

An immuno-dominant variable surface antigen-like protein was up-regulated in clone R17-12. This type of protein has been identified in other pathogenic protists, such as *E. histolytica* and *T. vaginalis* (Edman *et al.*, 1990; Carlton *et al.*, 2007). In *E. histolytica*, amino acid sequence divergence was found between pathogenic and non pathogenic strains and this protein was characterized as a potential diagnostic target for strain differentiation with restriction enzymes. Thus, these surface proteins which were found differentially expressed could be involved also in *T. gallinae* pathogenic mechanisms.

A GP63-like protein was also up-regulated in clone R17-12. This molecule is a zinc metalloproteinase of 63 kDa, being the most abundant surface glycoprotein of *Leishmania* sp. described as a leishmanolysin involved in the virulence mechanisms of the parasite (Joshi *et al.*, 1998; Yao *et al.*, 2003; Gomez *et al.*, 2009). Their function has been associated with the evasion of the complement-mediated lysis of recently inoculated promastigotes in the vertebrate host (Joshi *et al.*, 1998). Besides, this enzyme has been related to favour promastigote dermal migration by the destruction of fibronectin and collagen IV, that will facilitate their phagocytosis by macrophages and dissemination into other tissues (McGwire *et al.*, 2003). Recently, the production of exosomes in the parasite has been proved to be another virulence factor that modulates the immune response of the host, with transcription factors and protein tyrosine phosphatases regulated after macrophage invasion by GP63 (Atayde *et al.*, 2016). Furthermore, it has been used as a vaccine candidate with interesting results, capable of the activation of a cellular and humoral immune response in trials of visceral leishmaniasis in mice (Xu and Liew, 1995; Sachdeva *et al.*, 2009; Mazumder *et al.*, 2011a and b; Kaur *et al.*, 2016). First investigations in human patients revealed immunogenic properties of the protein with the stimulation of T-cells and humoral response (Nascimento *et al.*, 1990). Moreover, it has been evaluated for the diagnosis of cutaneous and visceral leishmaniasis in serum with good results in non-human primates and as a useful PCR target for *L. infantum* infections in dogs (Gicheru and Olobo, 1994; Guerbouj *et al.*, 2014). In *T. vaginalis* genome, more than 75 GP63-like proteins have been identified (Carlton *et al.*, 2007). One study discarded the role in trophozoite adhesion to HeLa cells of one *T. vaginalis* GP63 protein (Ma *et al.*, 2011). Besides,

GP63 proteins of *T. vaginalis* were found to cleave a protein kinase (mammalian target of rapamycin, mTOR) involved in cell growth, proliferation, motility, survival, protein synthesis and transcription using an experimental model with SiHa cells, a phenomenon that was also known for GP63 proteins of *Leishmania* spp. (Jaramillo *et al.*, 2011; Quan *et al.*, 2014).

Finally, a Ras (rat sarcome) protein of small guanosine triphosphatases (GTPases) was found more abundantly expressed in clone R17-12 (fold change 3.8). Membrane and cytoplasmic locations were referred for this protein with functions related to several signalling pathways, cell growth, proliferation and differentiation.

In summary, *T. gallinae* clonal cultures have exhibited distinctive virulence factors in the proteomic analysis. Most of the differentially expressed proteins had sequence identities with *T. vaginalis* proteins, although four of them obtained *T. gallinae* matches. The fewer proteomic studies carried out in *T. gallinae* in comparison with *T. vaginalis* explain this lower identification rate.

The analysis of parasitic surface proteins has demonstrated the expression of virulence factors on clonal cultures, providing valuable information for the understanding of the pathogenesis of the infection.

6. CONCLUSIONS

1. Oropharyngeal *Trichomonas* spp. infection was been detected in 23 of the 53 species analysed in this study. In 16 of those host species, genetic sequences of the parasite were reported for the first time.

2. Seven genotypes of the parasite, three of them being novel reports, have been detected for the ITS1/5.8S rRNA/ITS2 region (ITS). Genotypes *T. gallinae*-1 and *T. gallinae*-2 were widely distributed among wild birds, while the others were described in particular species: European turtle doves with genotypes *T. canistomae*-like-1, *T. tenax*-like-2 and *Trichomonas* sp.-1, goshawks with genotype *T. canistomae*-like-2 and Egyptian vultures with genotype *T. vaginalis*-like-5.

3. Risk factors for the development of lesions of avian trichomonosis have been identified: ITS genotype *T. gallinae*-1 for all the bird species analysed and non-strict ornithophagous diet in predator birds. The development of resistance to the disease seems to be influenced by the number of reinfections.

4. The MLST analysis of Bonelli's eagle isolates revealed that nestlings were infected with pathogenic genotypes of the parasite. The special monitoring avoided the death of a high number of birds. Trichomonosis is an important mortality cause for nestlings that must be evaluated in the conservation programmes of this endangered species.

5. European turtle doves harbour novel genotypes of the ITS, small subunit of rRNA and Fe-hydrogenase genes. This diversity indicates that they could be introducing new strains of the parasite due to its migratory behaviour.

6. A classification system of macroscopical lesions compatible with avian trichomonosis was implemented. Its incorporation into the evaluation process of the birds will facilitate the prognosis and provide a better understanding among professionals.

7. *In vitro* virulence assays of clonal cultures on LMH cells revealed a high pathogenicity of genotypes *T. gallinae*-1 and *T. gallinae*-2, although the first one demonstrated higher virulence at 24 and 48 hours post-infection. This fact agrees with the *in vivo* observations, where *T. gallinae*-1 genotype is isolated more frequently from animals with lesions.

8. A high number of proteins have been detected differentially expressed between *T. gallinae* clones with distinct genotype. Several peptidases, BspA-like proteins and proteins of the cytoskeleton involved in trophozoite movement, during their process of adhesion to the host cells and vesicle-mediated transport, and recognized as virulence factors for *T. vaginalis* and *T. gallinae,* were detected in both clones. Proteins like immuno-dominant variable surface antigen-like, GP63-like and a Ras protein, related with virulence mechanisms or with the chronic character of the infection in other protozoa, were found more abundantly expressed in the clone with genotype *T. gallinae*-1.

6. CONCLUSIONES

1. Se ha detectado la infección por *Trichomonas* spp. en cavidad orofaríngea en 23 de las 53 especies de aves muestreadas en este estudio. Además, se han publicado secuencias genéticas de este protozoo en 16 hospedadores por primera vez.

2. En la región ITS1/5.8S rRNA/ITS2 (ITS) se detectaron siete genotipos del parásito, tres de ellos nuevas descripciones. Los genotipos *T. gallinae*-1 y *T. gallinae*-2 estuvieron ampliamente distribuidos entre las diferentes especies de aves silvestres analizadas, mientras que el resto fueron aislados únicamente en ciertos hospedadores: *T. canistomae*-like-1, *T. tenax*-like-2 y *Trichomonas* sp.-1 en tórtolas europeas, *T. canistomae*-like-2 en azores y *T. vaginalis*-like-5 en alimoches.

3. Se han identificado factores de riesgo que favorecen el desarrollo de lesiones de tricomonosis aviar: la parasitación por el genotipo ITS *T. gallinae*-1 en todas las especies de aves estudiadas y una dieta ornitófaga no estricta en aves rapaces. El desarrollo de resistencia frente a la enfermedad parece estar relacionado con la frecuencia de reinfecciones.

4. El análisis MLST de aislados de águila perdicera demostró que los pollos estaban infectados con genotipos patógenos del parásito. Se evitó la muerte de un elevado número de aves gracias a las medidas especiales de seguimiento. La tricomonosis es una causa importante de mortalidad en pollos que debe ser evaluada en los programas de conservación de esta especie amenazada.

5. Las tórtolas europeas albergan nuevos genotipos de la región ITS, subunidad menor ribosomal y Fe-hidrogenasa. Esta diversidad refleja la posibilidad de que nuevos genotipos del parásito estén siendo introducidos, debido al carácter migratorio de la especie.

6. Se elaboró un sistema de clasificación de lesiones macroscópicas compatibles con tricomonosis aviar. Su incorporación dentro del proceso de evaluación de las aves facilitará la asignación de un pronóstico y mejorará el intercambio de información entre profesionales.

7. Los ensayos de virulencia *in vitro* con cultivos clonales en células LMH indicaron una elevada patogenicidad de los genotipos *T. gallinae*-1 y *T. gallinae*-2, si bien el primero de ellos demostró una mayor virulencia a las 24 y 48 horas post-infección. Este hecho concuerda con las observaciones *in vivo*, donde este genotipo se aísla más frecuentemente en aves con lesiones.

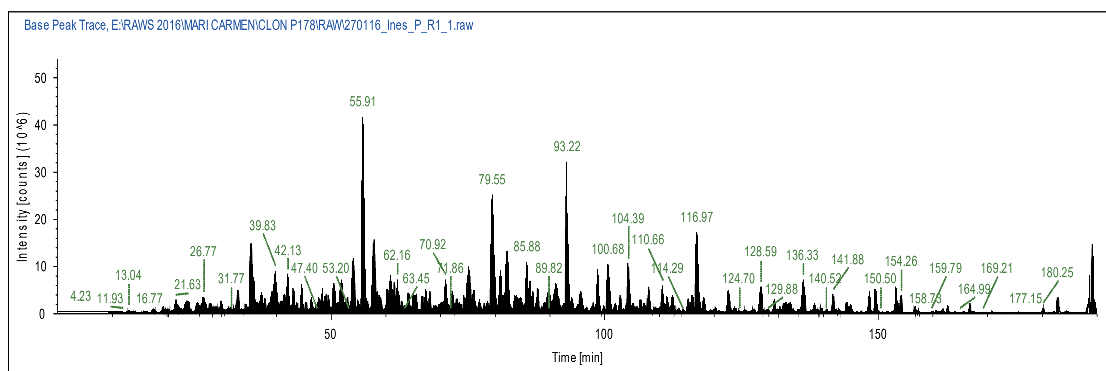
8. Se ha encontrado un elevado número de proteínas expresadas diferencialmente entre clones de *T. gallinae* con distinto genotipo. Varias peptidasas, BspA-like proteínas y proteínas del citoesqueleto involucradas en el movimiento de los trofozoítos, en procesos de adhesión y transporte mediado por vesículas, y reconocidas como factores de virulencia en *T. vaginalis* y *T. gallinae,* se detectaron en ambos clones. Proteínas como el antígeno inmuno-dominante variable de superficie-like, GP63-like y una proteína de la familia Ras, relacionadas con mecanismos de virulencia o con el carácter crónico de la infección en otros protozoos, se encontraron en mayor abundancia en el clon con genotipo *T. gallinae*-1.

APPENDICES

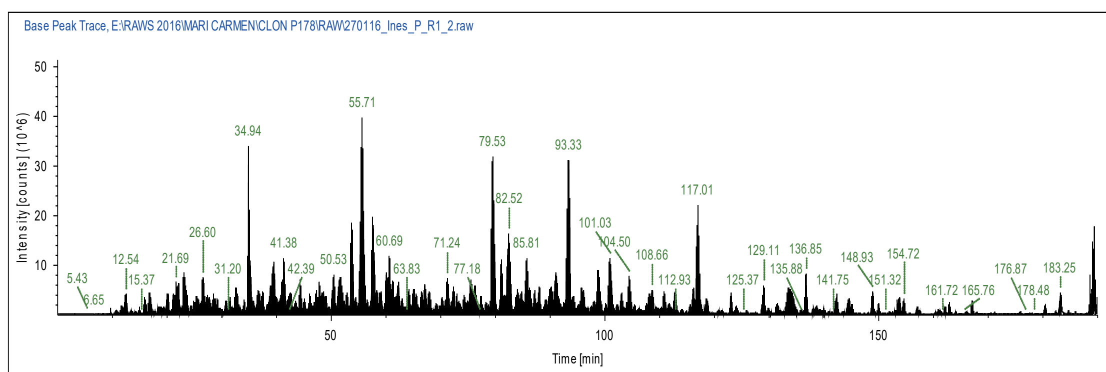
APPENDIX I – LC-MS/MS chromatograms from cell membrane and organelle membrane protein fraction of *T. gallinae* clonal cultures P178-13 C7 P11 and R17-12 C1 P9.

Clone P178-13 C7

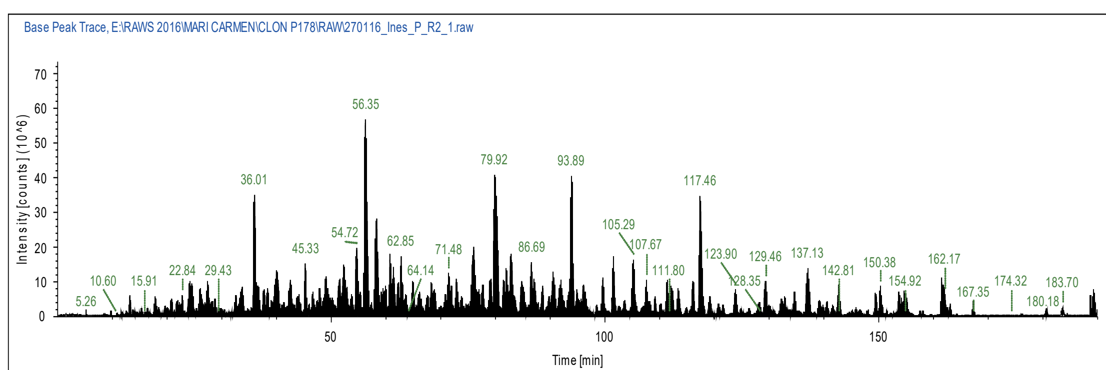
BIOLOGICAL REPLICATE 1 – TECHNICAL REPLICATE 1



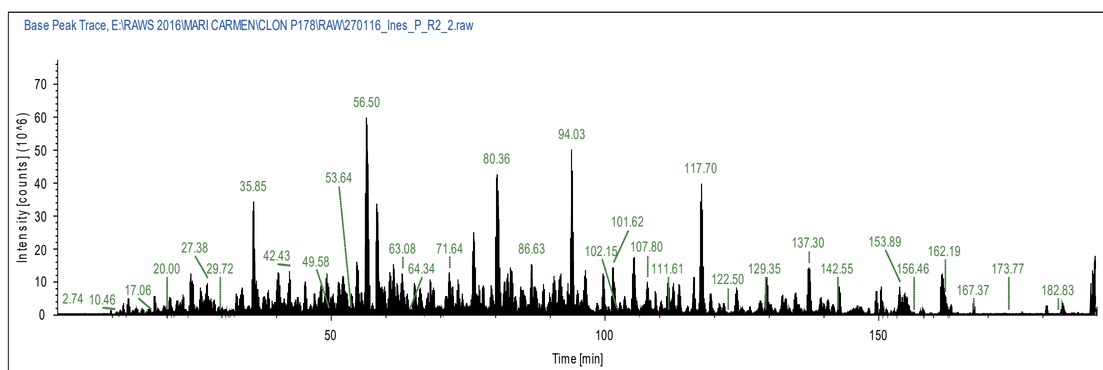
BIOLOGICAL REPLICATE 1 – TECHNICAL REPLICATE 2



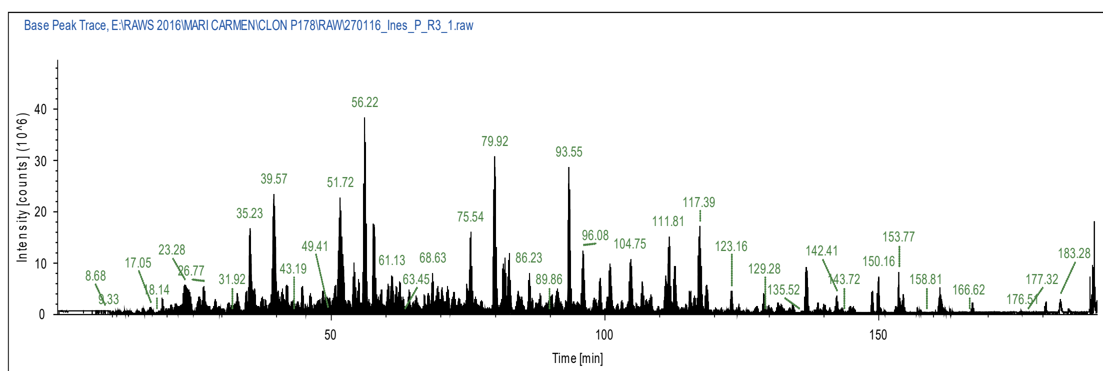
BIOLOGICAL REPLICATE 2 – TECHNICAL REPLICATE 1



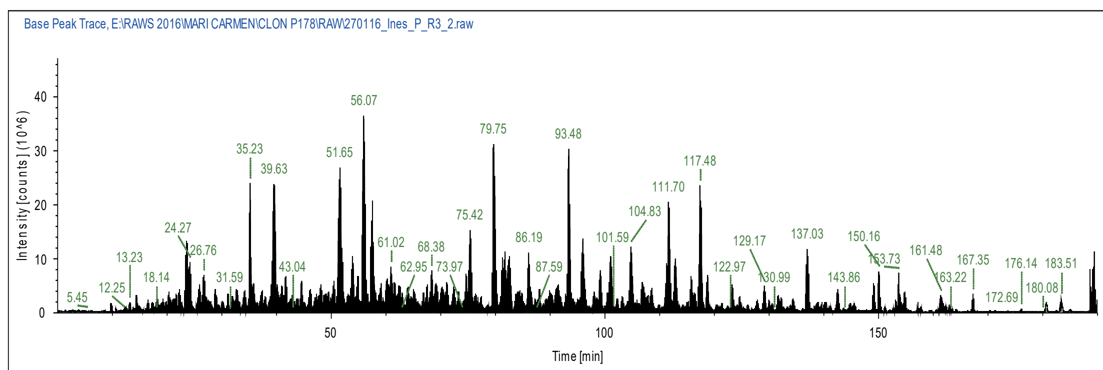
BIOLOGICAL REPLICATE 2 – TECHNICAL REPLICATE 2



BIOLOGICAL REPLICATE 3 – TECHNICAL REPLICATE 1



BIOLOGICAL REPLICATE 3 – TECHNICAL REPLICATE 2



Clone R17-12 C1

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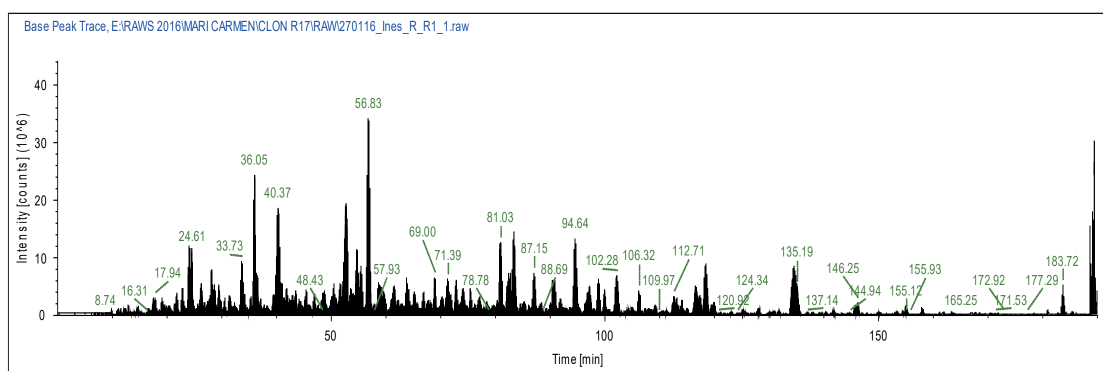

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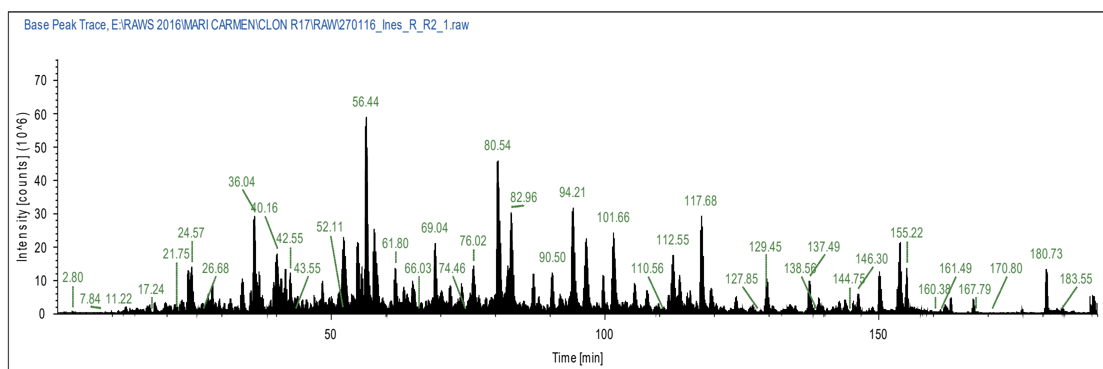

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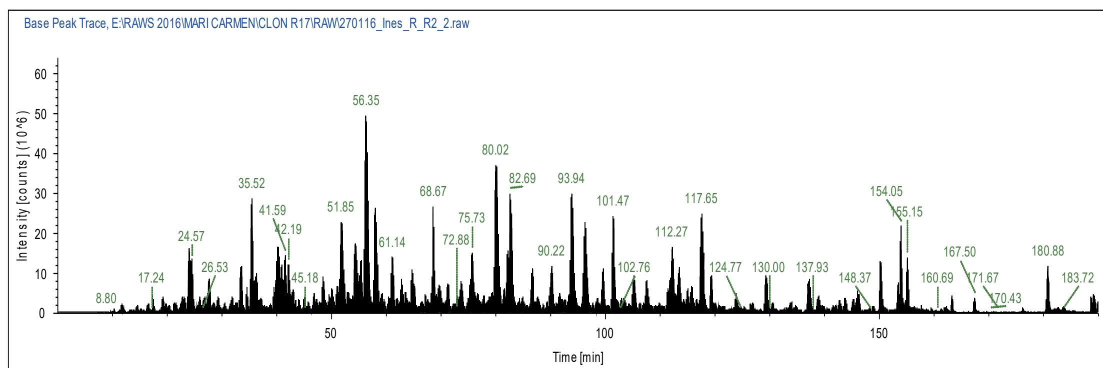

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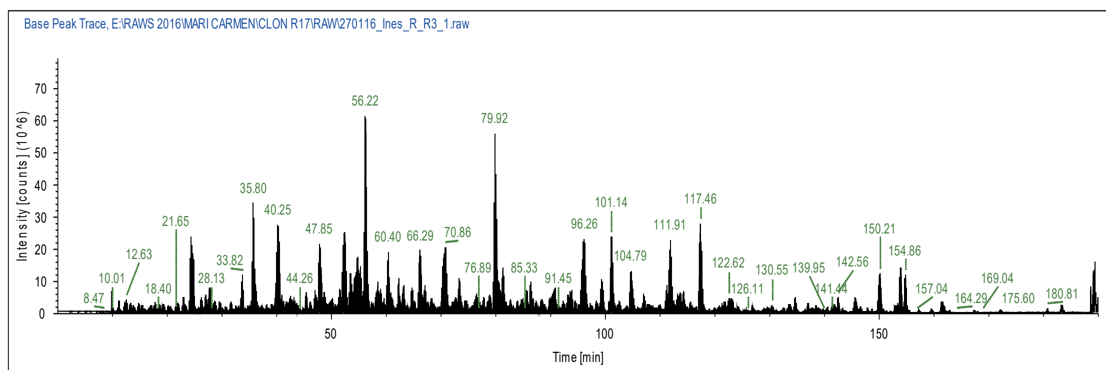
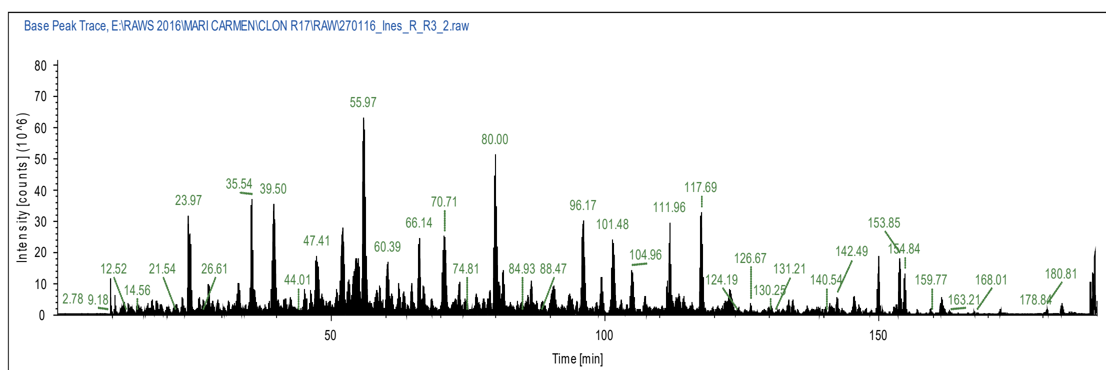


BIOLOGICAL REPLICATE 3 – TECHNICAL REPLICATE 2



APPENDIX II - List of 170 identified and relatively quantified proteins from membrane and organelle membrane fraction of *T. gallinae* clonal cultures that were more abundant in clone P178-13 C7 with ≥ 3 fold change and statistical significance (ANOVA, p-value < 0.01). P: number of identified peptides and number of unique, non-conflicting peptides in parentheses. Score: total protein score (sum of individual peptide scores).

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
ATPase	Cellular metabolism	14.38	ATPase, AAA family protein [*Trichomonas vaginalis* G3]	2 (2)	30.29	5.54E+05	3.85E+04	1.45E-06	gi|121886218
Binding of large substrates		10	hypothetical protein TVAG_109510 [*Trichomonas vaginalis* G3]	2 (2)	29.24	4.60E+05	4.60E+04	3.33E-04	gi|121905439
		3.83	hypothetical protein TVAG_028120 [*Trichomonas vaginalis* G3]	2 (2)	30.5	2.67E+04	6960.72	1.76E-04	gi|121907365
Calcium signal modulators		6.04	calcium-translocating P-type ATPase, PMCA-type family protein [*Trichomonas vaginalis* G3]	2 (2)	71.75	5.25E+04	8685.17	2.13E-05	gi|121889617
Cell adhesion	Cell adhesion	31.76	hypothetical protein TVAG_130090 [*Trichomonas vaginalis* G3]	2 (2)	32.04	3.18E+04	1000.03	1.82E-03	gi|121915109
		7.25	conserved hypothetical protein [*Trichomonas vaginalis* G3]	2 (2)	27.97	4.96E+05	6.84E+04	8.08E-06	gi|121890028
		3.65	hypothetical protein TVAG_457560 [*Trichomonas vaginalis* G3]	2 (2)	28.54	7.44E+04	2.04E+04	3.02E-04	gi|121917345
Cell stress response	Cellular metabolism	8.13	Heat shock 70 kDa protein, mitochondrial precursor, putative [*Trichomonas vaginalis* G3]	2 (2)	74.39	2.02E+05	2.48E+04	1.66E-03	gi|121914923
		3.22	Hsp90 protein [*Trichomonas vaginalis* G3]	6 (6)	326.25	3.83E+04	1.19E+04	1.97E-05	gi|121917639
Cytoskeleton	Cellular component	12.34	hypothetical protein TVAG_208940 [*Trichomonas vaginalis* G3]	3 (3)	45.13	5.98E+04	4845.66	9.04E-06	gi|121910773
		12.08	Dynein heavy chain family protein [*Trichomonas vaginalis* G3]	2 (2)	31.34	5.87E+04	4861.8	2.78E-07	gi|121914899

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Cytoskeleton	Cellular component	9.25	hypothetical protein TVAG_110590 [*Trichomonas vaginalis* G3]	4 (4)	54.08	2.93E+04	3163.73	7.36E-05	gi|121915660
		8.62	hypothetical protein TVAG_454480 [*Trichomonas vaginalis* G3]	2 (2)	33.83	1.15E+04	1337.43	4.29E-04	gi|121898727
		6	hypothetical protein TVAG_252220 [*Trichomonas vaginalis* G3]	3 (3)	42.53	2.22E+05	3.70E+04	6.78E-05	gi|121910564
		5.74	hypothetical protein TVAG_169860 [*Trichomonas vaginalis* G3]	3 (3)	54.45	5.49E+04	9577.77	1.16E-04	gi|121912858
		5.38	hypothetical protein TVAG_329890 [*Trichomonas vaginalis* G3]	2 (2)	27.58	9.73E+04	1.81E+04	8.69E-06	gi|121887190
		4.75	profilin, putative [*Trichomonas vaginalis* G3]	2 (2)	64.27	6.67E+04	1.40E+04	2.17E-05	gi|121906940
		4.16	hypothetical protein TVAG_021520 [*Trichomonas vaginalis* G3]	2 (2)	40.01	1.35E+05	3.23E+04	6.41E-04	gi|121915416
		3.84	hypothetical protein TVAG_477720 [*Trichomonas vaginalis* G3]	2 (2)	45.1	2.78E+05	7.25E+04	4.09E-03	gi|121882914
Cytoskeleton, metal ion binding		6.23	putative alpha-actinin [*Histomonas meleagridis*]	3 (3)	48.12	7720.57	1239	2.59E-03	gi|262358541
DNA replication	DNA replication	14.09	possible regulator of nonsense transcripts, putative [*Trichomonas vaginalis* G3]	3 (3)	81.75	4.87E+05	3.46E+04	7.25E-07	gi|121916893
		4.74	polymerase zeta subunit, putative [*Trichomonas vaginalis* G3]	2 (2)	36.13	2.96E+04	6239.31	1.58E-06	gi|121909895
		4.7	Helicase, putative [*Trichomonas vaginalis* G3]	2 (2)	36.9	4.43E+04	9427.08	2.77E-07	gi|121911876
		4.09	Ribonucleotide reductase, all-alpha domain containing protein [*Trichomonas vaginalis* G3]	5 (5)	255.06	1.61E+05	3.94E+04	2.53E-04	gi|121915211

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
DNA replication	DNA replication	3.87	exonuclease family protein [*Trichomonas vaginalis* G3]	3 (3)	52.5	5.95E+05	1.54E+05	1.70E-06	gi|121905927
Endocytosis	Cellular metabolism	7.4	Dynamin central region family protein [*Trichomonas vaginalis* G3]	3 (3)	64.69	5.79E+04	7821.44	5.98E-05	gi|121887114
Energetic metabolism	Cellular metabolism	41.08	Oligosaccharyl transferase STT3 subunit family protein [*Trichomonas vaginalis* G3]	2 (2)	63.86	1.33E+04	323.32	7.69E-08	gi|121902670
		22.13	lactate dehydrogenase family protein [*Trichomonas vaginalis* G3]	2 (2)	43.1	1.21E+05	5473.69	3.85E-07	gi|121889193
		15.72	Adenylate kinase family protein [*Trichomonas vaginalis* G3]	2 (2)	27.55	5.16E+04	3283.89	1.36E-07	gi|121914240
		8.93	dnaK protein [*Trichomonas vaginalis* G3]	4 (4)	101.55	1.64E+05	1.84E+04	5.84E-08	gi|121907663
		7	glucose-6-phosphate 1-dehydrogenase family protein [*Trichomonas vaginalis* G3]	7 (7)	285.86	8.47E+04	1.21E+04	2.95E-07	gi|121883413
		4.49	glucose-6-phosphate isomerase family protein [*Trichomonas vaginalis* G3]	3 (3)	89.42	7.67E+04	1.71E+04	1.22E-06	gi|121908707
		4.47	galactokinase family protein [*Trichomonas vaginalis* G3]	3 (3)	134.69	2.73E+04	6100.32	3.28E-07	gi|121903545
		4.24	ABC transporter family protein [*Trichomonas vaginalis* G3]	3 (3)	99.04	3.27E+04	7707.22	7.94E-05	gi|121889755
		3.94	Alpha amylase, catalytic domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	94.07	1.35E+04	3431.11	9.70E-04	gi|121915021
		3.78	glycosyl transferase, group 1 family protein [*Trichomonas vaginalis* G3]	2 (2)	43.52	2.36E+04	6251.76	5.41E-05	gi|121889369
		3.64	6-phosphogluconate dehydrogenase, decarboxylating family protein [*Trichomonas vaginalis* G3]	2 (2)	99.56	1.10E+05	3.03E+04	6.83E-06	gi|121896682

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Energetic metabolism	Cellular metabolism	3.54	3'5'-cyclic nucleotide phosphodiesterase family protein [*Trichomonas vaginalis* G3]	3 (3)	60.29	4.57E+05	1.29E+05	7.28E-04	gi|121889147
		3.22	ATP-dependent DNA helicase, RecQ family protein [*Trichomonas vaginalis* G3]	3 (2)	54.81	2.05E+05	6.37E+04	2.82E-03	gi|121888663
Gluconeogenesis		3.05	phosphoenol pyruvate carboxykinase. putative [*Trichomonas vaginalis* G3]	10 (10)	517.55	2.79E+06	9.16E+05	2.24E-03	gi|121888730
Gluconeogenesis, glycolysis		5.08	glucose-6-phosphate isomerase family protein [*Trichomonas vaginalis* G3]	17 (17)	917.8	2.76E+05	5.44E+04	1.37E-04	gi|121906378
Glycolysis, oxidation-reduction homeostasis		3.46	GDP dissociation inhibitor family protein [*Trichomonas vaginalis* G3]	6 (6)	355.73	1.37E+05	3.96E+04	4.89E-05	gi|121879717
Hydrogenase, metal ion binding		3.58	hydrogenosomal Fe-hydrogenase, partial [*Trichomonas gallinae*]	10 (10)	704.23	2.57E+05	7.19E+04	8.81E-03	gi|507310429
Metal ion binding		8.23	hypothetical protein TVAG_276700 [*Trichomonas vaginalis* G3]	2 (2)	37.03	2.29E+05	2.79E+04	1.68E-05	gi|121891036
Metal ion binding, lyase		3.26	fructose-1.6-bisphosphate aldolase, putative [*Trichomonas vaginalis* G3]	12 (12)	714.5	1.64E+06	5.03E+05	2.12E-03	gi|121909882
Metal ion binding, oxidation-reduction homeostasis		21.01	putative NADPH-dependent butanol dehydrogenase [*Trichomonas vaginalis*]	2 (2)	206.35	4.42E+04	2102.87	2.18E-05	gi|4426910
		6.9	Pyridine nucleotide-disulphide oxidoreductase family protein [*Trichomonas vaginalis* G3]	3 (3)	178.07	2.07E+05	3.00E+04	9.64E-08	gi|121898098
Metal ion binding, transferase		3.47	Phosphoglucomutase/phosphomannomutase. alpha/beta/alpha domain I family protein [*Trichomonas vaginalis* G3]	8 (8)	414.71	2.67E+05	7.70E+04	3.13E-05	gi|121885206
Mitochondrial transport		6.98	hypothetical protein TVAG_548390 [*Trichomonas vaginalis* G3]	2 (2)	30.34	1.34E+05	1.91E+04	1.35E-04	gi|121841176
Nucleotide binding	DNA replication	3.23	heavy neurofilament protein. putative [*Trichomonas vaginalis* G3]	3 (3)	46.16	1.54E+04	4756.37	5.43E-05	gi|121898592

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Oxidation-reduction homeostasis	Cell adhesion	19.6	hypothetical protein TVAG_008090 [*Trichomonas vaginalis* G3]	2 (2)	36.49	1.26E+05	6403.58	3.63E-07	gi|121897688
	Cellular metabolism	4.14	thioredoxin-disulfide reductase family protein [*Trichomonas vaginalis* G3]	2 (2)	113.87	1.21E+05	2.93E+04	1.62E-03	gi|121893127
Peptidase		4.3	Clan CA, family C19, ubiquitin hydrolase-like cysteine peptidase [*Trichomonas vaginalis* G3]	2 (2)	29.13	1.45E+05	3.38E+04	1.12E-08	gi|121883839
		3.67	Clan CA, family C1, cathepsin L-like cysteine peptidase, partial [*Trichomonas gallinae*]	6 (6)	286.99	1.28E+06	3.49E+05	1.69E-03	gi|386364444
		3.23	hypothetical protein TVAG_051850 [*Trichomonas vaginalis* G3]	2 (2)	43.6	3.61E+04	1.12E+04	1.35E-04	gi|121889871
Phospholipid synthesis		3.07	hypothetical protein TVAG_407170 [*Trichomonas vaginalis* G3]	6 (6)	348.73	2.85E+05	9.28E+04	2.69E-05	gi|121895175
Protein bindin		5.2	hypothetical protein TVAG_436440 [*Trichomonas vaginalis* G3]	2 (2)	37.45	6.51E+04	1.25E+04	4.47E-04	gi|121916037
Protein metabolism		16.09	PIKK family atypical protein kinase [*Trichomonas vaginalis* G3]	2 (2)	44.69	5892.6	366.31	7.63E-04	gi|121912614
		12.54	CAMK family protein kinase [*Trichomonas vaginalis* G3]	2 (2)	31.32	8977.61	715.69	5.04E-03	gi|121906466
		10.98	PIKK family atypical protein kinase [*Trichomonas vaginalis* G3]	3 (3)	51.02	1.66E+05	1.52E+04	4.81E-04	gi|121910679
		9.98	CAMK family protein kinase [*Trichomonas vaginalis* G3]	2 (2)	32.25	1.62E+04	1619.25	1.01E-03	gi|121912207
		7.91	Glutamine synthetase, catalytic domain containing protein [*Trichomonas vaginalis* G3]	3 (3)	107.13	7.43E+04	9390.65	1.67E-08	gi|121892813
		6.83	hypothetical protein TVAG_107620 [*Trichomonas vaginalis* G3]	2 (2)	62.43	2.16E+04	3163.05	2.65E-06	gi|121896349

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Protein metabolism	Cellular metabolism	5.94	glycosyl transferase, group 2 family protein [*Trichomonas vaginalis* G3]	4 (4)	195.85	2.82E+04	4751.28	1.67E-05	gi|121911637
		4.67	TKL family protein kinase [*Trichomonas vaginalis* G3]	2 (2)	43.23	2.84E+04	6084.74	1.34E-05	gi|121902296
		4.63	AGC family protein kinase [*Trichomonas vaginalis* G3]	2 (2)	54.75	1.69E+05	3.65E+04	9.97E-03	gi|121890243
		4.59	Clan CA, family C1, cathepsin L-like cysteine peptidase [*Trichomonas vaginalis* G3]	2 (2)	43.69	2.27E+04	4950.94	3.18E-03	gi|121908100
		4.49	Clan MH, family M20, peptidase T-like metallopeptidase [*Trichomonas vaginalis* G3]	3 (3)	123.12	1.34E+05	2.97E+04	1.24E-04	gi|121901617
		3.41	Aminotransferase, classes I and II family protein [*Trichomonas vaginalis* G3]	2 (2)	164.19	1.55E+05	4.54E+04	3.92E-05	gi|121909674
		3.32	Clan CA, family C19, ubiquitin hydrolase-like cysteine peptidase [*Trichomonas vaginalis* G3]	2 (2)	27.44	5.97E+04	1.80E+04	1.48E-03	gi|121906634
		3.16	Copine family protein [*Trichomonas vaginalis* G3]	3 (3)	53.44	6.76E+04	2.14E+04	2.31E-03	gi|121901567
		3.11	co-chaperone Hsc20 family protein [*Trichomonas vaginalis* G3]	2 (2)	70.46	3.19E+04	1.03E+04	0.01	gi|121889907
		3.03	Family T1, proteasome beta subunit, threonine peptidase [*Trichomonas vaginalis* G3]	3 (3)	94.65	3.75E+04	1.24E+04	1.47E-04	gi|121916758
		3.01	conserved hypothetical protein [*Trichomonas vaginalis* G3]	2 (2)	44.55	8215.11	2729.95	5.01E-06	gi|121890884
Protein targeting to Golgi		8.23	hypothetical protein TVAG_201650 [*Trichomonas vaginalis* G3]	2 (2)	29.22	6770.17	822.38	2.96E-04	gi|121910323

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Protein targeting to Golgi	Cellular metabolism	3.75	viral A-type inclusion protein. putative [*Trichomonas vaginalis* G3]	4 (4)	58.73	5.37E+04	1.43E+04	3.17E-04	gi|121893459
Protein-protein interaction		46.22	hypothetical protein TVAG_147380 [*Trichomonas vaginalis* G3]	2 (2)	32.88	4.88E+05	1.05E+04	1.01E-05	gi|121880636
		5.62	hypothetical protein TVAG_093700 [*Trichomonas vaginalis* G3]	3 (3)	41.23	4.05E+04	7208.38	1.75E-06	gi|121917422
		4.06	hypothetical protein TVAG_075500 [*Trichomonas vaginalis* G3]	2 (2)	31.87	1.96E+05	4.82E+04	1.13E-05	gi|121918033
Ribosomal large subunit assembly	Cellular component	6.02	hypothetical protein TVAG_464510 [*Trichomonas vaginalis* G3]	2 (2)	37.7	4.56E+04	7570.82	3.91E-06	gi|121903946
		3.36	hypothetical protein TVAG_470660 [*Trichomonas vaginalis* G3]	5 (5)	89.09	1.69E+05	5.03E+04	2.62E-05	gi|121901205
		3.28	hypothetical protein TVAG_476450 [*Trichomonas vaginalis* G3]	3 (3)	47.7	1.98E+05	6.02E+04	2.32E-03	gi|121917945
Ribosomal protein		3.47	40S ribosomal protein S16. putative [*Trichomonas vaginalis* G3]	2 (2)	143.1	5.03E+04	1.45E+04	7.03E-04	gi|121885138
RNA processing		7.59	conserved hypothetical protein [*Trichomonas vaginalis* G3]	2 (2)	56.49	4.09E+04	5381.27	2.58E-06	gi|121899537
Signal transduction	Signal transduction	4.26	hypothetical protein TVAG_271930 [*Trichomonas vaginalis* G3]	2 (2)	41.4	3.53E+04	8275.3	2.75E-05	gi|121907112
		4.21	small GTP-binding protein, putative [*Trichomonas vaginalis* G3]	2 (2)	237.3	3.06E+05	7.28E+04	1.97E-03	gi|121880468
		3.95	GTPase-activator protein, putative [*Trichomonas vaginalis* G3]	2 (2)	29.36	2.40E+05	6.07E+04	3.09E-04	gi|121892626
		3.67	hypothetical protein TVAG_530150 [*Trichomonas vaginalis* G3]	2 (2)	38.53	1.52E+05	4.13E+04	1.15E-04	gi|121877483

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Signal transduction	Signal transduction	3.04	Adenylate and Guanylate cyclase catalytic domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	34.97	3.13E+04	1.03E+04	6.87E-04	gi|121912340
Transaldolase	Cellular metabolism	5.52	Transaldolase A, putative [*Trichomonas vaginalis* G3]	2 (2)	62.98	2.40E+04	4347.89	8.56E-05	gi|121896456
Transaminase		4.3	aminotransferase, classes I and II family protein [*Trichomonas vaginalis* G3]	3 (3)	158.74	1.17E+05	2.72E+04	9.78E-03	gi|121901152
Transcription		10.47	hypothetical protein TVAG_285400 [*Trichomonas vaginalis* G3]	2 (2)	37.86	1.72E+04	1639.41	8.33E-05	gi|121859384
		5.69	RNA polymerase II largest subunit, partial [*Trichomonas gallinae*]	2 (2)	87.66	8257.66	1452.08	2.60E-04	gi|327242119
		4.79	viral A-type inclusion protein, putative [*Trichomonas vaginalis* G3]	2 (2)	37.11	1.34E+04	2797.47	2.36E-04	gi|121897262
		3.32	histone H4-3 [*Trichomonas vaginalis* G3]	2 (2)	184.78	2.99E+05	9.01E+04	3.19E-03	gi|121889898
Transferase		3.56	UTP--glucose-1-phosphate uridylyltransferase family protein [*Trichomonas vaginalis* G3]	5 (5)	168.95	2.17E+04	6092.98	2.44E-06	gi|121904545
Translation	Translation	6.9	hypothetical protein TVAG_184380 [*Trichomonas vaginalis* G3]	2 (2)	36.51	3.65E+05	5.29E+04	1.93E-04	gi|121905611
		5.81	Adenylate and Guanylate cyclase catalytic domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	32.96	6.12E+04	1.05E+04	1.87E-05	gi|121914781
		5.68	hypothetical protein TVAG_016880 [*Trichomonas vaginalis* G3]	9 (9)	453.74	1.17E+06	2.06E+05	9.26E-05	gi|121899865
		3.86	hypothetical protein TVAG_266630 [*Trichomonas vaginalis* G3]	4 (4)	259.78	6.39E+04	1.66E+04	4.27E-06	gi|121912479
		3.66	Type III restriction enzyme, res subunit family protein [*Trichomonas vaginalis* G3]	2 (2)	46.03	7.36E+05	2.01E+05	8.63E-03	gi|121898850

<table>
<tr><th>Predicted function</th><th>Category</th><th>Fold change</th><th>Description</th><th>P</th><th>Score</th><th>P178-13</th><th>R17-12</th><th>p-value</th><th>Accession number</th></tr>
<tr><td>Translation, metal ion binding</td><td>Translation</td><td>7.63</td><td>alanyl-tRNA synthetase, partial [Trichomonas vaginalis]</td><td>5 (5)</td><td>187.01</td><td>1.31E+05</td><td>1.71E+04</td><td>8.44E-06</td><td>gi|51102334</td></tr>
<tr><td>Translation, transmembrane transport</td><td>Translation, cellular metabolism</td><td>3.92</td><td>Mitochondrial carrier protein [Trichomonas vaginalis G3]</td><td>9 (9)</td><td>479.27</td><td>6.94E+05</td><td>1.77E+05</td><td>4.36E-04</td><td>gi|121889868</td></tr>
<tr><td rowspan="4">Transmembrane transport</td><td rowspan="4">Cellular metabolism</td><td>5.45</td><td>ABC transporter family protein [Trichomonas vaginalis G3]</td><td>4 (4)</td><td>149.49</td><td>2.76E+04</td><td>5066.61</td><td>3.12E-07</td><td>gi|121883001</td></tr>
<tr><td>4.22</td><td>Beige/BEACH domain containing protein [Trichomonas vaginalis G3]</td><td>4 (4)</td><td>71.58</td><td>1.55E+05</td><td>3.67E+04</td><td>7.34E-06</td><td>gi|121889875</td></tr>
<tr><td>4.12</td><td>hydrogenosomal carrier protein. partial [Trichomonas gallinae]</td><td>14 (14)</td><td>744.55</td><td>2.07E+06</td><td>5.02E+05</td><td>5.36E-04</td><td>gi|30315255</td></tr>
<tr><td>3.81</td><td>phospholipid-translocating P-type ATPase, flippase family protein [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>49.93</td><td>1.47E+05</td><td>3.84E+04</td><td>1.56E-05</td><td>gi|121897029</td></tr>
<tr><td rowspan="7">Unknown</td><td rowspan="7">Unknown</td><td>71.88</td><td>hypothetical protein TVAG_073850 [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>27.16</td><td>3.60E+05</td><td>5001.77</td><td>2.10E-06</td><td>gi|121904064</td></tr>
<tr><td>38.77</td><td>hypothetical protein TVAG_468600 [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>28.26</td><td>6.93E+05</td><td>1.79E+04</td><td>6.75E-07</td><td>gi|121894474</td></tr>
<tr><td>34.83</td><td>hypothetical protein TVAG_437390 [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>30.01</td><td>9.06E+05</td><td>2.60E+04</td><td>3.27E-06</td><td>gi|121916130</td></tr>
<tr><td>33.62</td><td>hypothetical protein TVAG_107130 [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>39.66</td><td>1.78E+04</td><td>529.75</td><td>2.08E-05</td><td>gi|121891496</td></tr>
<tr><td>30.06</td><td>hypothetical protein TVAG_398490 [Trichomonas vaginalis G3]</td><td>3 (3)</td><td>47.12</td><td>5.19E+05</td><td>1.73E+04</td><td>9.12E-06</td><td>gi|121886424</td></tr>
<tr><td>29.82</td><td>surface antigen BspA-like [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>29.65</td><td>2.77E+05</td><td>9299.22</td><td>8.57E-07</td><td>gi|121886631</td></tr>
<tr><td>26.1</td><td>hypothetical protein TVAG_237830 [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>33.96</td><td>5.68E+04</td><td>2176.18</td><td>1.82E-05</td><td>gi|121916980</td></tr>
</table>

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Unknown	Unknown	25.28	hypothetical protein TVAG_431160 [*Trichomonas vaginalis* G3]	2 (2)	36.23	2.02E+04	797.16	1.28E-06	gi|121896835
		23.96	hypothetical protein TVAG_343940 [*Trichomonas vaginalis* G3]	2 (2)	32.59	1.42E+05	5940.33	2.39E-07	gi|121906390
		12.33	viral A-type inclusion protein. putative [*Trichomonas vaginalis* G3]	2 (2)	27.25	1.58E+05	1.28E+04	1.76E-06	gi|121895325
		11.09	hypothetical protein TVAG_474360 [*Trichomonas vaginalis* G3]	3 (3)	66.75	4.52E+04	4075.46	9.98E-07	gi|121899160
		10.63	hypothetical protein TVAG_145680 [*Trichomonas vaginalis* G3]	2 (2)	31.61	8.98E+04	8450.01	2.49E-04	gi|121903522
		10.31	hypothetical protein TVAG_050140 [*Trichomonas vaginalis* G3]	2 (2)	38.37	2.33E+04	2258.32	4.77E-06	gi|121902205
		10.08	hypothetical protein TVAG_411320 [*Trichomonas vaginalis* G3]	2 (2)	43.82	1.38E+05	1.36E+04	3.38E-06	gi|121910013
		10.06	hypothetical protein TVAG_346840 [*Trichomonas vaginalis* G3]	2 (2)	35.55	1.03E+05	1.03E+04	1.52E-06	gi|121892389
		9.83	Trichohyalin. putative [*Trichomonas vaginalis* G3]	2 (2)	34.76	2.48E+05	2.53E+04	4.74E-06	gi|121890270
		9.34	unknown [*Trichomonas vaginalis*]	2 (2)	50.84	2.48E+04	2658.03	1.56E-04	gi|8886406
		8.69	hypothetical protein TVAG_123890 [*Trichomonas vaginalis* G3]	5 (5)	91.09	2.15E+05	2.48E+04	2.21E-07	gi|121900942
		8	Bap-like [*Trichomonas vaginalis* G3]	4 (4)	61.57	1.63E+05	2.04E+04	5.06E-07	gi|121916013
		7.7	viral A-type inclusion protein. putative [*Trichomonas vaginalis* G3]	3 (3)	46.1	6.05E+04	7854.07	2.06E-05	gi|121909496
		7.01	hypothetical protein TVAG_324880 [*Trichomonas vaginalis* G3]	3 (3)	43.43	3.55E+05	5.07E+04	3.81E-06	gi|121896216

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Unknown	Unknown	6.99	viral A-type inclusion protein. putative [*Trichomonas vaginalis* G3]	2 (2)	37.21	2.57E+06	3.67E+05	4.14E-05	gi|121899611
		6.9	hypothetical protein TVAG_484970 [*Trichomonas vaginalis* G3]	2 (2)	35.41	3.85E+04	5571.69	3.74E-05	gi|121880058
		6.86	hypothetical protein TVAG_080900 [*Trichomonas vaginalis* G3]	3 (3)	70.53	4.95E+05	7.22E+04	1.94E-04	gi|121900462
		6.86	hypothetical protein TVAG_354770 [*Trichomonas vaginalis* G3]	2 (2)	33.78	1.51E+04	2207.47	4.52E-05	gi|121903451
		6.67	hypothetical protein TVAG_092160 [*Trichomonas vaginalis* G3]	2 (2)	32.51	2.92E+05	4.37E+04	2.55E-06	gi|121885070
		6.36	hypothetical protein TVAG_090740 [*Trichomonas vaginalis* G3]	4 (4)	69.63	3.51E+05	5.52E+04	3.33E-05	gi|121893329
		6.29	hypothetical protein TVAG_034750 [*Trichomonas vaginalis* G3]	2 (2)	44.13	3.11E+04	4946.4	1.14E-06	gi|121889632
		5.77	hypothetical protein TVAG_420130 [*Trichomonas vaginalis* G3]	2 (2)	35.41	9.36E+04	1.62E+04	3.57E-04	gi|121904444
		5.48	hypothetical protein TVAG_454150 [*Trichomonas vaginalis* G3]	3 (3)	59.95	9.60E+04	1.75E+04	1.22E-05	gi|121912769
		5.41	viral A-type inclusion protein, putative [*Trichomonas vaginalis* G3]	2 (2)	39.76	4.59E+04	8474.23	8.81E-04	gi|121904864
		5.21	ankyrin repeat protein. putative [*Trichomonas vaginalis* G3]	2 (2)	30.77	1.19E+04	2276.93	6.16E-06	gi|121912087
		5.06	hypothetical protein TVAG_420220 [*Trichomonas vaginalis* G3]	2 (2)	32.07	2.24E+05	4.43E+04	9.75E-04	gi|121904453
		4.99	hypothetical protein TVAG_249590 [*Trichomonas vaginalis* G3]	2 (2)	34.48	4.54E+04	9097.28	1.04E-04	gi|121917123
		4.72	hypothetical protein TVAG_374940 [*Trichomonas vaginalis* G3]	3 (3)	83.67	2.95E+04	6247.27	2.64E-06	gi|121885719

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Unknown	Unknown	4.66	hypothetical protein TVAG_425440 [*Trichomonas vaginalis* G3]	2 (2)	26.83	6.65E+04	1.43E+04	4.10E-06	gi|121882729
		4.6	conserved hypothetical protein [*Trichomonas vaginalis* G3]	2 (2)	28.07	3.64E+04	7908.32	1.35E-06	gi|121903532
		4.41	hypothetical protein TVAG_248380 [*Trichomonas vaginalis* G3]	2 (2)	36.55	1.79E+04	4056.04	4.11E-05	gi|121906564
		4.39	conserved hypothetical protein [*Trichomonas vaginalis* G3]	2 (2)	36.44	5.76E+04	1.31E+04	9.43E-06	gi|121910686
		4.37	hypothetical protein TVAG_103950 [*Trichomonas vaginalis* G3]	3 (3)	54.5	7.85E+04	1.79E+04	7.59E-07	gi|121888190
		3.95	hypothetical protein TVAG_009150 [*Trichomonas vaginalis* G3]	2 (2)	28.39	8.08E+04	2.04E+04	1.32E-07	gi|121881711
		3.86	hypothetical protein TVAG_391180 [*Trichomonas vaginalis* G3]	2 (2)	28.47	4.60E+04	1.19E+04	1.88E-05	gi|121915950
		3.83	hypothetical protein TVAG_429790 [*Trichomonas vaginalis* G3]	2 (2)	77.15	1.63E+05	4.27E+04	4.88E-03	gi|121889097
		3.81	Leucine Rich Repeat family protein [*Trichomonas vaginalis* G3]	2 (2)	36.22	7.94E+04	2.09E+04	5.73E-04	gi|121911542
		3.76	hypothetical protein TVAG_231340 [*Trichomonas vaginalis* G3]	2 (2)	39.8	5.29E+04	1.41E+04	6.26E-05	gi|121895616
		3.69	hypothetical protein TVAG_250870 [*Trichomonas vaginalis* G3]	2 (2)	36.5	3.37E+04	9124.25	4.60E-04	gi|121889502
		3.52	hypothetical protein TVAG_497970 [*Trichomonas vaginalis* G3]	2 (2)	37.47	2.26E+05	6.43E+04	1.68E-05	gi|121885514
		3.5	hypothetical protein TVAG_034380 [*Trichomonas vaginalis* G3]	3 (3)	51.15	1.64E+05	4.68E+04	1.72E-03	gi|121901242
		3.48	hypothetical protein TVAG_132560 [*Trichomonas vaginalis* G3]	2 (2)	29.99	5.76E+05	1.65E+05	2.02E-03	gi|121886642

<table>
<tr><th>Predicted function</th><th>Category</th><th>Fold change</th><th>Description</th><th>P</th><th>Score</th><th>P178-13</th><th>R17-12</th><th>p-value</th><th>Accession number</th></tr>
<tr><td rowspan="10">Unknown</td><td rowspan="10">Unknown</td><td>3.47</td><td>hypothetical protein TVAG_286280 [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>70.56</td><td>3.36E+04</td><td>9673.65</td><td>2.13E-04</td><td>gi|121900413</td></tr>
<tr><td>3.44</td><td>hypothetical protein TVAG_440200 [Trichomonas vaginalis G3]</td><td>3 (3)</td><td>211.22</td><td>3.89E+05</td><td>1.13E+05</td><td>1.66E-03</td><td>gi|121891682</td></tr>
<tr><td>3.42</td><td>viral A-type inclusion protein. putative [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>33.23</td><td>5.68E+04</td><td>1.66E+04</td><td>1.11E-05</td><td>gi|121898845</td></tr>
<tr><td>3.4</td><td>hypothetical protein TVAG_066230 [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>29.36</td><td>2.55E+05</td><td>7.50E+04</td><td>4.71E-04</td><td>gi|121885580</td></tr>
<tr><td>3.22</td><td>hypothetical protein TVAG_222340 [Trichomonas vaginalis G3]</td><td>3 (3)</td><td>61.94</td><td>1.25E+05</td><td>3.87E+04</td><td>7.55E-05</td><td>gi|121883477</td></tr>
<tr><td>3.16</td><td>hypothetical protein TVAG_085630 [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>38.97</td><td>1.30E+05</td><td>4.11E+04</td><td>9.40E-04</td><td>gi|121895549</td></tr>
<tr><td>3.11</td><td>hypothetical protein TVAG_295140 [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>46.18</td><td>2.83E+04</td><td>9097.47</td><td>0.01</td><td>gi|121914059</td></tr>
<tr><td>3.08</td><td>hypothetical protein TVAG_013740 [Trichomonas vaginalis G3]</td><td>3 (3)</td><td>58.34</td><td>1.09E+05</td><td>3.54E+04</td><td>2.40E-03</td><td>gi|121916831</td></tr>
<tr><td>3.05</td><td>hypothetical protein TVAG_415360 [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>34.89</td><td>5.79E+04</td><td>1.90E+04</td><td>1.44E-06</td><td>gi|121897998</td></tr>
<tr><td>3.05</td><td>viral A-type inclusion protein. putative [Trichomonas vaginalis G3]</td><td>2 (2)</td><td>27.95</td><td>3.60E+05</td><td>1.18E+05</td><td>9.26E-03</td><td>gi|121896827</td></tr>
<tr><td>Vacuole protein</td><td>Cellular component</td><td>8.21</td><td>hypothetical protein TVAG_238570 [Trichomonas vaginalis G3]</td><td>3 (3)</td><td>45.97</td><td>1.82E+05</td><td>2.21E+04</td><td>4.93E-04</td><td>gi|121915699</td></tr>
<tr><td>Vesicle-mediated transport</td><td rowspan="3">Exocytosis</td><td>7.17</td><td>Clathrin and VPS domain-containing protein, partial [Trichomonas vaginalis G3]</td><td>8 (8)</td><td>349.57</td><td>1.08E+05</td><td>1.51E+04</td><td>1.06E-04</td><td>gi|121873597</td></tr>
<tr><td rowspan="2">Vesicle-mediated transport, intracellular protein transport</td><td>7.11</td><td>Clathrin and VPS domain-containing protein [Trichomonas vaginalis G3]</td><td>6 (6)</td><td>208.37</td><td>1.46E+05</td><td>2.06E+04</td><td>2.48E-05</td><td>gi|121898439</td></tr>
<tr><td>3.74</td><td>Adaptin N terminal region family protein [Trichomonas vaginalis G3]</td><td>6 (6)</td><td>218.66</td><td>3.07E+04</td><td>8213.41</td><td>2.85E-05</td><td>gi|121909323</td></tr>
</table>

APPENDIX III - List of 132 identified and relatively quantified proteins from membrane and organelle membrane fraction of *T. gallinae* clonal cultures that were more abundant in clone R17-12 C1 with ≥ 3 fold change and statistical significance (ANOVA, *p*-value < 0.01). P: number of identified peptides and number of unique, non-conflicting peptides in parentheses. Score: total protein score (sum of individual peptide scores). ER: endoplasmic reticulum.

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Binding of large substrates	Cellular metabolism	3.39	hypothetical protein TVAG_269050 [*Trichomonas vaginalis* G3]	2 (2)	29.17	1.19E+04	4.01E+04	1.35E-03	gi|121903411
		3.23	hypothetical protein TVAG_067470 [*Trichomonas vaginalis* G3]	2 (2)	27.16	5.57E+04	1.80E+05	5.48E-04	gi|121911914
Calcium signal modulators		4.06	EF hand family protein [*Trichomonas vaginalis* G3]	2 (2)	70.33	2.55E+04	1.03E+05	4.41E-07	gi|121885510
		3.36	Calmodulin, putative [*Trichomonas vaginalis* G3]	3 (3)	202.21	1.01E+04	3.40E+04	5.73E-07	gi|121905996
		3.29	Centrin, putative [*Trichomonas vaginalis* G3]	3 (3)	83.18	1.15E+04	3.79E+04	1.69E-05	gi|121913299
Carbohydrate metabolism		6.54	malate dehydrogenase: SUBUNIT=A, putative [*Trichomonas vaginalis* G3]	4 (4)	245.34	2.16E+05	1.41E+06	5.90E-06	gi|121896029
		3.46	malate dehydrogenase, putative [*Trichomonas vaginalis* G3]	3 (3)	156.4	1.55E+05	5.36E+05	2.12E-05	gi|121915536
Cell adhesion	Cell adhesion	18.96	Immuno-dominant variable surface antigen-like [*Trichomonas vaginalis* G3]	3 (3)	53.94	4.89E+04	9.28E+05	2.95E-04	gi|121916712
		7.78	GP63-like [*Trichomonas vaginalis* G3]	2 (2)	38.73	6.63E+04	5.16E+05	3.85E-07	gi|121892295
Cell signal transduction or membrane trafficking	Cellular metabolism	4.99	C2 domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	36.07	7828.24	3.91E+04	5.52E-03	gi|121897227
Chromatin associated protein	Cellular component	8.37	hypothetical protein TVAG_176990 [*Trichomonas vaginalis* G3]	2 (2)	45.75	1.85E+04	1.55E+05	5.47E-07	gi|121893061

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Cytoskeleton	Cellular component	17.87	Dynein heavy chain family protein [*Trichomonas vaginalis* G3]	2 (2)	28.74	1.32E+04	2.36E+05	1.41E-05	gi|121917287
		15.03	conserved hypothetical protein [*Trichomonas vaginalis* G3]	2 (2)	72.72	2663.79	4.00E+04	7.90E-06	gi|121905200
		10.23	Erythrocyte membrane-associated giant protein antigen 332 containing protein [*Trichomonas vaginalis* G3]	3 (3)	58.8	1.48E+04	1.51E+05	7.82E-08	gi|121905491
		9.23	Dynein heavy chain family protein [*Trichomonas vaginalis* G3]	2 (2)	32.88	3.82E+04	3.52E+05	8.11E-03	gi|121890380
		6.89	conserved hypothetical protein [*Trichomonas vaginalis* G3]	2 (2)	42.44	1.02E+04	7.06E+04	5.96E-05	gi|121905453
		5.21	Dynein heavy chain family protein [*Trichomonas vaginalis* G3]	2 (2)	34.21	2.46E+04	1.28E+05	5.03E-04	gi|121905610
		4.18	alpha-tubulin [*Trichonympha agilis*]	6 (4)	481.79	4.81E+04	2.01E+05	4.92E-03	gi|496528628
		3.8	hypothetical protein TVAG_166580 [*Trichomonas vaginalis* G3]	2 (2)	63.65	1277.02	4855.76	1.56E-05	gi|121916503
		3.57	alpha-tubulin 2, partial [*Tritrichomonas foetus*]	3 (1)	115.83	5022.1	1.79E+04	9.13E-05	gi|33465441
		3.28	Dynein heavy chain family protein [*Trichomonas vaginalis* G3]	3 (3)	65.61	3.71E+04	1.22E+05	4.13E-06	gi|121917695
		3.15	Tctex-1 family protein [*Trichomonas vaginalis* G3]	4 (4)	142.29	8039.24	2.53E+04	1.17E-06	gi|121902586
Deaminase	Cellular metabolism	7.31	Vps52 / Sac2 family protein [*Trichomonas vaginalis* G3]	2 (2)	29.81	4624.93	3.38E+04	1.77E-06	gi|121902006
DNA repair	DNA replication	38.78	PIKK family atypical protein kinase [*Trichomonas vaginalis* G3]	2 (2)	34.52	3292.42	1.28E+05	2.36E-09	gi|121892589

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
DNA repair	DNA replication	13.26	PIKK family atypical protein kinase [*Trichomonas vaginalis* G3]	2 (2)	31.22	2490.17	3.30E+04	5.19E-05	gi|121889168
DNA replication		6.13	XRN 5'-3' exonuclease N-terminus family protein [*Trichomonas vaginalis* G3]	2 (2)	38.94	3998.07	2.45E+04	2.43E-04	gi|121913977
		3.59	ATP-dependent DNA helicase. RecQ family protein [*Trichomonas vaginalis* G3]	2 (2)	34.18	1.04E+04	3.74E+04	2.96E-04	gi|121909374
Endopeptidase	Cellular metabolism	5.93	Clan SB, family S8, subtilisin-like serine peptidase [*Trichomonas vaginalis* G3]	2 (2)	47.09	1.63E+04	9.69E+04	3.44E-04	gi|121885232
Energetic metabolism		7.31	Adenylate and Guanylate cyclase catalytic domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	38.65	1.20E+04	8.79E+04	0.01	gi|121890765
		6.73	Starch binding domain containing protein [*Trichomonas vaginalis* G3]	3 (3)	79.81	1.98E+04	1.33E+05	8.92E-04	gi|121903627
		4.11	Adenylate and Guanylate cyclase catalytic domain containing protein [*Trichomonas vaginalis* G3]	3 (3)	40.7	1.08E+04	4.42E+04	3.60E-03	gi|121917245
		3.98	hypothetical protein TVAG_164890 [*Trichomonas vaginalis* G3]	17 (17)	1307.4 2	5.60E+05	2.23E+06	8.24E-07	gi|121886677
		3.24	Phosphorylase family protein [*Trichomonas vaginalis* G3]	3 (3)	94.45	9127.21	2.96E+04	1.04E-04	gi|121891200
		3.18	hypothetical protein TVAG_021890 [*Trichomonas vaginalis* G3]	2 (2)	98.37	1.90E+04	6.04E+04	8.14E-06	gi|121915453
		3.07	4-alpha-glucanotransferase family protein [*Trichomonas vaginalis* G3]	10 (10)	425.98	4.24E+04	1.30E+05	6.46E-07	gi|121895827
Exocytosis	Exocytosis	4.52	hypothetical protein TVAG_431000 [*Trichomonas vaginalis* G3]	2 (2)	30.31	1.38E+04	6.22E+04	4.30E-05	gi|121896819
		3.56	SNARE protein, putative [*Trichomonas vaginalis* G3]	2 (2)	94.78	1819.33	6479.78	2.51E-03	gi|121914867

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Glycolysis	Cellular metabolism	10.16	PEP-utilizing enzyme, TIM barrel domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	74.26	1978.28	2.01E+04	1.22E-03	gi|121914977
		4.07	enolase 4, putative [*Trichomonas vaginalis* G3]	3 (2)	88.69	5589.54	2.28E+04	4.60E-06	gi|121908406
Hydrolase		10.34	hypothetical protein TVAG_034780 [*Trichomonas vaginalis* G3]	2 (2)	34.23	1553.6	1.61E+04	7.15E-04	gi|121917683
		3.8	Clan CA, family C19, ubiquitin hydrolase-like cysteine peptidase [*Trichomonas vaginalis* G3]	4 (4)	68.7	1.17E+04	4.46E+04	5.81E-05	gi|121884693
Hydrolase, protein phosphatase		5.06	protein phosphatase 2C, putative [*Trichomonas vaginalis* G3]	2 (2)	29.1	2.42E+04	1.23E+05	1.39E-03	gi|121905006
		3.37	phosphatase, putative [*Trichomonas vaginalis* G3]	3 (3)	110.32	6005.06	2.02E+04	2.59E-08	gi|121888987
Intracellular peptidase		13.16	Clan CA, family C19, ubiquitin hydrolase-like cysteine peptidase [*Trichomonas vaginalis* G3]	2 (2)	26.71	1.22E+04	1.60E+05	1.29E-07	gi|121898331
Kinase, transferase		6.9	pyruvate, phosphate dikinase family protein [*Trichomonas vaginalis* G3]	9 (9)	562.62	1.32E+04	9.12E+04	1.36E-08	gi|121904065
Membrane transport		14.96	Beige/BEACH domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	27.22	2461.94	3.68E+04	8.64E-08	gi|121916866
		12.66	Beige/BEACH domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	30.24	2100.93	2.66E+04	5.65E-05	gi|121893763
Nucleotide binding		6.27	hypothetical protein TVAG_069480 [*Trichomonas vaginalis* G3]	2 (2)	30.21	2.57E+04	1.61E+05	3.71E-05	gi|121901569
		3.3	hypothetical protein TVAG_447350 [*Trichomonas vaginalis* G3]	2 (2)	43.77	1.69E+04	5.56E+04	6.27E-05	gi|121911949
Nucleus regulation	DNA replication	7.86	SNF2 family N-terminal domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	28.25	1.23E+05	9.67E+05	1.25E-05	gi|121903240

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Peptidase	Cellular metabolism	4.88	Clan CA, family C2, calpain-like cysteine peptidase [*Trichomonas vaginalis* G3]	3 (3)	44.02	3.07E+04	1.50E+05	3.37E-04	gi|121907418
		18.33	hybrid-cluster protein, putative [*Trichomonas vaginalis* G3]	2 (2)	32.17	1467.22	2.69E+04	8.26E-06	gi|121908608
Phosphoric ester hydrolase		12.12	SacI homology domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	35.91	7081.01	8.59E+04	1.50E-05	gi|121895312
Proteasome regulation		5.92	hypothetical protein TVAG_105990 [*Trichomonas vaginalis* G3]	2 (2)	30.71	2130.53	1.26E+04	1.90E-03	gi|121882893
		5.61	hypothetical protein TVAG_461850 [*Trichomonas vaginalis* G3]	2 (2)	39.8	865.64	4857.22	5.06E-03	gi|121890016
Protein binding		17.97	hypothetical protein TVAG_201640 [*Trichomonas vaginalis* G3]	2 (2)	52.48	507.58	9119.92	4.14E-06	gi|121910322
Protein catabolism		11.64	hypothetical protein TVAG_202850 [*Trichomonas vaginalis* G3]	2 (2)	38.18	1.32E+04	1.53E+05	2.75E-03	gi|121900799
Protein kinase		10.21	TKL family protein kinase [*Trichomonas vaginalis* G3]	3 (3)	48.39	5.12E+04	5.23E+05	5.30E-07	gi|121890017
Protein metabolism		27.1	TKL family protein kinase [*Trichomonas vaginalis* G3]	2 (2)	36.59	4166.16	1.13E+05	3.45E-07	gi|121888267
		10.22	DnaJ domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	61.26	2597.33	2.66E+04	6.84E-05	gi|121911862
		5.05	Armadillo/beta-catenin-like repeat family protein [*Trichomonas vaginalis* G3]	2 (2)	26.7	1.56E+04	7.89E+04	2.14E-08	gi|121911734
Protein synthesis		29.42	NAC domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	125.14	2206.15	6.49E+04	2.64E-06	gi|121897587
		4.64	adenylate kinase proprotein [*Trichomonas vaginalis*]	5 (5)	466.86	6.03E+04	2.80E+05	1.47E-07	gi|463923
		4.52	ribosomal protein L10a [*Trichomonas vaginalis*]	2 (2)	136.77	4.43E+04	2.00E+05	2.10E-04	gi|4193363

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Protein synthesis	Cellular metabolism	3.69	hypothetical protein TVAG_021170 [*Trichomonas vaginalis* G3]	11 (11)	185	4.37E+04	1.61E+05	1.35E-03	gi|121915383
		3.02	elongation factor 1 alpha, partial [*Trichomonas vaginalis*]	11 (11)	526.1	4.75E+05	1.44E+06	9.55E-05	gi|329750813
Protein-protein interaction		24.43	ankyrin repeat protein. putative [*Trichomonas vaginalis* G3]	2 (2)	26.85	1.23E+04	3.00E+05	7.38E-04	gi|121912911
		17.68	Leucine Rich Repeat family protein [*Trichomonas vaginalis* G3]	2 (2)	33.96	1.31E+04	2.32E+05	2.80E-10	gi|121915949
		5.76	conserved hypothetical protein [*Trichomonas vaginalis* G3]	2 (2)	29.35	2.84E+04	1.63E+05	9.12E-03	gi|121880471
		5.61	B-box zinc finger family protein [*Trichomonas vaginalis* G3]	2 (2)	30.34	1.00E+04	5.62E+04	4.39E-09	gi|121906335
		5.58	hypothetical protein TVAG_295630 [*Trichomonas vaginalis* G3]	2 (2)	32.38	2193.21	1.22E+04	1.12E-03	gi|121895859
		5.47	hypothetical protein TVAG_272350 [*Trichomonas vaginalis* G3]	2 (2)	32.55	2393.81	1.31E+04	3.65E-03	gi|121891581
		5.22	erythrocyte binding protein. putative [*Trichomonas vaginalis* G3]	4 (4)	181.54	2.41E+04	1.26E+05	1.63E-08	gi|121898736
		3.73	ankyrin repeat protein. putative [*Trichomonas vaginalis* G3]	2 (2)	26.8	3.81E+04	1.42E+05	6.19E-06	gi|121900474
		3.14	hypothetical protein TVAG_072710 [*Trichomonas vaginalis* G3]	2 (2)	37.26	2.11E+04	6.62E+04	7.79E-04	gi|121884184
Ribosomal protein	Cellular component	4.87	hypothetical protein TVAG_183210 [*Trichomonas vaginalis* G3]	2 (2)	28.68	3040.94	1.48E+04	1.96E-04	gi|121918316
Signal transduction	Signal transduction	6.47	ARF GAP-like zinc finger-containing protein [*Trichomonas vaginalis* G3]	2 (2)	51.31	8180.38	5.29E+04	3.75E-06	gi|121912557
		3.8	Ras family protein [*Trichomonas vaginalis* G3]	4 (4)	273.64	3.51E+04	1.33E+05	4.79E-08	gi|121900431

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Translation	Translation	7.53	hypothetical protein TVAG_456340 [*Trichomonas vaginalis* G3]	2 (2)	31.98	1.58E+04	1.19E+05	6.78E-07	gi|121917224
		6.9	hypothetical protein TVAG_369910 [*Trichomonas vaginalis* G3]	2 (2)	30.06	5705.69	3.93E+04	2.06E-05	gi|121897649
		5.99	RhoGEF domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	37.87	6003.78	3.60E+04	1.62E-05	gi|121917299
		5.27	eukaryotic release factor 3 GTPase subunit [*Trichomonas vaginalis*]	3 (3)	116.13	3470.55	1.83E+04	7.97E-04	gi|8307949
		4.68	RhoGAP domain containing protein [*Trichomonas vaginalis* G3]	2 (2)	32.11	1.10E+05	5.12E+05	1.22E-05	gi|121898182
		3.99	translation initiation factor eIF-5A family protein [*Trichomonas vaginalis* G3]	2 (2)	108.14	6.24E+04	2.49E+05	2.43E-05	gi|121876621
Translation of a protein for the ER	Cellular component	4.91	SRP54-type protein. putative [*Trichomonas vaginalis* G3]	4 (4)	103.41	3.00E+04	1.48E+05	3.56E-03	gi|121902866
Unknown	Unknown	Infinity	hypothetical protein TVAG_178450 [*Trichomonas vaginalis* G3]	2 (2)	29.49	0	7837.09	1.72E-11	gi|121915008
		47.27	hypothetical protein TVAG_172100 [*Trichomonas vaginalis* G3]	2 (2)	34.84	660.81	3.12E+04	2.90E-07	gi|121916171
		36.76	hypothetical protein TVAG_281980 [*Trichomonas vaginalis* G3]	2 (2)	38.32	35.72	1313.32	5.59E-03	gi|121905674
		36.55	hypothetical protein TVAG_191350 [*Trichomonas vaginalis* G3]	3 (3)	46.76	969.61	3.54E+04	1.95E-07	gi|121899991
		28.38	hypothetical protein TVAG_306240 [*Trichomonas vaginalis* G3]	2 (2)	29.34	2.53E+04	7.19E+05	1.32E-08	gi|121913292
		15.37	hypothetical protein TVAG_079530 [*Trichomonas vaginalis* G3]	2 (2)	31.49	3142.31	4.83E+04	2.97E-05	gi|121903733
		15.36	hypothetical protein TVAG_274870 [*Trichomonas vaginalis* G3]	3 (3)	45.93	1.29E+04	1.99E+05	1.12E-04	gi|121905190

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Unknown	Unknown	14.89	hypothetical protein TVAG_226060 [*Trichomonas vaginalis* G3]	2 (2)	32.47	2202.86	3.28E+04	4.29E-06	gi|121886529
		13.24	hypothetical protein TVAG_198390 [*Trichomonas vaginalis* G3]	2 (2)	50.17	1.66E+04	2.19E+05	7.01E-07	gi|121916622
		9.82	hypothetical protein TVAG_452000 [*Trichomonas vaginalis* G3]	2 (2)	30.83	1779.86	1.75E+04	4.25E-04	gi|121914488
		9.32	hypothetical protein TVAG_311190 [*Trichomonas vaginalis* G3]	2 (2)	35.21	1147.38	1.07E+04	6.13E-05	gi|121885740
		9.21	hypothetical protein TVAG_394820 [*Trichomonas vaginalis* G3]	5 (5)	88.08	6.42E+04	5.92E+05	2.13E-03	gi|121904361
		8.68	hypothetical protein TVAG_094340 [*Trichomonas vaginalis* G3]	2 (2)	35.35	4652.59	4.04E+04	9.08E-06	gi|121917485
		8.54	hypothetical protein TVAG_223640 [*Trichomonas vaginalis* G3]	4 (4)	77.95	1.34E+05	1.14E+06	2.31E-04	gi|121902309
		8.4	viral A-type inclusion protein, putative [*Trichomonas vaginalis* G3]	2 (2)	40.75	4.78E+04	4.01E+05	3.14E-07	gi|121889035
		7.83	surface antigen BspA-like [*Trichomonas vaginalis* G3]	2 (2)	33.59	1.03E+04	8.08E+04	6.08E-08	gi|121908345
		7.07	hypothetical protein TVAG_475480 [*Trichomonas vaginalis* G3]	3 (3)	48.62	8793.86	6.22E+04	1.79E-06	gi|121917849
		6.7	hypothetical protein TVAG_097910 [*Trichomonas vaginalis* G3]	5 (5)	90.12	1.91E+04	1.28E+05	3.11E-04	gi|121908343
		6.15	hypothetical protein TVAG_165240 [*Trichomonas vaginalis* G3]	2 (2)	54.28	9903.05	6.09E+04	1.54E-04	gi|121911058
		6.1	hypothetical protein TVAG_278990 [*Trichomonas vaginalis* G3]	2 (2)	27.14	6225.32	3.80E+04	7.04E-04	gi|121880711
		5.71	hypothetical protein TVAG_339270, partial [*Trichomonas vaginalis* G3]	2 (2)	66.29	1.21E+04	6.90E+04	5.19E-06	gi|121893354

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Unknown	Unknown	5.51	hypothetical protein TVAG_211540 [*Trichomonas vaginalis* G3]	2 (2)	28.63	2.88E+04	1.59E+05	1.02E-05	gi|121901696
		5.34	hypothetical protein TVAG_453100 [*Trichomonas vaginalis* G3]	2 (2)	31.75	2.24E+04	1.19E+05	3.70E-07	gi|121899431
		5.03	hypothetical protein TVAG_258250 [*Trichomonas vaginalis* G3]	2 (2)	28.53	9797.97	4.93E+04	4.65E-06	gi|121905903
		5.02	hypothetical protein TVAG_014360 [*Trichomonas vaginalis* G3]	2 (2)	41.88	4538.25	2.28E+04	5.52E-04	gi|121916891
		4.94	viral A-type inclusion protein. putative [*Trichomonas vaginalis* G3]	2 (2)	66.12	2.31E+04	1.14E+05	1.37E-04	gi|121888635
		4.68	surface antigen BspA-like [*Trichomonas vaginalis* G3]	2 (2)	35.3	4.00E+04	1.87E+05	1.12E-08	gi|121904158
		4.56	hypothetical protein TVAG_450670 [*Trichomonas vaginalis* G3]	2 (2)	29.81	6571.51	2.99E+04	1.22E-05	gi|121898402
		4.37	hypothetical protein TVAG_053070 [*Trichomonas vaginalis* G3]	2 (2)	37.07	6.74E+04	2.95E+05	9.74E-05	gi|121894239
		4.29	hypothetical protein TVAG_234990 [*Trichomonas vaginalis* G3]	2 (2)	28.42	1.47E+04	6.28E+04	5.00E-07	gi|121912801
		4.2	conserved hypothetical protein [*Trichomonas vaginalis* G3]	2 (2)	27.32	5.01E+04	2.10E+05	1.70E-05	gi|121882494
		4.15	hypothetical protein TVAG_182030 [*Trichomonas vaginalis* G3]	2 (2)	31.56	5606.64	2.33E+04	3.68E-08	gi|121894117
		4.04	hypothetical protein TVAG_410430 [*Trichomonas vaginalis* G3]	2 (2)	38.16	1.17E+04	4.74E+04	2.48E-07	gi|121906124
		4.01	hypothetical protein TVAG_042770 [*Trichomonas vaginalis* G3]	2 (2)	27.67	4.14E+04	1.66E+05	8.24E-04	gi|121887453
		4	hypothetical protein TVAG_101120 [*Trichomonas vaginalis* G3]	2 (2)	39.06	1.80E+04	7.21E+04	5.98E-08	gi|121914597

Predicted function	Category	Fold change	Description	P	Score	P178-13	R17-12	*p*-value	Accession number
Unknown	Unknown	3.92	hypothetical protein TVAG_124350 [*Trichomonas vaginalis* G3]	2 (2)	38.81	8475.14	3.32E+04	2.03E-04	gi|121884217
		3.66	hypothetical protein TVAG_083270 [*Trichomonas vaginalis* G3]	2 (2)	39.28	1.45E+04	5.32E+04	7.45E-05	gi|121913667
		3.6	hypothetical protein TVAG_108950 [*Trichomonas vaginalis* G3]	3 (3)	62.25	4.53E+04	1.63E+05	2.32E-05	gi|121896591
		3.51	hypothetical protein TVAG_252720 [*Trichomonas vaginalis* G3]	2 (2)	29.49	1.12E+04	3.93E+04	2.44E-04	gi|121910614
		3.47	hypothetical protein TVAG_031830 [*Trichomonas vaginalis* G3]	3 (2)	54.04	1.53E+05	5.31E+05	4.95E-07	gi|121898598
		3.45	hypothetical protein TVAG_181960 [*Trichomonas vaginalis* G3]	2 (2)	30.5	4116.95	1.42E+04	3.19E-05	gi|121894110
		3.34	hypothetical protein TVAG_472390 [*Trichomonas vaginalis* G3]	3 (3)	59.72	8958.55	2.99E+04	2.42E-06	gi|121894522
		3.33	hypothetical protein TVAG_098730 [*Trichomonas vaginalis* G3]	2 (2)	45.64	6053.97	2.01E+04	1.86E-04	gi|121901143
		3.23	hypothetical protein TVAG_476440 [*Trichomonas vaginalis* G3]	2 (2)	33.54	1.00E+04	3.24E+04	1.88E-03	gi|121917944
		3.13	viral A-type inclusion protein. putative [*Trichomonas vaginalis* G3]	4 (4)	82	2.54E+04	7.95E+04	2.46E-04	gi|121899272
Vesicle-mediated transport	Exocytosis	4.89	Adaptin N terminal region family protein [*Trichomonas vaginalis* G3]	3 (3)	78.26	2.51E+04	1.23E+05	2.09E-03	gi|121908762
		3.51	CMGC family protein kinase [*Trichomonas vaginalis* G3]	2 (2)	48.38	2.85E+04	1.00E+05	1.94E-03	gi|121882186

APPENDIX IV – Publication from the Doctoral Thesis.

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ORIGINAL ARTICLE

Genetic characterization of oropharyngeal trichomonad isolates from wild birds indicates that genotype is associated with host species, diet and presence of pathognomonic lesions

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Oropharyngeal trichomonad isolates of wild birds from Spain were studied. A total of 1688 samples (1214 of predator birds and 474 of prey species) from wildlife recovery centres and scientific bird-ringing campaigns were analysed from 2011 to 2013. The overall infection prevalence was 20.3% (11.4% in predator birds and 43.3% in prey species). Pathognomonic lesions were present in 26% of the infected birds (57.3% in predator birds and 4.9% in prey species). The most commonly parasitized species were the goshawk (*Accipiter gentilis*, 74.5%) and the rock pigeon (*Columba livia*, 79.4%). Host species in which the parasite has not been previously analysed by polymerase chain reaction and sequencing in Spain are also reported: *Columba palumbus*, *Streptopelia turtur*, *Pica pica*, *A. gentilis*, *Accipiter nisus*, *Asio otus*, *Bubo bubo*, *Buteo buteo*, *Circus aeruginosus*, *Circus cyaneus*, *Falco naumanni*, *Falco peregrinus*, *Neophron percnopterus*, *Otus scops*, *Pernis apivorus* and *Strix aluco*. Sequence analysis of the ITS1/5.8S/ITS2 region revealed five different genotypes and also some mixed infections. A relationship between genotype and host species was observed, but only two genotypes (ITS-OBT-Tg-1and ITS-OBT-Tg-2) were widely distributed. Genotype ITS-OBT-Tg-1 was most frequently found in predator birds and statistically associated with pathognomonic lesions. Non-strict ornithophagous species were at higher risk to develop disease than ornithophagous ones. Genotypes ITS-OBT-Tcl-1 and ITS-OBT-Tcl-2 are new reports, and ITS-OBT-Tvl-5 is reported for the first time in Spain. They showed higher genetic homology to *Trichomonas canistomae* and *Trichomonas vaginalis* than to *Trichomonas gallinae*, indicating the possibility of new species within this genus.

Introduction

Avian trichomonosis is a parasitic disease caused mainly by the protozoan *Trichomonas gallinae* (Rivolta, 1878), although it has been recently established that other trichomonads can produce the disease (Anderson *et al.*, 2009; Girard *et al.*, 2014a, b). This flagellated parasite infects the upper digestive tract of birds, mainly the oropharynx, crop and oesophagus, where it is attached to the mucosa and multiplies by binary fission. The parasite induces erosions, ulcers and caseous necrotic granulomas in the epithelium of the digestive tract. Clinical signs of infection can vary from asymptomatic to dysphagia, regurgitation, sialorrhea/ptyalism and even death by starvation, because birds become unable to feed. Some isolates/species are able to induce necrotic foci on internal organs, such as the liver, and severe cases can be lethal as early as 4 days after infection (revised in Amin *et al.*, 2014). The reservoir hosts for *T. gallinae* are columbid species. The transmission is maintained in the wild by predation and necrophagy from raptors or by sharing contaminated food or water sources between columbids and other birds (finches; Lawson *et al.*, 2011a, b). The most frequent presentation is an endemic process with high morbidity but low mortality rates on adult columbids, although occasional outbreaks of the disease causing high mortality have been reported (revised in Amin *et al.*, 2014). In fact, trichomonosis has been detected as one of the concerns for the endangered pink pigeon (*Columba mayeri*) in Mauritius and for Bonelli's eagle (*Aquila fasciata*) in Spain and Portugal (Höfle *et al.*, 2000; Real *et al.*, 2000; Bunbury *et al.*, 2007). Recently, it has been

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described as an emerging disease for passerines, as trichomonosis has been reported in epidemic focuses involving finch species in the UK, Canada, Slovenia and Austria as well as other European countries (reviewed in Amin *et al*., 2014).

Prevalence values vary depending on the species and geographic location, but high levels of infection (86%) have been found in columbids such as the Eurasian collared dove (*Streptopelia decaocto*) and the European turtle dove (*Streptopelia turtur*) in England and the rock pigeon (*Columba livia*) (44.8%) in Spain (Sansano-Maestre *et al.*, 2009; Lennon *et al.*, 2013). High prevalence in raptors has been reported with values of 85% in ornithophagous species like the Cooper's hawk (*Accipiter cooperii*) and 65.1% in goshawks (*Accipiter gentilis*) (Krone *et al.*, 2005). This parasite constitutes an important cause of infectious mortality for raptor nestlings, especially in urban areas where columbids are their main prey (Cooper & Petty, 1988; Rutz *et al.*, 2006; Molina-López *et al.*, 2011; Chi *et al.*, 2013).

The most frequently used region to study intra-specific variation within the *Trichomonadidae* is ITS1/5.8S/ITS2, composed of the 5.8S large subunit of the ribosomal RNA and the adjacent introns ITS1 and ITS2 (Kleina *et al*., 2004). This region is widely used for phylogenetic purposes and is recognized as a strong molecular tool to determine inter-specific and intra-specific diversity (revised in Amin *et al*., 2014).

A recent study characterized isolates of *T. gallinae* and described 12 different genotypes of the ITS1/5.8S/ITS2 region across the USA (Gerhold *et al.*, 2008). In Europe, other studies have revealed the existence of six variants for this region (Grabensteiner *et al.*, 2010). Two genotypes were predominant in wild birds in Spain, while in Britain three genotypes have so far been found (Sansano-Maestre *et al*., 2009; Chi *et al.*, 2013).

One of the latest epidemic reports in columbids, with 15 to 20% mortality rate, was produced by two different pathogenic isolates in the Caribbean, one of them described as *Trichomonas*-like parabasalid (Stimmelmayr *et al.*, 2012). Ecco *et al.* (2012) found five different pathogenic trichomonads on raptors and passerines in Brazil, belonging to genotypes of *T. gallinae*, *Trichomonas vaginalis*-like and similar to the newly described species *Simplicimonas similis*.

The existence of isolates with higher similarity to *T. vaginalis* and *Trichomonas tenax* in Europe, Brazil and the USA has also been reported, indicating the possibility of different species of trichomonads in the avian population (Gerhold *et al.*, 2008; Grabensteiner *et al.*, 2010; Ecco *et al.*, 2012). Recently, Girard *et al.* (2014a) identified a new species (*Trichomonas stableri*) of the parasite involved in mortality events of band-tailed pigeons (*Patagioenas fasciata monilis*) in the USA. This latest discovery reflects the large diversity of oral trichomonads present on bird species and points out the need for further investigations in this area.

The genetic variability of the parasite and the correlation with the clinical presentation have been poorly studied. Previous studies carried out in Spain and the UK, employing sequencing of the ITS1/5.8S/ITS2 region, have shown a relationship between the genetic variant and the presence of pathognomonic lesions (Sansano-Maestre *et al.*, 2009; Lawson *et al.*, 2011a; Chi *et al*., 2013; Girard *et al.*, 2014b). In fact, only one clonal strain was detected in the British outbreaks, where it was estimated that 500,000 birds died directly from trichomonosis (Robinson *et al.*, 2010; Lawson *et al.*, 2011a).

The objectives of our research were to provide information about the occurrence and presence of genetic variants of oropharyngeal trichomonads among wild bird species in samples obtained from the birds at scientific ringing campaigns, clinical cases and admissions from some of the most important wildlife recovery centres in Spain. The relationships between genotype, host species, diet and presence of pathognomonic lesions have been analysed.

Materials and Methods

Sampling and geographic location. Before sampling, a letter asking for collaboration was sent to some of the most important wildlife recovery centres of Spain (those having a large number of admissions) and bird-ringing scientists of nearby areas (Valencian Community, Murcia and Madrid). The geographical location of the centres is shown in Figure 1. A total of 1688 samples were analysed from 2011 to 2013, except two samples from goshawks obtained in 2007 that were included in the analysis because they showed a distinct genotype.

Wildlife recovery centres. Wildlife recovery centres located in the provinces of Alicante, Barcelona, Cuenca, Murcia, Toledo and Zaragoza submitted samples from the avian patients that showed trichomonosis-like lesions (Figure 2). Samples were taken with a sterile cotton swab previously moistened with physiologic saline solution that was gently rubbed over the oropharyngeal cavity and crop. The diagnosis was made by culture using InPouch™ TF Feline pockets (BioMed Diagnostics Inc, White City, Oregon, USA) (Bunbury *et al.*, 2007; Anderson *et al.*, 2009; Girard *et al.*, 2014a). InPouch™ pockets were inoculated with the oral swab and incubated at 37ºC during 48 h prior to the submission of the samples to the laboratories of the Veterinary Faculties of University CEU Cardenal Herrera (Moncada, Valencia) or University Complutense of Madrid, Spain.

A systematic review of all admissions was carried out at the wildlife recovery centres of Grupo de Rehabilitación de la Fauna Autóctona y su Hábitat (GREFA) in Madrid, "La Granja de El Saler" in Valencia and the breeding centre for lesser kestrel (*Falco naumanni*) of Medio Ambiente (DEMA) in Badajoz. Trypticase–maltose–yeast extract (TYM) medium was used for the screening of oral trichomonads (Diamond, 1957).

Scientific ringing campaigns. Adults and juvenile birds of both raptors and passerine granivorous species were tested by culture using TYM medium. Samples were submitted to the above-mentioned laboratories during the 24 h following collection. Scientific ringing campaigns were scheduled by local ornithological organizations: Naturalistas del Sureste (ANSE) in the province of Murcia; Pit-Roig, Albufera and Monticola ringing groups from the CMA-SEO/Birdlife in the provinces of Valencia and Madrid; and Sociedad Valenciana de Ornitología in the province of Valencia. Special authorizations were obtained for the capture and ringing of raptor nestlings (goshawk and Eurasian eagle-owl, *Bubo bubo*) that were included in different research projects. Due to the recent outbreaks and spread of the infection among Passeriformes in northern Europe (Lawson *et al.*, 2011b), some of these species were also studied: greenfinch (*Carduelis chloris*), chaffinch (*Fringilla coelebs*) and Eurasian blue tit (*Cyanistes caeruleus*).

Parasite culture. Oropharyngeal swab samples were inoculated in the medium (TYM/Inpouch™), incubated at 37°C and examined daily during 15 days using an inverted microscope to control the presence of motile trophozoites. Subsequent passages in TYM medium were done to perform axenization of the parasite. The composition of the medium was as follows: 20 g trypticase (Sigma-Aldrich, St. Louis, MO, USA), 10 g D(+)-maltose (Sigma-Aldrich), 10 g yeast extract (Sigma-Aldrich), 1 g L-cysteine (Sigma-Aldrich), 0.1 g ascorbic acid (Sigma-Aldrich) and 10% inactivated foetal bovine serum (Sigma-Aldrich) per litre. After mixing all of the ingredients, the pH was adjusted to 6 and sterilized by filtration through a filter of 0.22 μm (Millipore, Billerica, MA, USA). Antibiotics and antimycotic supplements were used to avoid contamination: 24 ml nystatin/l (10,000 IU/ml; Sigma-Aldrich) and 36 mg/l each of ticarcillin, vancomycin and ceftiofur (Sigma-Aldrich) were added. Storage at –80ºC

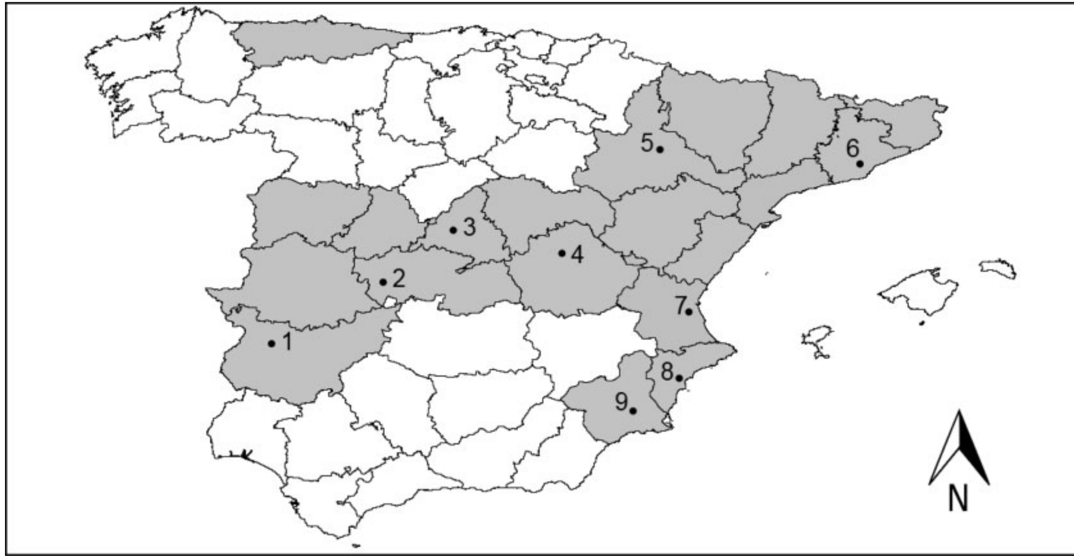

Figure 1. *Map of Spain showing the origin of the sampled birds. Shaded areas correspond to areas of coverage of recovery centres (admissions). Dots indicate the main wildlife recovery centres: 1, DEMA (Almendralejo, Badajoz); 2, CERI (Sevilleja de la Jara, Toledo); 3, GREFA (Majadahonda, Madrid); 4, El Ardal (Albendea, Cuenca); 5, La Alfranca (Pastriz, Zaragoza); 6, Torreferrusa (Santa Perpétua de Mogoda, Barcelona); 7, La Granja (El Saler, Valencia); 8, Santa Faz (Santa Faz, Alicante); 9, El Valle (La Alberca, Murcia).*

using 5% dimethyl sulphoxide (Sigma-Aldrich) in TYM medium was used to preserve the isolates for further analysis.

DNA extraction. Genomic DNA was extracted from parasite cultures using a commercial DNA extraction kit (DNeasy Blood and Tissue Extraction Kit; QIAGEN, Valencia, California, USA). Parasites were recovered from 1 ml culture (ranging from 40,000 to 1,200,000 trophozoites) and centrifuged at $350 \times g$ for 3 min. The supernatant was discarded and the pellet was washed twice in phosphate-buffered saline, pH 7.4. The pellet was processed following the manufacturer's instructions. DNA was stored at –20°C until use.

Polymerase chain reaction of the fragment ITS1/5.8S/ITS2. Polymerase chain reaction (PCR) analysis was carried out using primers TFR1 (5′-TGCTTCAGCTCAGCGGGTCTTCC-3′) and TFR2 (5′-CGGTAGGT-GAACCTGCCGTTGG-3′) (Felleisen, 1997). The reaction was done in a final volume of 50 µl, containing 5 µl of 10 × buffer, 1.5 mM $MgCl_2$, 2 mM dNTP, 2 µM each primer, 2.5 iu Taq polymerase (MP, Thomas Scientific, Swedesboro, NJ, USA) and 5 µl genomic DNA. The PCR protocol started with an initial step to activate the enzyme at 95°C for 9 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 66°C for 30 sec, extension at 72°C for 30 sec and a final extension step at 72°C for 15 min. Electrophoresis was done in a 1.5% agarose gel stained with ethidium bromide (0.5 µl/ml) at 80 V for 35 min. Ten microlitres of each sample were loaded and gels were observed under ultraviolet light.

Sequence analysis and phylogenetic trees. PCR amplification products were purified with a commercial kit (MinElute PCR Purification Kit; QIAGEN) and submitted for sequencing to the laboratories Sistemas Genómicos, S.A. (Paterna, Valencia, Spain). The reaction was completed in an automatic sequencer (3730XL DNA Analyzer; Applied Biosystems, Foster City, CA, USA) with the ABI PRISM® BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems). Chromatograms were manually checked in both directions and assembled using Lasergene SeqMan software version 7.0.0 (DNASTAR, Madison, WI, USA). Nucleotide Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi/) version 2.2.29 was used to compare the identity of the sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Parameters were set for the Mega BLAST algorithm (optimization for highly similar sequences) and low complexity region filter.

A total of 83 isolates were analysed by PCR and sequencing of the ITS1/5.8S/ITS2 region (Tables 1 and 2). Table 3 presents the genotypes previously described by other authors and the new genotypes found in this study. We used a codification system based on BLAST sequence homology results to clarify the genetic group considering the genotypes found in the literature. The code (ITS-OBT-Tx-n) indicates the region of the genome (ITS, ITS1/5.8S/ITS2), source (OBT, oropharyngeal bird

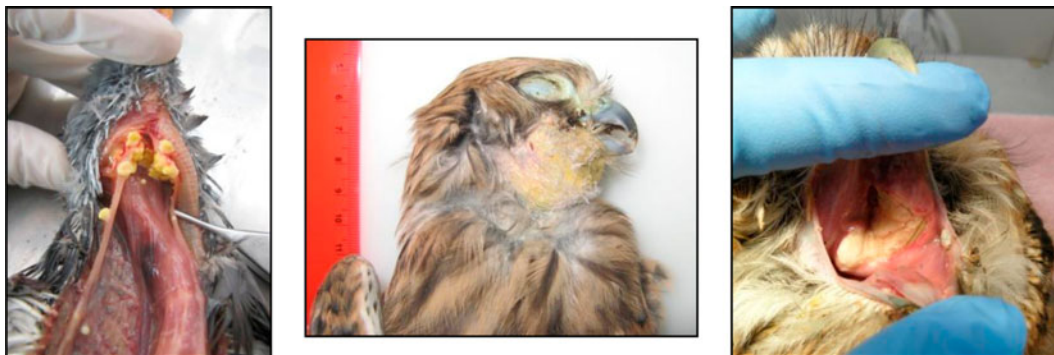

Figure 2. *From left to right: pathognomonic lesions of avian trichomonosis in wood pigeon (*Columba palumbus*), common kestrel (*Falco tinnunculus*) and Eurasian tawny owl (*Strix aluco*)*. Isolates P349/12, R164/12 and R198/13, respectively. *Photograph by Amalia García Talens*

Table 1. *Species of birds analysed in this study.*

Species	Common name	*n*	*P* (%)	*L* (%)	Type of host	Diet
Accipiter gentilis	Goshawk	55	74.5	0	Predator	O
Accipiter nisus	Eurasian sparrowhawk	18	27.8	27.8	Predator	O
Aegypius monachus	Monk vulture	30	0	0	Predator	NO
Aquila adalberti	Spanish imperial eagle	3	0	0	Predator	NO
Aquila chrysaetos	Golden eagle	25	4	8	Predator	NO
Aquila fasciata	Bonelli's eagle	21	0	4.8	Predator	NO
Aquila pennata	Booted eagle	19	21.1	21.1	Predator	NO
Ardea cinerea	Grey heron	1	0	0	Predator	NO
Asio flammeus	Short-eared owl	2	0	0	Predator	NO
Asio otus	Long-eared owl	11	27.3	27.3	Predator	NO
Athene noctua	Little owl	80	0	0	Predator	NO
Bubo bubo	Eagle owl	57	5.3	5.3	Predator	NO
Buteo buteo	Common buzzard	34	8.8	11.8	Predator	NO
Ciconia ciconia	White stork	1	0	0	Predator	NO
Circaetus gallicus	Short-toed eagle	3	0	0	Predator	NO
Circus aeruginosus	Marsh harrier	11	18.2	9.1	Predator	NO
Circus cyaneus	Hen harrier	7	28.6	0	Predator	NO
Circus pygargus	Montagu's harrier	35	0	0	Predator	NO
Columba livia	Rock pigeon	34	79.4	0	Prey	
Columba palumbus	Wood pigeon	10	70	60	Prey	
Coturnix coturnix	Common quail	1	0	0	Prey	
Falco columbarius	Merlin	3	0	0	Predator	NO
Falco naumanni	Lesser kestrel	249	4.4	0.8	Predator	NO
Falco peregrinus	Peregrine falcon	24	4.2	12.5	Predator	O
Falco subbuteo	Hobby	2	0	0	Predator	NO
Falco tinnunculus	Common kestrel	373	14.2	9.4	Predator	NO
Gallinula chloropus	Moorhen	1	0	0	Prey	
Garrulus glandarius	Eurasian jay	2	0	0	Predator	NO
Gyps fulvus	Griffon vulture	50	0	2	Predator	NO
Ixobrychus minutus	Little bittern	1	0	0	Predator	NO
Larus fuscus	Lesser black-backed gull	1	0	nd	Predator	NO
Melopsittacus undulatus	Budgerigar	1	100	100	Prey	
Milvus migrans	Black kite	11	0	9.1	Predator	NO
Milvus milvus	Red kite	9	0	11.1	Predator	NO
Myiopsitta monachus	Monk parakeet	3	0	0	Prey	
Neophron percnopterus	Egyptian vulture	2	50	0	Predator	NO
Otus scops	Eurasian scops owl	12	8.3	8.3	Predator	NO
Passerine species[a]		132	0	0	Prey	
Pernis apivorus	European honey-buzzard	4	25	0	Predator	NO
Pica pica	Magpie	4	25	25	Predator	NO
Streptopelia decaocto	Eurasian collared dove	289	57.8	1	Prey	
Streptopelia turtur	European turtle dove	3	100	0	Prey	
Strix aluco	Tawny owl	18	16.7	44.4	Predator	NO
Tyto alba	Barn owl	36	5.6	8.3	Predator	NO
Total		1688	20.3	5.3		

Total number of samples (*n*), percentage of positive cultures (*P*) and birds with pathognomonic gross lesions for trichomonosis (*L*). Diet (ornithophagous [O]/non-strict ornithophagous [NO]) and type of bird (predator/prey) are also shown. [a]Passerine species included goldfinch (*Carduelis carduelis*), greenfinch (*Carduelis chloris*), blue tit (*Cyanistes caeruleus*), reed bunting (*Emberiza schoeniclus*), common chaffinch (*Fringilla coelebs*), great tit (*Parus major*), house sparrow (*Passer domesticus*), European serin (*Serinus serinus*), spotless starling (*Sturnus unicolor*) and blackbird (*Turdus merula*).

Trichomonadida), organism homology group (Tcl, *Trichomonas canistomae*-like; Tg, *T. gallinae*; Ts, *T. stableri*; Trichomonadida spp.; Tt, *T. tenax*; Tvl, *T. vaginalis*-like) and subgroup number (n = 1, 2, etc.; a new number was employed when genetic homology was less than 99%). Sequences less than 200 base pairs were not included in the table.

To construct the phylogenetic trees, sequences were selected by their different genotype and the presence of gross lesions. A total of seven isolates were used, with GenBank accession numbers KF993691, KF993692, KF993700, KF993701 and KF993705 to KF993707 (Supplementary Table 1). Sequences of the ITS1/5.8S/ITS2 region published by other authors available in the GenBank database and with a minimum length of 290 nucleotides were included (Felleisen, 1997; Kutisova *et al.*, 2005; Gaspar da Silva *et al.*, 2007; Gerhold *et al.*, 2008; Anderson *et al.*, 2009; Sansano-Maestre *et al.*, 2009; Grabensteiner *et al.*, 2010; Robinson *et al.*, 2010; Chi *et al.*, 2013; Girard *et al.*, 2014a) (Supplementary Table 2). A sequence of *Pentatrichomonas hominis* was also added as an out-group reference (Cepicka *et al.*, 2005). The analysis involved 26 nucleotide sequences. Codon positions included were first + second + third + noncoding. All of the positions containing gaps and missing data were eliminated. There was a total of 256 positions in the final dataset. Multiple alignment of the sequences was done employing the Clustal W algorithm of MEGA software version 5.05 (Tamura *et al.*, 2011). The average Jukes-Cantor distance was 0.094. Evolutionary distances were computed with the maximum composite likelihood method for the neighbour-joining tree and

Table 2. *Information about the* T. gallinae *isolates sequenced in this study.*

Species	Isolate	Lesion	Genetic group
Accipiter gentilis	RM3/12	No	ITS-OBT-Tg-2
	RM10/12	No	
	R256/11	No	
	R257/11	No	
	R43/12	No	
	R45/12	No	
	R57/12	No	
Aquila pennata	R134/12	Yes	
Asio otus	R137/12	Yes	
Buteo buteo	RM11/12	No	
	RM3/13	No	
Circus aeruginosus	R221/11	No	
Circus cyaneus	RM5/13	No	
Columba palumbus	PM1/12	Yes	
	P178/13	No	
	P179/13	No	
Falco naumanni	R11/13	No	
	R59/13	No	
	R66/13	No	
Falco peregrinus	RM1/12	Yes	
Falco tinnunculus	R100/11	No	
	R24/12	No	
	R25/12	No	
	R42/12	No	
Pernis apivorus	R237/13	No	
Streptopelia decaocto	PM2/12	Yes	
	P2/12	No	
	P139/12	No	
	P321/12	No	
	P329/12	No	
	P31/13	No	
	P33/13	No	
	P34/13	No	
	P38/13	No	
	P94/13	No	
Accipiter gentilis	RM7/13	No	ITS-OBT-Tg-1
	R64/11	No	
Accipiter nisus	RM13/12	No	
Aquila pennata	R213/11	Yes	
Asio otus	RM14/12	Yes	
Bubo bubo	R16/12	Yes	
	R17/12	Yes	
Buteo buteo	RM8/13	Yes	
Circus cyaneus	RM9/12	No	
Falco tinnunculus	RM5/12	Yes	
	RM6/12	Yes	
	RM7/12	Yes	
	RM8/12	Yes	
	R55/11	Yes	
	R72/11	Yes	
	R97/11	Yes	
	R99/11	Yes	
	R125/11	ND	
	R147/11	ND	
	R176/11	Yes	
	R179/11	Yes	
	R180/11	Yes	
	R189/11	Yes	
	R49/12	Yes	
	R127/12	Yes	
	R131/12	Yes	
	R191/13	Yes	

Table 2 (*Continued*)

Species	Isolate	Lesion	Genetic group
	R193/13	Yes	
	R198/13	Yes	
Melopsittacus undulatus	P112/13	Yes	
Pica pica	OM1/12	Yes	
Otus scops	R210/13	Yes	
Streptopelia decaocto	P96/11	No	
	P95/13	No	
Strix aluco	RM12/12	Yes	
Tyto alba	R183/13	Yes	
Streptopelia turtur	P243/12	No	ITS-OBT-Tcl-1
	P262/12	No	
	P196/13	No	
Accipiter gentilis	R30/07	No	ITS-OBT-Tcl-2
	R31/07	No	
Neophron percnopterus	R235/13	No	ITS-OBT-Tvl-5
Accipiter gentilis	R44/12	No	MIXED INFECTION (ITS-OBT-Tg-1/ITS-OBT-Tg-2)
Accipiter nisus	RM15/12	Yes	
Asio otus	R176/13	Yes	
Columba palumbus	PM2/13	No	
	P349/12	Yes	
Falco tinnunculus	R186/13	Yes	

ND, not determined.

with the Tamura-3 parameter model for the maximum likelihood tree (Figure 3 and Supplementary Figure 1). Bootstrap of 2000 replicates was employed to estimate tree reliability.

Statistical analysis. Analysis of risk factors was performed using categorical data of host bird species (predator bird/prey), presence of pathognomonic gross lesions for trichomonosis (other causes of lesions were not investigated), genotype (ITS-OBT-Tg-1/ITS-OBT-Tg-2) and diet in predator birds (ornitophagous/non-strict ornitophagous). The presence of lesions was not determined in two birds; therefore, two samples were excluded from the statistical analysis. Win Episcope software version 2.0 (http://www.clive.ed.ac.uk/winepiscope/) was used for the estimation of odds ratios (ORs) and 95% confidence intervals (CIs). The presence of interaction and confounding among variables were explored by stratification of the samples and estimation of the OR in each stratum and estimation of the Mantel–Haenszel test (OR_{MH}). Birds infected by more than one genotype (n = 6) and birds with non-frequent genotypes (genotypes ITS-OBT-Tcl-1, ITS-OBT-Tcl-2, ITS-OBT-Tvl-5, n = 5) were not employed in the stratification.

Sequence data. New sequences and sequences found in hosts not described previously were submitted to GenBank database with accession numbers KF993680 to KF993707 and KJ776739 to KJ776743 (Supplementary Table 1).

Results

Sampling and presence of lesions. We processed cultures from 1688 birds from 53 different species. Table 1 presents detailed information, including the number of samples, percentage of positive cultures and number of birds that displayed gross lesions. A total of 1214 of the samples were from predators: 17 species of the order Accipitriformes, seven of the order Strigiformes and five of the order Falconiformes. Within this group we have included samples of lesser black-backed gull (*Larus fuscus*, n = 1), white stork (*Ciconia ciconia*, n = 1), grey heron (*Ardea cinerea*, n = 1) and little bittern (*Ixobrychus minutus*, n = 1), as they are able to predate columbid chicks.

A total of 474 samples were obtained from different prey species, including 13 species of the order Passeriformes, four of the order Columbiformes, two of the order Psittaciformes, one of the order Galliformes and one of the order Gruiformes.

We found 20.3% of the birds positive for *Trichomonas* sp. infection, 11.4% of predator birds and 43.3% among prey species (60.7% if only columbid hosts are considered). No positive birds were found in finch species. Pathognomonic gross lesions of trichomonosis were present in 26% of the analysed birds, 57.3% of predator birds and 4.9% of prey species. The highest percentage of parasitized predator birds was observed in the goshawk (74.5%), and among prey species the rock pigeon showed the highest percentage of infection (79.4%), both species without birds displaying gross lesions.

Phylogenetic analysis. Five genotypes have been found in this study among the 83 sequences from the isolates; two of them reported frequently in the literature (ITS-OBT-Tg-1 and ITS-OBT-Tg-2; see Table 3), one of them reported previously in two papers (genotype ITS-OBT-Tvl-5; Grabensteiner *et al.*, 2010; Kelly-Clark *et al.*, 2013), and two new genotypes not reported previously (ITS-OBT-Tcl-1 and ITS-OBT-Tcl-2; Tables 2 and 3, Figure 3, Supplementary Table 1, and Supplementary Figure 1).

The phylogenetic trees constructed support the cluster of oral bird trichomonad isolates found in the literature into six clades (Figure 3 and Supplementary Figure 1): one grouping *T. gallinae* isolates (two sub-clades), one highly similar to *T. canistomae*, one highly similar to *T. tenax*, one grouping sequences KC529665 and FN433473, one including only sequence EU215359, and the last grouping sequences highly similar to *T. vaginalis* (three sub-clades). Sequences from our isolates belong to different genetic groups included in three of the clades: two sequences in the

Table 3. *Genotypes of the ITS1/5.8SS/ITS2 region of oral* Trichomonas *sp. isolated from birds described by other authors and genetic group codification used in this paper (ITS-OBT-Tx-n).*

Genotype						
Gerhold *et al.*	Grabensteiner *et al.*	Chi *et al.*	Sansano-Maestre *et al.*	Genbank sequences (author)	Organism sequence homology (% of BLAST identity, E-value)	Genetic group
	IV[a]	A[a]	B[a]	AY349182 (Kleina *et al.*)	*T. gallinae* AY349182 (Kleina *et al.*) (100%, 1e-154)	ITS-OBT-Tg-1
				EF208019 (Gaspar da Silva *et al.*)	*T. gallinae* AY349182 (Kleina *et al.*) (100%, 4e-108)	
A[a]				EU215369 (Gerhold *et al.*)[a]	*T. gallinae* EU881911 (Sansano-Maestre *et al.*) (100%, 9e-146)	
				EU290649 (Anderson *et al.*)[a]	*T. gallinae* EU881911 (Sansano-Maestre *et al.*) (100%, 1e-159)	
				FN433476 (Grabensteiner *et al.*)[a]	*T. gallinae* AY349182 (Kleina *et al.*) (100%, 1e-154)	
				GQ150752 (Robinson *et al.*)[a]	*T. gallinae* AY349182 (Kleina *et al.*) (100%, 5e-107)	
				HG008050 (Ganas *et al.*)[a]	*T. gallinae* AY349182 (Kleina *et al.*) (100%, 1e-138)	
				KC215387 (Girard *et al.*)[a]	*T. gallinae* JN007005 (Reinmann *et al.*) (100%, 0.0)	
B[a]				EU215368 (Gerhold *et al.*)[a]	*T. gallinae* KC215387 (Girard *et al.*) (99%, 1e-170)	
				EU881911,13[a],15,16[a] (Sansano-Maestre *et al.*)	*T. gallinae* KC215387 (Girard *et al.*) (99%, 0.0; 100%, 3e-171; 100%, 3e-171; 100%, 3e-171)	
D, E	I	C	A	EU215363-64 (Gerhold *et al.*)	*T. gallinae* U86614 (Felleisen) (100%, 3e-125; 100%, 4e-154)	ITS-OBT-Tg-2
				FN433475 (Grabensteiner *et al.*)	*T. gallinae* U86614 (Felleisen) (100%, 4e-154)	
C				EU215362 (Gerhold *et al.*)	*T. gallinae* U86614 (Felleisen) (99%, 0.0)	
				EU881912, 14, 17 (Sansano-Maestre *et al.*)	*T. gallinae* U86614 (Felleisen) (99%, 0.0); EU215362 (Gerhold *et al.*) (99%, 5e-169; 99%, 5e-169)	
	V			FN433477 (Grabensteiner *et al.*)	*T. gallinae* EU881912 (Sansano-Maestre *et al.*) (99%, 9e-151)	
				U86614 (Felleisen)	*T. gallinae* EU881912 (Sansano-Maestre *et al.*) (99%, 0.0)	
	II[a]			FN433474 (Grabensteiner *et al.*)[a]	*T. tenax* (KF164607) (99%, 2e-147)	ITS-OBT-Ttl-1
				KF993705 (this paper)	*T. canistomae* (AY244652) (97%, 1e-144)	ITS-OBT-Tcl-1
				KF993706 (this paper)	*T. canistomae* (AY244652) (97%, 1e-134)	ITS-OBT-Tcl-2
	III[a]			FN433473 (Grabensteiner *et al.*)[a]	*T. canistomae* (AY244652) (91%, 3e-110)	ITS-OBT-*Trichomonas* sp.-1
				KC529665 (Chi *et al.*)	*T. gallinae* FN433473 (Grabensteiner *et al.*) (99%, 4e-150)	ITS-OBT-*Trichomonas* sp.-2
I				EU215361 (Gerhold *et al.*)	*T. vaginalis* (U86613) (Felleisen) (99%, 0.0)	ITS-OBT-Tvl-1
L				EU215366 (Gerhold *et al.*)	*T. vaginalis* (U86613) (Felleisen) (99%, 2e-167)	ITS-OBT-Tvl-2
H				EU215360 (Gerhold *et al.*)	*T. vaginalis* (U86613) (Felleisen) (98%, 8e-172)	ITS-OBT-Tvl-3
J				EU215365 (Gerhold *et al.*)	*T. vaginalis* (U86613) (Felleisen) (98%, 2e-168)	ITS-OBT-Tvl-4

Table 3 (*Continued*)

Genotype						
Gerhold *et al.*	Grabensteiner *et al.*	Chi *et al.*	Sansano-Maestre *et al.*	Genbank sequences (author)	Organism sequence homology (% of BLAST identity, E-value)	Genetic group
	VI			FN433478 (Grabensteiner *et al.*)	*T. vaginalis* (FJ813603) (97%, 6e-138)	ITS-OBT-Tvl-5
				KF214774 (Kelly-Clark *et al.*)	Not yet released in GenBank	
				KF993707 (this paper)	*T. vaginalis* (FJ813603) (97%, 2e-152)	
G				EU215359 (Gerhold *et al.*)	*T. vaginalis* (U86613) (Felleisen) (92%, 1e-145)	ITS-OBT-*Trichomonas* sp.-3
K[a]				EU215367 (Gerhold *et al.*)	*T. stableri* (KC215389) (100%, 7e-167)	ITS-OBT-Ts-1
				JX089392 (Ecco *et al.*)[a]	*T. stableri* (KC215389) (100%, 1e-48)	
				KC215389 (Girard *et al.*)[a]	*T. stableri* (KC215390) (99%, 0.0)	
				KC215390 (Girard *et al.*)[a]	*T. stableri* (KC215389) (99%, 0.0)	
F				EU215358 (Gerhold *et al.*)	*T. stableri* (KC215389) (94%, 4e-104)	ITS-OBT-Ts-2
				JX089388 (Ecco *et al.*)[a]	*Simplicimonas* sp. (HQ334182) (99%, 8e-100)	ITS-OBT-*Simplicimonas* sp.
				EU290650 (Anderson *et al.*)	*Histomonas* sp. (HQ334185) (83%, 4e-45)	ITS-OBT-*Trichomonadida* spp.

ITS, ITS1/5.8S/ITS2 region; OBT, oropharyngeal bird Trichomonadida; Tx, organism homology group (Tcl, *T. canistomae*-like; Tg, *T. gallinae*; Ts, *T. stableri*; Tt, *T. tenax*; Trichomonadida spp.; Tvl, *T. vaginalis*-like); n, subgroup number (new numbers were used when homology was less than 99%). References for the authors mentioned in the table: Anderson, Anderson *et al.* (2009); Chi, Chi *et al.* (2013); Ecco, Ecco *et al.* (2012); Felleisen, Felleisen (1997); Ganas, Ganas *et al.* (2014); Gaspar da Silva, Gaspar da Silva *et al.* (2007); Gerhold, Gerhold *et al.* (2008); Girard, Girard *et al.* (2014a); Grabensteiner, Grabensteiner *et al.* (2010); Kelly-Clark, Kelly-Clark *et al.* (2013); Kleina, Kleina *et al.* (2004); Reinmann, Reinmann *et al.* (2012); Robinson, Robinson *et al.* (2010); Sansano-Maestre, Sansano-Maestre *et al.* (2009). [a]Associated with gross lesions.

T. gallinae clade, two sequences in the *T. canistomae*-like clade and one sequence in the *T. vaginalis*-like clade.

Genotype ITS-OBT-Tg-2 (GenBank numbers KF993680 to KF993692, KJ776740 and KJ776743) was found in predator birds and columbids, including mainly birds without gross lesions but also a minor amount of birds with lesions (5/35). Isolates of *T. gallinae* from columbids published by various authors were in this group: Felleisen (1997) (U86614), Sansano-Maestre *et al.* (2009) (EU881912), Gerhold *et al.* (2008) (EU215362-64) and Grabensteiner *et al.* (2010) (FN433475). Also, an isolate from a canary (*Serinus canaria*) of Grabensteiner *et al.* (2010) (FN433477) was classified in this genotype.

Genotype ITS-OBT-Tg-1 (GenBank numbers KF993693 to KF993704, KJ776739, KJ776741 and KJ776742) was reported mostly in predator birds with pathognomonic lesions (n = 27/33 predator birds), but also four isolates with this genotype were obtained from asymptomatic predator birds. Additionally, the sequences obtained from a passerine with lesions (magpie, *Pica pica*) and a psittacine with clinical signs (budgerigar, *Melopsittacus undulatus*) were included in this clade. This group contained sequences of *T. gallinae* from symptomatic birds including columbids of Gerhold *et al.* (2008) (EU215369), Grabensteiner *et al.* (2010) (FN433476) and Girard *et al.* (2014a) (KC215387), predator birds of Gerhold *et al.* (2008) (EU215368) and Sansano-Maestre *et al.* (2009) (EU881913, EU881916), and the emerging strain of finches across Europe of Robinson *et al.* (2010) (GQ150752) and Ganas *et al.* (2014) (HG008050) (Table 3).

Three newly described genotypes in Spain are reported here: genotypes ITS-OBT-Tcl-1, ITS-OBT-Tcl-2 and ITS-OBT-Tvl-5 (GenBank accession numbers KF933705, KF933706 and KF933707, respectively). Remarkably, this is the first description of genotypes ITS-OBT-Tcl-1 and ITS-OBT-Tcl-2 in the literature. All of them were isolated from birds without macroscopic lesions. Genotype ITS-OBT-Tcl-1 was obtained from European turtle doves, genotype ITS-OBT-Tcl-2 from goshawk nestlings and genotype ITS-OBT-Tvl-5 from an Egyptian vulture (*Neophron percnopterus*).

Genotypes ITS-OBT-Tcl-1 and ITS-OBT-Tcl-2 are highly similar (they differ only in three nucleotides, positions 48, 230 and 305), and are clustered together in a clade with *T. canistomae* (AY244652, 97% identity, E-values of 1e-144 and 1e-134, respectively).

Genotype ITS-OBT-Tvl-5 showed 100% homology with a sequence obtained from a bearded vulture (*Gypaetus barbatus*) of Grabensteiner *et al.* (2010) (FN433478) and a bald eagle (*Haliaeetus leucocephalus*) of Kelly-Clark *et al.* (2013) (KF214774). The maximum number of nucleotide changes between the five genotypes found in this study was 33 (ITS-OBT-Tg-1 vs. ITS-OBT-Tvl-5), and the minimum variation was three (ITS-OBT-Tcl-1 vs. ITS-OBT-Tcl-2).

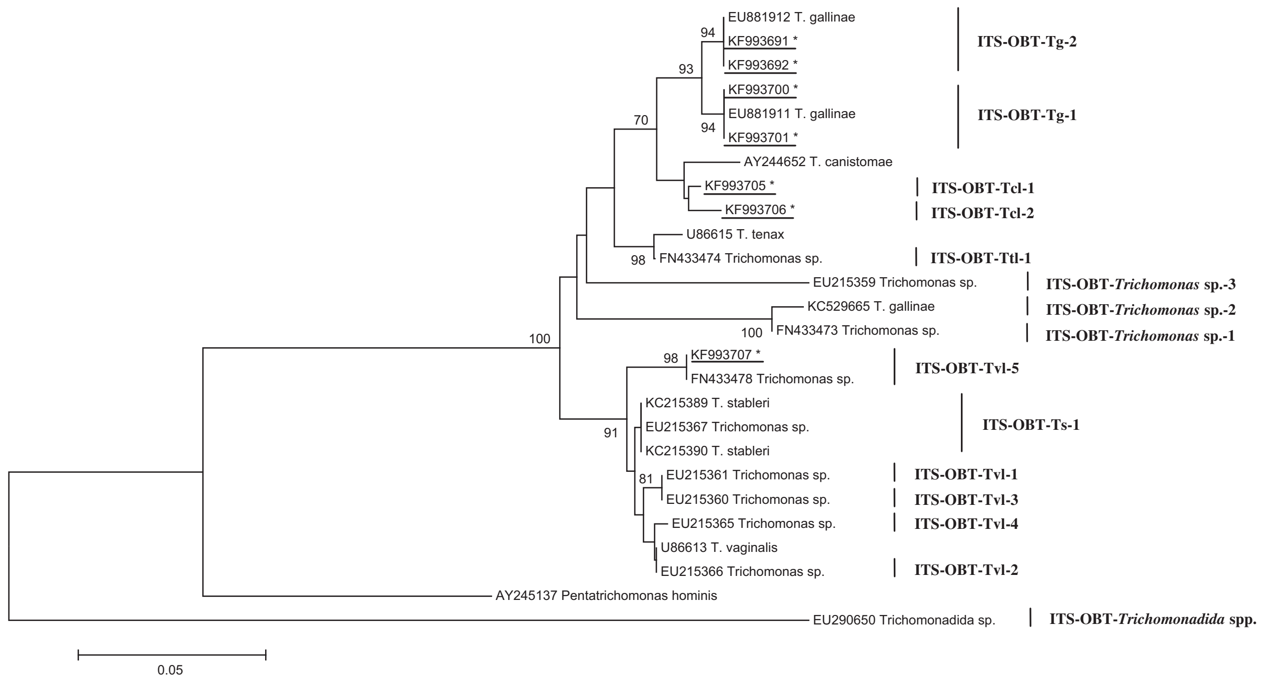

Figure 3. *Phylogenetic tree using the region ITS1/5.8S/ITS2 of* Trichomonas *sp. by the neighbour-joining method. Bootstrap values lower than 70 are not shown (*n *= 2000 replicates). Sequences from other authors are indicated with GenBank accession number and organism name. Sequences reported in this paper are only shown with the GenBank accession number underlined and marked with an asterisk.*

Risk factors: genotype, type of host and diet. Genotypes ITS-OBT-Tg-1 and ITS-OBT-Tg-2 were the most frequent among the isolates and there was an association between genotype and type of host, genotype ITS-OBT-Tg-1 being associated with predator birds in general and genotype ITS-OBT-Tg-2 associated with prey species (OR = 4, 95% CI = 1.4 to 12).

OR values indicated that infection by genotype ITS-OBT-Tg-1 is a risk factor for the development of pathognomonic lesions in comparison with the other genotypes (ITS-OBT-Tg-2, ITS-OBT-Tcl-1, ITS-OBT-Tcl-2, ITS-OBT-Tvl-5; OR = 41.9, 95% CI = 13.8 to 127.5). Moreover, the diet (ornithophagous/non-strict ornithophagous) of the birds and the type of host (predator bird/prey) were also risk factors to develop symptomatic infections. Considering only birds positive for *Trichomonas* sp. culture, predator birds were at higher risk to develop lesions than prey species (OR = 4.3, 95% CI = 1.5 to 12.8). In the same way, the association between the diet (ornithophagous/non-strict ornithophagous) of predator birds (considering the goshawk, the Eurasian sparrowhawk *Accipiter nisus* and the peregrine falcon *Falco peregrinus* as strict ornithophagous) and the development of pathognomonic lesions in birds positive for *Trichomonas* sp. culture indicated that non-strict ornithophagous species were at higher risk to develop gross lesions than ornithophagous birds (OR = 14.9, 95% CI 3.7 to 60.3).

Influence of genotype on pathognomonic lesions. Since the variables diet and host type were associated with the genotype found, OR_{MH} was calculated to more precisely estimate the association between genotype ITS-OBT-Tg-1 and the presence of gross lesions. The analysis indicated that after removing the influence of confounding variables (type of host and diet), there was still a higher risk to develop symptomatic trichomonosis if genotype ITS-OBT-Tg-1 was present (OR_{MH} = 35.4, 95% CI = 11.7 to 107.3, host type stratification; and OR_{MH} = 37.6, 95% CI = 10.5 to 134.4, diet in predator bird stratification). The influence of the genotype was also observed when separate strata (prey/predator bird) were analysed (OR_{prey} = 10.5, 95% CI = 1.2 to 91.9; $OR_{predator\ bird}$ = 59.4, 95% CI = 15.4 to 228.6); therefore, being a predator bird increased the risk to develop gross lesions if genotype ITS-OBT-Tg-1 was present. The influence of the genotype could be observed only in non-strict ornithophagous birds, possibly due to the scarce numbers of birds displaying pathognomonic lesions in ornithophagous species (n = 2) ($OR_{ornithophagous}$ = 3.3, 95% CI = 0.8 to 14.6; $OR_{non\text{-}strict\text{-}ornitophagous}$ = 195, 95% CI = 116.2 to 327.2).

Influence of type of host on pathognomonic lesions. Although there was a higher risk to develop gross lesions in predator birds than in prey species in each of the strata (genotype ITS-OBT-Tg-1/ITS-OBT-Tg-2) analysed separately ($OR_{genotype\ ITS\text{-}OBT\text{-}Tg\text{-}1}$ = 6.9, 95% CI = 6.3 to 7.5; $OR_{genotype\ ITS\text{-}OBT\text{-}Tg\text{-}2}$ = 1.2, 95% CI = 1.1 to 1.4), the results were not consistent when the OR_{MH} test was used. The influence of type of host in the development of gross lesions was not as strong as the genotype of the isolate (OR_{MH} = 2.5, 95% CI = 0.6 to 10.1, genotype stratification).

Influence of diet in predator bird species on pathognomonic lesions. Non-strict ornithophagous birds were at higher risk to develop gross lesions than ornithophagous birds, although the risk was less intense when the effect of the variable genotype was removed by stratification (OR_{MH} = 8.3, 95% CI = 1.2 to 58, genotype stratification). After stratification by genotype, it could be observed that the effect of the diet was more intense when genotype ITS-OBT-Tg-1 was present ($OR_{genotype\ ITS\text{-}OBT\text{-}Tg\text{-}1}$ = 90, 95% CI = 74.9 to 108; $OR_{genotype\ ITS\text{-}OBT\text{-}Tg\text{-}2}$ = 1.5, 95% CI = 1.1 to 2.2).

Discussion

No positive cultures were recovered from finch species and no outbreaks or dead birds were noticed during the course of the study. The fact that birds were from ornithological ringing campaigns might be the reason for this, since mainly healthy individuals were sampled as they were capable of performing the migration routes required for each species. Besides, the artificial food supply for small passerines is not extended practice in Spain as it could be in the UK. However, we cannot exclude the presence of the infection in finch birds in our country, since our sample size was small and from a limited geographic area ($n = 132$).

We found five genotypes in this study (ITS-OBT-Tg-1, ITS-OBT-Tg-2, ITS-OBT-Tcl-1, ITS-OBT-Tcl-2 and ITS-OBT-Tvl-5). Genotypes ITS-OBT-Tcl-1 and ITS-OBT-Tcl-2 form a distinctive and different cluster, with no previous isolates that we could find in the literature. This is the first time they have been reported and therefore constitute newly discovered genotypes. BLAST analysis indicated that the highest similarity was obtained with a *Trichomonas* sp. isolate from a dog (AJ784785) and *T. canistomae* (AY244652), in comparison with other *T. gallinae* isolates. Girard *et al.* (2014b) also reported *T. vaginalis*-like genotypes that were present only in some species and not in others, suggesting a restricted host range or the possibility of more recently evolved organisms. Genotype ITS-OBT-Tvl-5 is another newly described genotype in Spain, and to our knowledge the first detection of this genotype in the Egyptian vulture. BLAST analysis indicated maximum similarity with a sequence obtained from a bearded vulture, another scavenger species, published by Grabensteiner *et al.* (2010) (FN433478) and from a bald eagle (Kelly-Clark *et al.*, 2013) (KF214774). These findings suggest there are some oral trichomonads that might be classified into an intermediate position or even that they could be new species of this genus. Nevertheless, more studies need to be done in order to confirm this last suggestion (Martínez-Díaz *et al.*, 2014).

The statistical results considering genotype and lesions agree with the results published by Sansano-Maestre *et al.* (2009) and Chi *et al.* (2013), who found genotype ITS-OBT-Tg-1 in birds with pathognomonic lesions and more prevalent among predator birds. There are many isolates from other studies that showed 100% homology to genotype ITS-OBT-Tg-1, but in many cases the authors did not report whether the birds displayed gross lesions or evident clinical signs. However, some of them were implied in epidemic episodes of avian trichomonosis, like GQ150752 (Robinson *et al.,* 2010; the UK), HG008050 (Ganas *et al.*, 2014; Austria and Slovenia), and KC215387 (Girard, *et al.*, 2014a, b; the USA). Others were recovered from birds with gross lesions like a rock pigeon (Grabensteiner *et al.*, 2010) (FN433476), a barn owl (*Tyto alba*) (Sansano-Maestre *et al.,* 2009) (EU881913), a mourning dove (*Zenaida macroura*) (Gerhold *et al*., 2008) (EU215369) and a broad-winged hawk (*Buteo platypterus*) (99% homology; Gerhold, *et al*., 2008) (EU215368). These findings show that this genotype is found among many species, including raptors, columbids and other avian species like corvids and finches over the world. Actually, this is the most frequently isolated genotype from birds with clinical signs and seems to be much more related to the presence of pathognomonic lesions than the others, as previous studies have reported (Sansano-Maestre *et al*., 2009; Chi *et al*., 2013; Girard *et al*., 2014b). On the other hand, the absence of oropharyngeal lesions might be explained either because some samples could have been taken at an early time in the infection and the clinical signs had not developed yet or because birds could have been immunized previously and be asymptomatic carriers of the parasite for a certain time (revised in Forrester & Foster, 2008), as occurs in other trichomonad infections (*Trichomonas foetus* infections in cattle, as an example).

Regarding the relationship between the presence of pathognomonic lesions and the diet of predator birds, statistical results indicated that birds with non-strict ornithophagous diet are at higher risk of developing gross lesions than strict ornithophagous species. We would like to highlight here the case of the common kestrels, in which we have found an important percentage of birds with lesions (9.4%) genotype ITS-OBT-Tg-1 being the most frequent (21/25). This species is characterized by its generalist predator diet, including mainly insects, lizards, small mammals and birds (Navarro-López *et al*., 2014). Their habitat tolerance, being capable of reproducing in urban environments, has put this raptor species at risk of *T. gallinae* infection, due to the contact and proximity to columbid preys in these places. In contrast, we have found the case of the goshawk, in which no gross lesions were seen and the main genotype was ITS-OBT-Tg-2 (7/12). This raptor feeds mainly on birds of columbid species and lives in forested areas (Toyne, 1998). The absence of lesions may indicate the evolutionary history of the raptor and its diet, feeding on columbid prey that mainly harboured genotype ITS-OBT-Tg-2 of *T. gallinae*, with an adaptation to the parasite and the establishment of non-pathogenic infections. In this case, it could be possible that ornithophagous species develop a significant immune response acquired through their diet that would protect them against highly virulent strains (revised in Forrester & Foster, 2008). On the other hand, the low percentage of birds with gross lesions could be a consequence of the prevalence of non-pathogenic genotypes in columbids. Taking the statistical results, we can conclude that the genotype and diet of predator birds are risk factors for clinical trichomonosis, but the influence of the genotype is much higher than the influence of the diet.

Genotype ITS-OBT-Tg-2 was observed most frequently in columbids and raptors without visible gross lesions. This finding has been reported previously by other authors (Sansano-Maestre *et al*., 2009; Chi *et al*., 2013). In the present study, we found five isolates from birds with macroscopic lesions of genotype ITS-OBT-Tg-2: GenBank accession numbers KF993682 from a wood pigeon (*Columba palumbus*), KF993686 from a booted-eagle (*Aquila pennata*) recovered in a district of Valencia city, KF993687 from a long-eared owl (*Asio otus*) found predating on a canary cage, KF993690 from a peregrine falcon located in Madrid city, and KJ776743 from an Eurasian collared dove. The long-eared owl showed initial lesions at the oropharynx similar to avian trichomonosis and other infectious agents (i.e. candidiasis), but other pathogens were not investigated at the time of sampling. All of them were from birds recovered in urban areas or with initial lesions that could have acquired the infection by predation on infected columbids or, in the case of KF993682 and KJ776743, being one of the reservoir hosts. This fact could be the reason for detecting genotype ITS-OBT-Tg-2, commonly isolated from columbids.

Lesions in these five samples might also be a consequence of a mixed infection (genotypes ITS-OBT-Tg-1/ITS-OBT-Tg-2). The clinical isolates could suffer an

unexpected selection process due to different culture medium requirements and/or *in-vitro* multiplication rates of the variants during the protocol of isolation and axenization of the parasite. Also, the selection of a particular genotype over the others after several passages has been documented previously. The use of clonal cultures from freshly isolates could clarify this issue (Grabensteiner *et al.*, 2010).

A study has recently investigated the pH conditions of the oral cavity of Cooper's hawk and its influence on the persistence of the infection (Urban & Mannan, 2014). They found that nestlings had a suitable pH for the survival of the parasite in comparison with adults and fledglings, and, as a result, they had a higher incidence of infection by *Trichomonas* sp. In the same sense, the association that we have found between genotype and host could be explained by the different growing requirements of each genotype and the particular conditions for the establishment of the parasite in each host species.

In this study, we also report avian hosts for the parasite in Spain, with no sequences of the ITS1/5.8S/ITS2 region published in GenBank. These species were: two columbids (*C. palumbus* and *S. turtur*), one corvid (*P. pica*) and 13 raptors (*A. gentilis*, *A. nisus*, *A. otus*, *B. bubo*, *Buteo buteo*, *Circus aeruginosus*, *Circus cyaneus*, *F. naumanni*, *F. peregrinus*, *N. percnopterus*, *Otus scops*, *Pernis apivorus*, *Strix aluco*). These data support the wide range of hosts for the parasite, including raptors with a predominant insectivore diet that can feed on columbids before or during the breeding season, like the European honey-buzzard (*P. apivorus*) and the common scops-owl (*O. scops*) (Roberts & Coleman, 2001).

As suggested by Amin *et al*. (2014), more studies are needed to determine the association between pathology and genetic variant, using multi-locus genetic markers and single copy genes. Also, there is a need to study new bird species in order to determine the diversity of oropharyngeal trichomonads present, as different genotypes have been described on different hosts (genotypes ITS-OBT-Tcl-1, ITS-OBT-Tcl-2 and ITS-OBT-Tvl-5) and novel species have been reported recently (Girard *et al*., 2014a; Martínez-Díaz *et al*., 2014). This new knowledge will help us to understand the epidemiology of the disease in order to control and limit the mortality of outbreaks found in wildlife.

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Supplemental data

Supplemental data for this article can be accessed here.

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APPENDIX V – Other publications related with the Doctoral Thesis.

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ORIGINAL PAPER

Trichomonas gypaetinii n. sp., a new trichomonad from the upper gastrointestinal tract of scavenging birds of prey

Rafael Alberto Martínez-Díaz · Francisco Ponce-Gordo · Irene Rodríguez-Arce · María Carmen del Martínez-Herrero · Fernando González González · Rafael Ángel Molina-López · María Teresa Gómez-Muñoz

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Abstract In the context of an epidemiological study carried out by several wildlife recovery centers in Spain, trichomonads resembling *Trichomonas gallinae* were found in the oropharyngeal cavity of 2 Egyptian vultures (*Neophron percnopterus*) and 14 cinereous vultures (*Aegypius monachus*) which did not show any symptoms of trichomonosis. In order to characterize them, these isolates along with seven other *T. gallinae* isolates obtained from different hosts and from different geographical origin were analyzed. Genetic analyses were performed by sequencing the small subunit ribosomal RNA (SSU-rRNA) and the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA regions. The morphological study of the isolates in both light and scanning electron microscopy was also performed. The sequences obtained in the genetic analysis coincide with previously published sequences of an isolate named as *Trichomonas* sp., obtained from a bearded vulture (*Gypaetus barbatus*), and showed clear differences to the *T. gallinae* sequences (97 and 90–91 % homology, respectively, for SSU-rRNA and ITS regions) and display higher similarity with *Trichomonas vaginalis* and *Trichomonas stableri* than with *T. gallinae*. Multivariate statistical analysis of the morphometric study also reveals significant differences between the trichomonads of vultures and the isolates of *T. gallinae*. The isolates from vultures presented smaller values for each variable except for the length of axostyle projection, which was higher. These results together with the different nature of their hosts suggest the possibility of a new species of trichomonad which we hereby name *Trichomonas gypaetinii*, whose main host are birds of the subfamily Gypaetinae.

Rafael Alberto Martínez-Díaz and Francisco Ponce-Gordo contributed equally to this work.

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Keywords Trichomonas · SSU-rRNA and ITS genotypes · Morphology · Vultures · Host range · New species

Introduction

The trichomonads are a group of protists included in the parabasalids, which are classified in the supergroup (suprakingdom-level group) Excavata (Cavalier-Smith 2002) and the phylum Metamonada (Cavalier-Smith 2003), although the monophyly of both supertaxa is still under intense debate (Cepicka et al. 2010; Hampl et al. 2009; Malik et al. 2011; Simpson et al. 2006). The combination of morphological and molecular data suggests the existence of six parabasalian lineages (Hypotrichomonadea, Trichomonadea, Tritrichomonadea, Cristamonadea, Trichonymphea, and Spirotrichonymphea) ranked as classes by Cepicka et al. (2010). The class Trichomonadea includes anaerobic (aerotolerant) organisms without mitochondria and peroxisomes, but with hydrogenosomes (specialized organelles involved in metabolic processes that extend glycolysis) (Kulda 1999). They are characterized by a single karyomastigont with 2–6 flagella and by the absence of a comb-like structure and an infrakinetosomal body (Cepicka et al. 2010).

The family Trichomonadidae is included in the order Trichomonadida; their species have a B-type costa which always underlies the lamelliform undulating membrane (Amos et al. 1979; Brugerolle 1976; Poirier et al. 1990). This group contains free-living species and endobionts of animals and humans. They are mostly parasitic or commensal flagellates inhabiting oxygen-poor environments. Among these microorganisms, *Trichomonas vaginalis* is the causative agent of human trichomonosis, *Tritrichomonas foetus* is recognized as a significant cause of bovine infertility, and *Trichomonas gallinae* is the main aethiologic agent of avian trichomonosis.

T. gallinae commonly inhabits the upper part of the digestive tract in a wide range of birds, but can also affect other organs depending on the virulence of the strain. Pigeons and doves are the main host of this parasite, but it is also present in other columbiformes, galliformes, psittaciformes, passeriformes, strigiformes, and falconiformes (Amin et al. 2014; Forrester and Foster, 2008; Seddiek et al. 2014). *T. gallinae* can cause granulomatous lesions occupying the oral cavity and esophageal lumen, which may occlude leading to the death of the birds as a result of severe starvation. However, it is well known that virulence of the strains shows high variability, and the parasite can be present in both healthy and sick animals. Data on prevalence found in different hosts by different authors throughout the world vary greatly but only few molecular studies have yet been performed in order to investigate the genetic polymorphism among isolates of this parasite in different bird species. Studies carried out on the gene 5.8S ribosomal RNA (rRNA) and the surrounding internal transcribed spacer (ITS) regions have proved to be very useful for the taxonomy of the Trichomonadidae family and of the genus *Trichomonas* (Anderson et al. 2009; Chi et al. 2013; Ecco et al. 2012; Felleisen 1997; Gaspar da Silva et al. 2007; Gerhold et al. 2008; Grabensteiner et al. 2010; Hayes et al. 2003; Kleina et al. 2004; Lawson et al. 2011; Mostegl et al. 2012; Munsch et al. 2009; Sansano-Maestre et al. 2009; Walker et al. 2003).

There are evidences indicating that *T. gallinae* is not the sole etiologic agent of avian trichomonosis, and that the incorporation of molecular tools is critical in the investigation of infectious causes of mortality in birds. Recently, Girard et al. (2014a) have proposed a new species, named *Trichomonas stableri*, which causes similar clinical and pathologic features to *T. gallinae* infections.

Within a large epidemiological study on avian trichomonosis performed by our group, trichomonads resembling *T. gallinae* were found in the oropharyngeal cavity in 2 Egyptian vultures (*Neophron percnopterus*) and 14 cinereous vultures (*Aegypius monachus*). A preliminary genetic characterization showed that these isolates differ significantly from *T. gallinae*. The objective of this work is the morphological and molecular characterization of these isolates.

Material and methods

Source of samples and laboratory cultures

Samples were obtained from a wide epidemiological study on avian trichomonosis performed at different wildlife recovery centers in Spain, involving more than 1700 samples from 54 different bird species. The positive samples from vultures (n= 16) were from different geographical origin (Fig. 1). For comparison purposes, we have included seven isolates obtained from other avian hosts. The characteristics of all the isolates studied are shown in Table 1. All the isolates were obtained between 2012 and 2013 from the oropharynx of two Eurasian collared doves (*Streptopelia decaocto*), one common kestrel (*Falco tinnunculus*), one tawny owl (*Strix aluco*), one common magpie (*Pica pica*), one hen harrier (*Circus cyaneus*), one common buzzard (*Buteo buteo*), two Egyptian vultures (*N. percnopterus*) and fourteen cinereous vultures (*A. monachus*). All of the birds were initially diagnosed by microscopic examination, showing natural infections by trichomonads with varying clinical signs ranging from asymptomatic to lethal infections.

The parasites were isolated in tryptose/yeast/maltose medium (TYM) (Amin et al. 2010), with antibiotics (240 U/ml nystatin and 36 μg/ml of tircarcillin, vancomycin, and ceftiofur), and supplemented with 10 % fetal calf serum (Sigma, St. Louis, MO, USA). Cultures were maintained at 37 °C, and subcultures were made every 48 h. Parasites were

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Fig. 1 Geographical location of the samples from vultures

subcultured once or twice before the manipulation was performed. They were preserved by freezing at −80 °C with 5 % dimethylsulfoxide (DMSO) until use.

DNA extraction and PCR amplification of the SSU-rRNA and ITS1/5.8S/ITS2 regions

DNA extraction was performed from the initial isolates and from 1 ml of the cultures in logarithmic growth phase. DNeasy blood and tissue extraction kit (Qiagen Ltd., Hilden, Germany) was used following the manufacturer's instructions. DNA was eluted in a final volume of 80 µl. The PCR amplification of the SSU-rRNA gene followed the protocol of Ganas et al. (2014) and that of Felleisen (1997) for the ITS1/5.8S/ITS2 region. Oligonucleotide primers, HM-LONG-F (AGGAAG CACACTATGGTCATAG) and HM-LONG-R (CGTTAC CTTGTTACGACTTCTCCTT) for the SSU-rRNA and TFR1 (TGCTTCAGTTCAGCGGGTCTTCC) and TFR2 (CGGTAGGTGAACCTGCCGTTGG) for the ITS1/5.8S/ ITS2 region, were obtained from Qiagen (Qiagen Ltd., Crawley, UK). PCR reactions were undertaken using HotStarTaq Master Mix kit (Qiagen Ltd., Hilden, Germany). Twenty-five microliters of reaction volume was used, including 12.5 µl of HotStarTaq Master mix, 8 µl of water suitable for PCR, 1 µl of each primer in 10 µM solution, and 2.5 µl of DNA sample. The amplifications were performed in a thermal cycler GeneAmp 2700 (Applied Biosystems) using the following protocol for both ribosomal targets (SSU-rRNA and ITS1/5.8S/ITS2 regions): 15 min at 95 °C initial denaturation; 40 cycles of denaturation for 30 s at 94 °C, annealing for 1 min at 55 °C, extension for 2 min at 72 °C, and a final extension step at 72 °C for 10 min. The amplified product was observed under UV light after ethidium bromide staining of a 1.5 % agarose gel.

Sequencing and phylogenetic analysis of the SSU-rRNA and ITS1/5.8S/ITS2 regions

Amplified PCR products were purified using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare), and sequenced in both directions in Sistemas Genómicos (Paterna, Valencia, Spain), applying the same primers used for the amplification. The chromatograms obtained were manually checked and sequences were aligned using the SeqMan Pro program of the Lasergene DNA suite v. 7 (DNAStar Inc). Sequences of each isolate were compared with the sequences deposited in the GenBank, EMBL, and DDBJ databases, using the BLAST algorithm as implemented in the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/).

A multiple alignment was performed using the free software MEGA version 6.06 and BioEdit Sequence Aligment Editor 7.0.9.0, by the Clustal W method (Tamura et al. 2013). The Neighbor-Joining method with the maximum likelihood composite model was used to generate the corresponding phylogenetic tree, using a bootstrap value of 2000 replicates; *Pentatrichomonas hominis* was used as out-group.

Light microscopy and morphometric study

One milliliter of each culture was centrifuged at 1500 rpm for 3 min, the supernatant discarded, and the pellet diluted in 50 µl of fresh medium. The parasites were stained with Diff-Quick (Medion Diagnostic AG).

For the morphometric study, 30 trophozoites of each preparation were randomly selected. Seventeen parameters related to the cell body, nucleus, anterior flagella, undulating membrane, and axostyle were measured using the software Images Plus 2.0 ML (Motic China Group Co., Ltd.). The parameters considered were the length, width, perimeters, and surfaces of the cell body and the nucleus; the length of the anterior flagella, recurrent flagellum and costa; the total length of the axostyle and length of the axostyle projection (Fig. 2).

Statistical analysis

Statistical analysis of the morphometric data was performed using the IBM SPSS Statistics software version 21. The different isolates were grouped according to their ITS sequences. A multivariate analysis of variance (MANOVA) was conducted to assess if there were differences between the genetic groups on a linear combination of the morphological parameters measured. To avoid problems of collinearity, the Pearson's correlation coefficients were calculated to identify the highly correlated variables ($r \geq 0.700$, $p < 0.01$) and exclude them from the analysis. Univariate ANOVA tests were made to determine the variables that show differences

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Table 1 Characteristics of the studied isolates

Isolate	Host	Common name	Wild/captive	Geographical location	GenBank Acc N° SSU-rRNA	GenBank Acc N° ITS1/5.8S/ITS2
PM2–12*	*Streptopelia decaocto*	Eurasian collared dove	Wild	Madrid	KM246608	KJ776743
RM2–12*	*Falco tinnunculus*	Common kestrel	Wild	Barcelona	KM246604	KJ776741
RM12–12*	*Strix aluco*	Tawny owl	Wild	Madrid	KM246606	KF993703
OM1–12*	*Pica pica*	Common magpie	Wild	Zaragoza	KM246605	KF993693
P2–12*	*Streptopelia decaocto*	Eurasian collared dove	Wild	Valencia	KM246607	KM246600
RM9–12*	*Circus cyaneus*	Hen harrier	Wild	Ávila	KM246603	KF993702
RM11–12*	*Buteo buteo*	Common buzzard	Wild	Madrid	KM246609	KM246599
RM4–12*	*Neophron percnopterus*	Egyptian vulture	Captive	Madrid	KM246611	KM246602
R235–13*	*Neophron percnopterus*	Egyptian vulture	Wild	Valencia	***	KF993707
13–3061*	*Aegypius monachus*	Cinereous vulture	Wild	Madrid	KM246610	KM246601
13–3123*	*Aegypius monachus*	Cinereous vulture	Wild	Cáceres	***	***
13–3430*	*Aegypius monachus*	Cinereous vulture	Wild	Almería	***	***
13–3064**	*Aegypius monachus*	Cinereous vulture	Wild	Segovia	***	***
13–3124**	*Aegypius monachus*	Cinereous vulture	Wild	Cáceres	***	***
13–3277**	*Aegypius monachus*	Cinereous vulture	Wild	Cáceres	***	***
13–3278**	*Aegypius monachus*	Cinereous vulture	Wild	Cáceres	***	***
13–3279**	*Aegypius monachus*	Cinereous vulture	Wild	Cáceres	***	***
13–3341**	*Aegypius monachus*	Cinereous vulture	Wild	Ciudad Real	***	***
13–3342**	*Aegypius monachus*	Cinereous vulture	Wild	Ciudad Real	***	***
13–3343**	*Aegypius monachus*	Cinereous vulture	Wild	Valencia	***	***
13–3365**	*Aegypius monachus*	Cinereous vulture	Wild	Valencia	***	***
13–3375**	*Aegypius monachus*	Cinereous vulture	Wild	Madrid	***	***
13–3429**	*Aegypius monachus*	Cinereous vulture	Wild	Sevilla	***	***

*Morphological and genetically characterized isolates. **Genetically characterized isolates. ***The sequences from all vulture isolates were identical to those submitted (KM246601, KM246602, KM246610, and KM246611), and they were not submitted as it was considered not necessary

among the genetic groups ($p<0.01$), and Scheffé was used as post hoc test to identify their possible grouping.

Scanning electron microscopy

From each of the genetic variants (as identified by their ITS sequences), one isolate was analyzed by scanning electron microscopy (SEM). The trichomonad cells were obtained from centrifugation of 2.5 ml of a 48 h subculture at 1500 rpm for 3 min. The supernatant was removed and the pelleted cells were washed two times with 5 ml phosphate buffered saline (PBS) and finally diluted in 0.5 ml PBS. The cells were collected on the surface of a filter of 0.22-μm pore size, and all further processing was carried out directly on the filter. The fixation was made with 2.5 % glutaraldehyde and 4 % paraformaldehyde in PBS for 3 h at 4 °C, and then the cells were dehydrated by washing them with acetone of increasing concentration (30, 50, 70, 80, 90, and 100 %) (two steps of 15 min in each concentration). The final processing (critical point drying and metallization) was performed by the technicians at the Centro Nacional de Microscopía Electrónica (CNME). The observation and capture of the digital images

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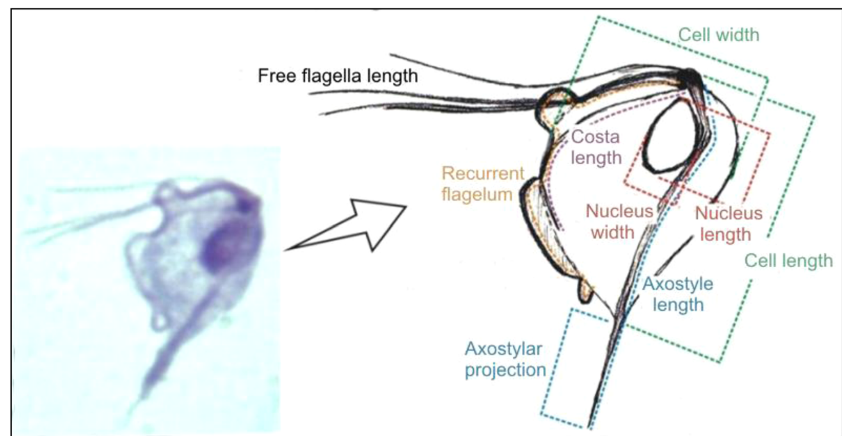

Fig. 2 Morphological parameters analyzed in the present study

were performed in a JEOL JSM-6400 scanning electron microscope at the CNME.

Results and discussion

Analysis of the SSU-rRNA and ITS1/5.8S/ITS2 regions

The amplification and sequencing of the SSU-rRNA region resulted in assembled sequences ranging from 1433 to 1446 bp; they have been deposited in the GenBank/EMBL/DDBJ databases under the accession numbers indicated in the Table 1. All sequences from vultures were identical; for this reason, we have uploaded only one sequence from *Aegypius* and two from *Neophron*. The phylogenetic analysis revealed the presence of some variability between our isolates (Fig. 3; Online Resource 1), being all the vulture isolates clustered in a clearly separate branch with a *Trichomonas sp.* strain found in a bearded vulture (Grabensteiner et al. 2010). All of our other isolates clustered with *T. gallinae* already studied genotypes (Gerhold et al. 2008).

Sequences of the ITS1-5.8SrRNA-ITS2 regions were 324–326 bp long, as previously described by Felleisen (1997) for *T. gallinae*. They have been uploaded to the GenBank/EMBL/DDBJ databases under the accession numbers shown in Table 1. The sequence analysis of our samples (Fig. 4; Online Resource 2) showed again that the vulture isolates were clustered in a separate branch with a sequence of *Trichomonas* sp. from a bearded vulture (Grabensteiner et al. 2010). All the other isolates were grouped with other sequences of *T. gallinae*, commonly found in several epidemiological studies (Chi et al. 2013; Girard et al. 2014b; Martínez-Herrero et al., submitted; Robinson et al. 2010; Sansano-Maestre et al. 2009). These sequences can be grouped in two genotypes (A and B) (Sansano-Maestre et al. 2009), which are included as subgroups of the *T. gallinae*-like clade proposed by Grabensteiner et al. (2010).

The sequence of the SSU-rRNA and ITS regions of the vulture isolates showed 97 and 90–91 % homology, respectively, with the *T. gallinae* sequences obtained in this study or available in the Genbank/EMBL/DDBJ databases. For the ITS region, the vulture isolates displayed higher similarity (up to 97 %) with other *Trichomonas* species as *T. vaginalis* and *T. stableri*, being genetically closer to these instead of *T. gallinae*. These differences, especially in the ITS region, suggest that the isolates from the vultures are more than a genotypic variant of *T. gallinae*. Recently, Kelly-Clark et al. (2013) has described a trichomonad in a clinically healthy rehabilitated American bald eagle (*Haliaeetus leucocephalus*) in Canada. The authors indicated that the sequence they obtained was identical to that of Grabensteiner et al. (2010), and they describe the organism as oval- or pyriform-shaped cells resembling trichomonads, but there is no precise description of its morphology.

Light-microscopic morphology

The morphology of the different isolates was examined in fast metachromatic staining preparations. Only five of the vulture isolates (RM4–12, R235–13, 13–3061, 13–3123, and 13–3430) were analyzed; the other vulture isolates failed to grow and it was not possible to obtain stained slides from them.

According to the descriptions of various authors, the trophozoites of *T. gallinae* vary in shape and size. The cell body varies from ovoid to pyriform with a mean size of about 7–11 μm (Melhorn et al. 2009). The nucleus is oval, with a longitudinal diameter of about 2.5–3 μm, and it is located near the basis of the basal apparatuses of the flagella. The axostyle consists of a row of microtubules running from the region of the apical basal bodies to the posterior end of the cell, where it proceeds into a short peak. At the apical pole, four free flagella arise each from a typical basal body. These four free flagella have a length of about 11–13 μm. A fifth flagellum also originates at the apical pole, but it is closely linked to the cell surface in a lamelliform undulating membrane. This recurrent

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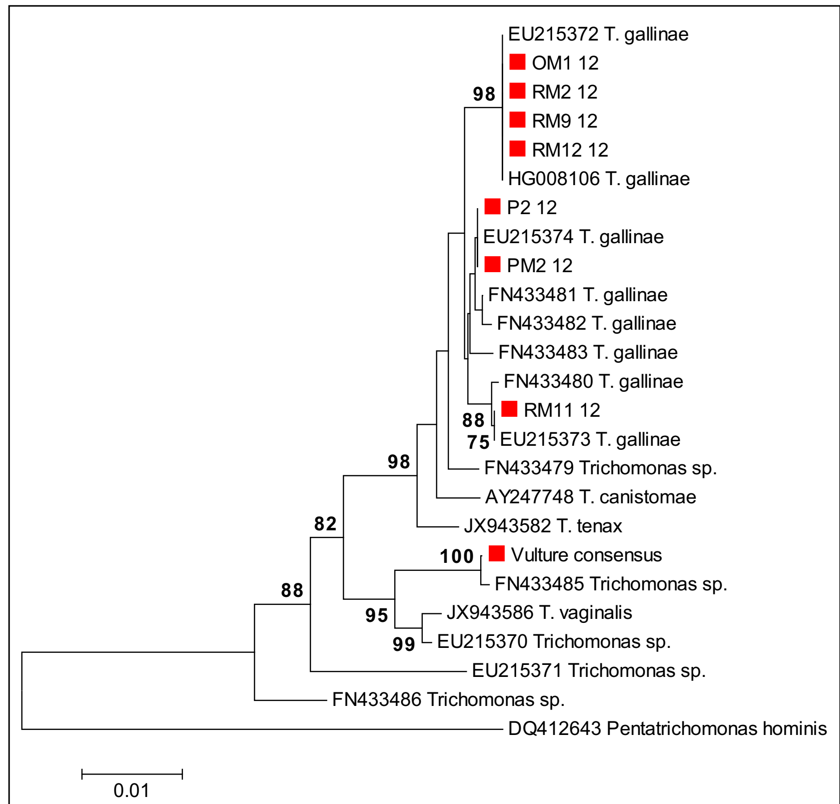

Fig. 3 Phylogenetic tree of the *Trichomonas* isolates based on the SSU-rRNA using the neighbor-joining method. Branch reliability was assessed using a bootstrap of 2000 replicates and shown as the frequency out of 100 (values lower than 70 are not shown). The scale represents nucleotide substitution per position

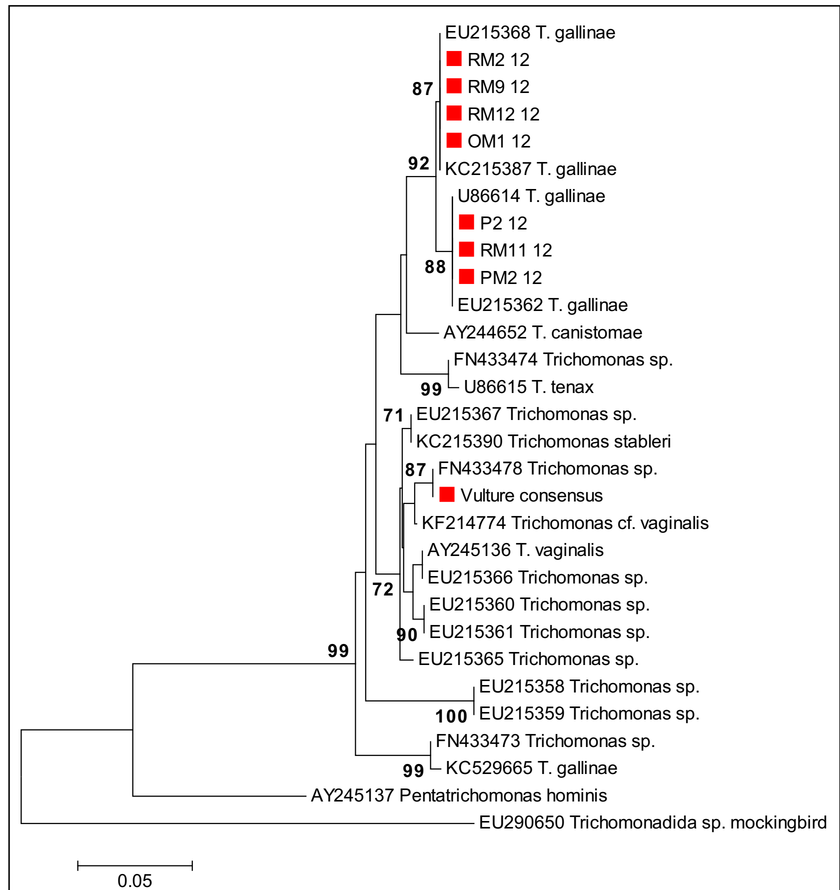

Fig. 4 Phylogenetic tree of the *Trichomonas* isolates based on the ITS1–5.8S rRNA-ITS2 region, using the neighbor-joining method. Branch reliability was assessed using a bootstrap of 2000 replicates and shown as the frequency out of 100 (values lower than 70 are not shown). The scale represents nucleotide substitution per position

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flagellum does not become free at the posterior end of the trophozoite and extends from two thirds up to three fourths of the cell surface (Melhorn et al. 2009).

The isolates studied in this work have a similar morphology to that described previously by Melhorn et al. (2009) (Fig. 5). The mean values of the morphological characteristics are also consistent with the values described by the above mentioned authors, being the greater length of the flagella the main discrepancy. In general, there is a wide morphological variability among isolates (Table 2; Online Resource 3).

The great morphological variability found in *T. gallinae* by other authors (Melhorn et al. 2009; Tasca and De Carli 2003) has been also observed in this work. In order to reduce the statistical type I error in our analysis, we have reduced the level of significance to $p<0.01$. The variables of perimeter and surface of both cell and nucleus were highly correlated with other variables (the respective length and width of the cell and the nucleus), and they were discarded from the MANOVA analysis. Also, the lengths of the free flagella were cross-correlated between them and they were substituted by a combined variable "total flagellar length".

For the statistical study, we initially considered three different groups according to the ITS sequences (A, B, and vulture isolates). The results of the MANOVA analysis with the variables finally selected indicated a significant difference between the genetic groups (Pillai's Trace test, $p<0.000$). Follow up univariate ANOVAs indicated that there were differences in relation to all the morphological criteria (F-tests, $p<0.000$) except the nucleus length and width ($p=0.021$ and 0.313, respectively). The results of the Scheffé post hoc tests (Table 3) showed that the genotypes A and B were morphologically indistinguishable while the vulture isolates presented smaller values for each variable except for the "length of axostyle projection", which was higher (Online Resource 4).

Scanning electron microscopy

Isolates RM11–12, RM12–12 (respectively corresponding to *T. gallinae* genotypes A and B), and the vulture isolate R235–13 were studied under SEM (Fig. 6). No differences were observed among the isolates, except the smaller size of the cells from vultures. The images highlight the plasticity of the trophozoites (from ovoid to pyriform). The four anterior flagella originated from a common region (the periflagellar canal) located at the apical pole of the cell. From this periflagellar canal, the recurrent flagellum emerges and forms an undulating membrane without a free end portion of the flagellum. The axostyle projection varied within each isolate, but it was clearly apparent its proportional greater size in the trichomonads from the vulture isolates due to their smaller cell size.

Special features of ***trichomonas*** from vultures

Even in the first observation in wet preparations of culture samples, the trophozoites of *Trichomonas* from vultures appeared to have smaller size than other trophozoites of *T. gallinae*. This perception was confirmed by the significant differences found in the results of the morphometric parameters measured.

Given the differences found in this study at the genetic, morphological, and host range level, we can propose that *Trichomonas* from vultures is not a genetic variant of *T. gallinae*, but corresponds to a different species. The genetic study of the ITS1–5.8SrRNA-ITS2 region by PCR-sequencing of the different isolates of this study revealed the presence of two genotypes previously described for *T. gallinae* (A and B), as well as the presence of a new variant obtained from 2 Egyptian vultures and 14 cinereous vultures. In addition, these sequences of the vultures' variant match a sequence described by Grabensteiner et al. (2010) from a bearded vulture (and identified as of *Trichomonas* sp.) from the Czech Republic. Kelly-Clark et al. (2013) found what they described as *T. vaginalis*-like in a rehabilitated American bald eagle from Canada, being genetically identical to the isolate of Grabensteiner et al. (2010). The presence of this parasite in a bald eagle is difficult to explain. Some authors claim that the diet of bald eagles can include birds and carrion (Anthony et al. 2008; Grubb and Lopez 2000), but basically feed on fish. The bird studied in Canada was housed individually within a flight-cage where biosecurity practices would reduce the likelihood of contamination by other species (cats, rodents, and wild birds), and it was fed a fish diet and its water was provided from a treated municipal groundwater source. Apart from this finding, it seems that this parasite is only present in necrophagous birds. In fact, to date, *T. gallinae* has never been conclusively reported in vultures; it has been reported only one time from an English Egyptian vulture in 1972 but without giving a precise identification (Forrester and Foster 2008).

The vultures (scavenging birds of prey) comprise of two groups of birds that are related only by evolutionary convergence since they are separated taxonomically: the Old World vultures (O. Falconiformes, Fam. Accipitridae, and Subfam. Gypaetinae) and the New World vultures (O. Falconiformes, and Fam. Cathartidae). In general, all the vultures feed primarily on dead animal remains. However, the eating habits of each type of vulture are quite different, which favors the survival of all of them if they coexist in the same niche (Del Hoyo et al. 1994). In general, the birds of prey who suffer infection by *T. gallinae* (Bonelli's eagle, common buzzard, etc.) acquire the parasite by feeding on other birds, especially columbiformes, main hosts of the protozoan. On the contrary, vultures do not feed by bird hunting. Cinereous vultures and griffon vultures usually feed on the remains of mammals ungulates such as deer or mouflon; in some places, carrion

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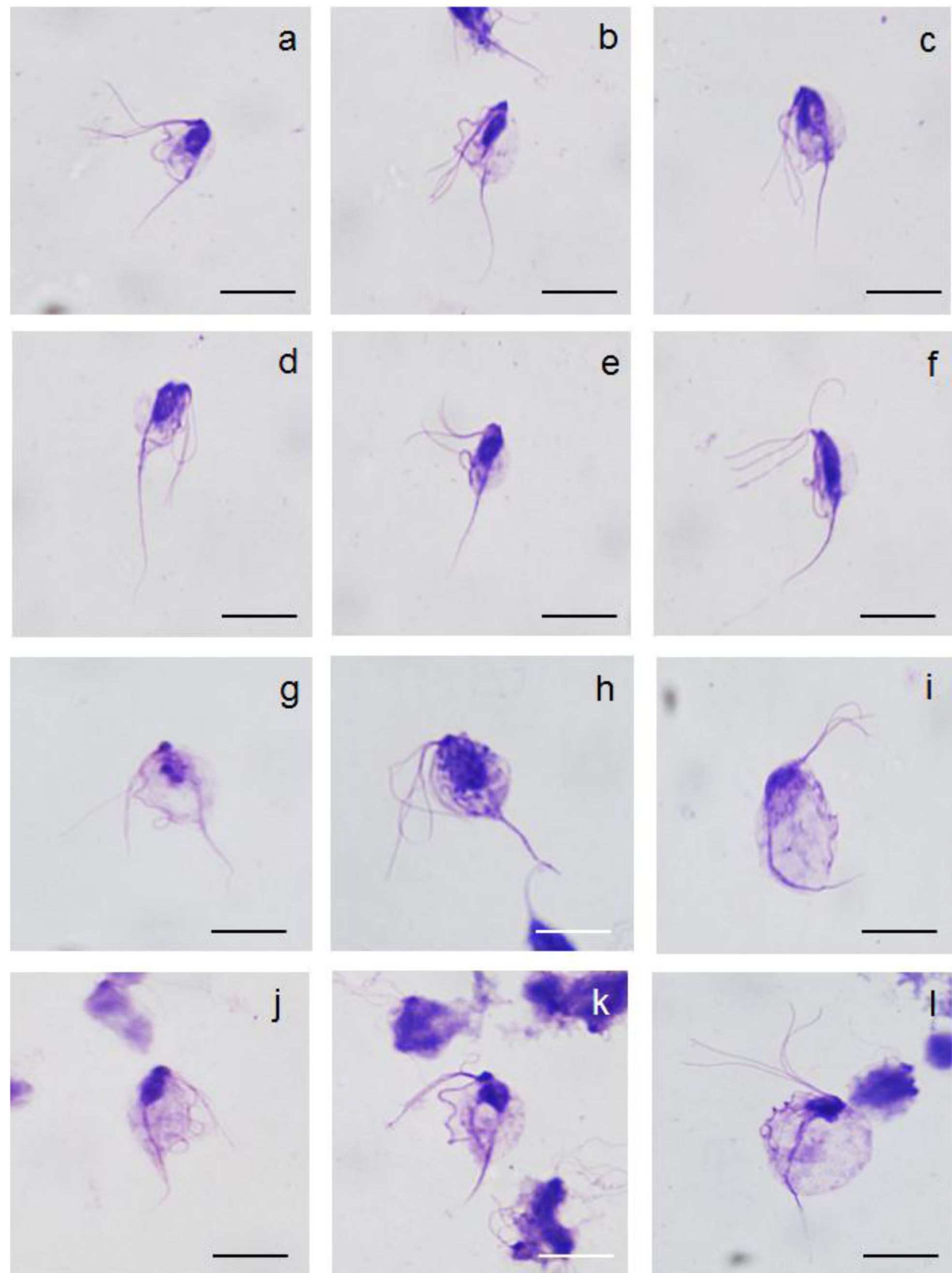

Fig. 5 Trophozoites from different isolates: **a**–**c** *Trichomonas* from Egyptian vultures (isolate RM4–12); **d**–**f** *Trichomonas* from cinereous vultures (isolate 13–3061); **g**–**i** *T. gallinae* genotype A (isolate PM2–12); **j**–**l** *T. gallinae* genotype B (isolate OM1–12). Fast metachromatic staining preparations. Scale bar 10 μm

left by shepherds is an important part of the diet of these birds. The cinereous vulture is more selective with the food than the griffon vulture, and it quickly goes to the corpse of the mammal to arrive earlier than other species of vultures to feed mainly on muscle avoiding the viscera. In some regions, the diet of cinereous vultures shows a high consumption of rabbits *Oryctolagus cuniculus*, particularly by the young vultures during the breeding season. However, adult vultures rely more on wild ungulates, as do the young vultures after fledging. Only occasionally, they feed on birds (Guzmán and Jiménez 1998). The Egyptian vulture has a more varied diet; the wild and domestic ungulates constitute most of their diet (up to 80 %), but they may also eat small mammals (rabbits and rats), birds (pigeons, chickens, and passerines), and even invertebrates (beetles, bugs, ants, and molluscs). In this case, it would be feasible the transmission through ingestion of birds, but these form a very minor part of its diet. The bearded vulture has a highly specialized feeding on skeletal remains; its extensive oral commissure and the presence of large amounts of acid secreting cells facilitate digestion of

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Table 2 Morphometrical data of the *Trichomonas gallinae* and the *Trichomonas* from Spanish vultures isolates. All values are expressed in micrometer. The different isolates were grouped according to their ITS sequences as *T. gallinae* A or B genotypes (following Sansano-Maestre et al. 2009) or vulture isolates. *The free flagella are ordered from the larger to the shorter one

Parameter	Mean±standard deviation (range)			
	T. gallinae A-genotype isolates	*T. gallinae* B-genotype isolates	*T. gallinae* isolates (A + B genotypes combined)	*Trichomonas* from vultures isolates
Cell length	10.9±1.7 (7.3–15.4)	10.6±1.9 (7.2–16.6)	10.7±1.8 (7.2–16.6)	8.1±1.1 (5.6–10.9)
Cell width	6.2±1.6 (3.5–11.6)	6.7±1.8 (3.4–12.7)	6.5±1.8 (3.4–12.7)	5.8±1.2 (2.6–8.4)
Cell perimeter	26.4±4.4 (19.0–41.0)	26.8±5.3 (17.5–44.4)	26.6±4.9 (17.5–44.4)	22.1±3.3 (13.9–29.1)
Cell surface	45.9±19.7 (17.1–124.8)	50.8±24.0 (18.0–150.4)	48.7±22.4 (17.1–150.4)	35.4±11.4 (10.7–64.4)
Nucleus length	3.6±0.7 (2.3–5.5)	3.3±0.6 (1.9–4.8)	3.4±0.7 (1.9–5.5)	3.4±0.5 (2.3–4.7)
Nucleus width	2.3±0.5 (1.0–3.7)	2.2±0.5 (1.1–3.3)	2.2±0.5 (1.0–3.3)	2.2±0.4 (1.1–3.2)
Nucleus perimeter	9.3±1.7 (4.5–13.8)	8.7±1.6 (4.6–12.9)	9.0±1.6 (4.5–13.8)	8.9±1.3 (6.1–11.7)
Nucleus surface	6.1±2.2 (2.2–12.6)	5.4±2.0 (2.2–11.3)	5.7±2.1 (2.2–12.6)	5.4±1.6 (2.2–9.9)
Length of flagellum 1*	18.2±2.0 (13.7–23.3)	17.4±2.5 (10.7–24.9)	17.8±2.3 (10.7–24.9)	14.4±1.4 (10.5–18.0)
Length of flagellum 2*	17.0±2.2 (11.6–22.7)	16.5±2.4 (10.2–23.1)	16.7±2.31 (10.2–23.1)	14.1±1.5 (9.1–18.4)
Length of flagellum 3*	15.1±1.9 (10.7–20.6)	14.5±2.4 (9.4–21.1)	14.8±2.2 (9.4–21.1)	12.5±1.6 (7.3–16.8)
Length of flagellum 4*	14.1±2.2 (7.9–20.4)	13.7±2.5 (7.4–20.5)	13.9±2.4 (7.4–20.5)	12.1±1.6 (7.2–16.2)
Length of recurrent flagellum	15.8±3.8 (5.3–26.3)	17.1±4.2 (8.6–27.2)	16.6±4.1 (5.3–27.2)	12.8±2.2 (6.9–19.4)
Length of costa	10.2±2.5 (3.9–16.1)	10.5±2.1 (6.0–15.2)	10.3±2.3 (3.9–16.1)	8.5±1.2 (5.1–12.5)
Length of axostyle	20.1±2.7 (15.9–28.5)	19.6±3.6 (12.2–30.6)	19.8±3.3 (12.2–30.6)	16.3±2.8 (8.6–22.9)
Length of the axostyle projection	6.7±2.1 (2.2–11.5)	6.4±2.2 (2.6–12.4)	6.6±2.2 (2.2–12.4)	7.6±1.9 (2.3–12.2)

bones. Wild and domestic ungulates are its main food sources. Furthermore, the special feeding of vultures in general leads to their physiology to present particular aspects which distinguish them from other birds (Del Hoyo et al. 1994).

Given all this, we can conclude that the tricomonad located in the upper digestive tract of vultures is not *T. gallinae*, but a genetically close species to *T. vaginalis* than to *T. gallinae*, and we have called it *Trichomonas gypaetinii*. Its features are as follows:

Trophozoite morphology: cell body varies from ovoid to pyriform with the morphologic characteristics of the genus *Trichomonas*. Sizes are shown in Table 2.
Diagnostic characters: small size, long, and very prominent axostyle; SSU-rRNA and ITS1-5.8S rRNA-ITS2

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Table 3 Results of the Scheffé post hoc tests with the variables that showed significant differences in univariate ANOVA analysis. The mean values shown correspond to the initial (non-transformed) variables (in μm). The genetic groups were established from the ITS sequences as *T. gallinae* genetic variants A and B (TgA and TgB) (Sansano-Maestre et al. 2009) and *Trichomonas* from vultures (Tv)

Transformed variable	Homogeneous group/s	Significance	Genetic group	Mean
Cell length	1	1.000	Tv	8.1
	2	0.424	TgB	10.6
			TgA	10.9
Cell width	1	0.159	Tv	5.8
			TgA	6.2
	2	0.067	TgA	6.2
			TgB	6.7
Nucleus length	1	0.016	TgB	3.3
			Tv	3.4
			TgA	3.6
Nucleus width	1	0.299	TgB	2.2
			Tv	2.2
			TgA	2.3
Total length of free flagella	1	1.000	Tv	15.1
	2	0.091	TgB	16.4
			TgA	16.7
Length of the recurrent flagellum	1	1.000	Tv	12.8
	2	0.033	TgA	15.8
			TgB	17.1
Length of costa	1	1.000	Tv	8.5
	2	0.310	TgA	10.2
			TgB	10.5
Length of axostyle	1	1.000	Tv	16.3
	2	0.350	TgB	19.6
			TgA	20.1
Axostyle projection	1	0.578	TgB	6.4
			TgA	6.7
	2	1.000	Tv	7.6

gene sequences; type of host (vultures, Subfam. Gypaetinae).
Host: *N. percnopterus* and *A. monachus*. According to genetic analysis made by other authors, *Gypaetus barbatus* and *Haliaeetus leucocephalus* could be also considered hosts of this protozoon (Grabensteiner et al. 2010; Kelly-Clark et al. 2013).
Site of infection: upper gastrointestinal tract.
Locality: obtained from *N. percnopterus* and *A. monachus* from Spain (Almería, Cáceres, Ciudad Real, Madrid, Segovia, Sevilla, Valencia). It has been also reported from *G. barbatus* from the Czech Republic (Grabensteiner et al. 2010) and *H. leucocephalus* from Canada (Kelly-Clark et al. 2013).
Pathology: not determined; non-pathogen so far.

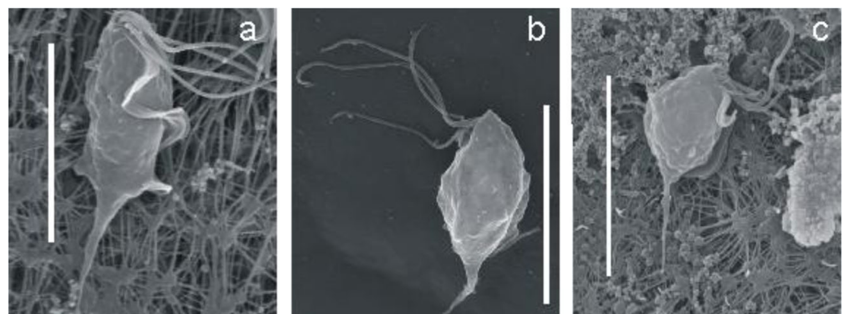

Fig. 6 Scanning electron micrographs of the *Trichomonas* trophozoites from different isolates: **a** *T. gallinae* genetic variant A (isolate RM11–12); **b** *T. gallinae* genetic variant B (isolate RM12–12); **c** *Trichomonas* from vultures (isolate RM4–12). All images are at the same magnification; scale bar 10 μm

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Etymology: the specific name refers to the group of avian hosts where we have found the parasites.

Further studies are needed to determine if other scavenger bird species may be hosts of this parasite.

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RAPD analysis and sequencing of ITS1/5.8S rRNA/ITS2 and Fe-hydrogenase as tools for genetic classification of potentially pathogenic isolates of *Trichomonas gallinae*

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ABSTRACT

Trichomonas gallinae is a worldwide parasite that causes oropharyngeal avian trichomonosis. During eight years, 60 axenic isolates were obtained from different bird species and characterized by three molecular methods: RAPD analysis and PCR-sequencing of ITS1/5.8S rRNA/ITS2 fragment and Fe-hydrogenase gene. We have found two genotypes of ITS1/5.8S rRNA/ITS2 widely distributed among bird populations, a new variant and also two sequences with mixed pattern. Genotype ITS-OBT-Tg-1 was associated with the presence of gross lesions in birds. We have found eight genotypes of the Fe-hydrogenase (A1, A2, C2, C2.1, C4, C5, C6 and C7), three of them are new reports (C5, C6 and C7), and also three sequences with mixed pattern. Subtype A1 of the Fe-hydrogenase was also related with the presence of lesions. RAPD analyses included most of the strains isolated from animals with lesions in one of the sub-clusters. Potentially pathogenic isolates of *T. gallinae* obtained in this study fulfill the following criteria with one exception: isolated from lesions + ITS-OBT-Tg-1 genotype + FeHyd A1 + RAPD sub-cluster I2.

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1. Introduction

Trichomonas gallinae is the parasitic protozoan responsible for avian trichomonosis, a worldwide emergent disease which affects many species of birds. The parasite usually lives in the upper digestive tract, where it multiplies asexually by means of binary fission. Columbiforms act as the main hosts of the protozoa, and are responsible for the wide distribution of the parasite in the wild. Direct transmission of the parasite is made during courtship between adults and from parents to their offspring by crop milk. Although the protozoan is not able to generate cystic forms, it can survive in water and regurgitated food for a short period of time (Purple et al., 2015). This was considered the main cause of transmission in the recent outbreaks and mortality events among passerine birds from Norway, Finland, Canada, Slovenia, The United Kingdom and many other European countries such as Germany, Austria and France. Also, the survival in carcasses is thought to be an important form of transmission to raptors (reviewed in Amin et al., 2014).

Recent findings using ITS1/5.8S rRNA/ITS2 sequencing revealed the existence of different genotypes of *T. gallinae* which affect the oral cavity of birds (Gerhold et al., 2008; Anderson et al., 2009; Sansano-Maestre et al., 2009; Grabensteiner et al., 2010; Chi et al., 2013; Martínez-Herrero et al., 2014). Two of the genotypes are predominant over the rest and one of them seems to be related with the mortality outbreaks of the disease in Europe (Chi et al., 2013; Lawson et al., 2011; Ganas et al., 2014), and in the USA (Girard et al., 2014a, b) and also to the development of gross lesions in affected individuals (Martínez-Herrero et al., 2014). Also, the study of the ITS1/5.8S rRNA/ITS2 sequence suggested to some authors the possibility that different species of trichomonads were able to colonize the upper digestive tract of birds (Anderson et al., 2009; Ecco et al., 2012; Girard et al., 2014a; Martínez-Díaz et al., 2015).

Sequencing of the Fe-hydrogenase gene (FeHyd) has also been used to investigate outbreaks of oral trichomonosis in Europe and in the USA, but the results were not consistent among the studies and the obtained sequences seemed to be more related to the host or geographic origin

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than to the presence of gross lesions and/or mortality (subtypes A1 and A2 in pathogenic isolates of Europe and the USA respectively and C4 in columbiforms of the UK, Lawson et al., 2011; Chi et al., 2013; Ganas et al., 2014; Girard et al., 2014b).

Other tool for DNA typing, like random amplified polymorphic DNA (RAPD), involves the amplification of random segments of genomic DNA using short single arbitrary primers and has been proved to be useful for the differentiation of trichomonad species (Cepicka et al., 2005;

Table 1
Axenic isolates of *T. gallinae* employed in this study. Year of sampling, location, presence of macroscopic lesions, host, ITS1/5.8S rRNA/ITS2 genotype, FeHyd genotype, RAPD cluster and GenBank accession numbers for FeHyd and ITS are indicated.

Isolate	Sampling year	Location	Lesion	Host	ITS genotype	FeHyd genotype	RAPD cluster	FeHyd GenBank accession numbers	ITS GenBank accession numbers
P124/05	2005	Alicante	No	*Columba livia*	ITS-OBT-Tg-2	C4	II5		
P69/05	2005	Castellón	No	*Columba livia*	ITS-OBT-Tg-2	C4	II2	**KP900037**	
P82/05	2005	Castellón	No	*Columba livia*	ITS-OBT-Tg-1	A1	I3	**KP900026**	
R18/05	2005	Alicante	No	*Aquila fasciata*	ITS-OBT-Tg-2	NOVEL C5	I2	**KP900039**	
R25/05	2005	Murcia	No	*Aquila pennata*	ITS-OBT-Tg-2	NOVEL C6	II6	**KP900041**	**EU881917**
P1/06	2006	Valencia	No	*Columba livia*	ITS-OBT-Tg-1	A2	I4		
P102/06	2006	Castellón	No	*Columba livia*	ITS-OBT-Tg-2	C4	II8		
P115/06	2006	Castellón	No	*Columba livia*	ITS-OBT-Tg-2	C4	II5		
P126/06	2006	Castellón	No	*Columba livia*	ITS-OBT-Tg-2	C4	II5		
P135/06	2006	Castellón	No	*Columba livia*	ITS-OBT-Tg-2	NOVEL C5	II6		
P16/06	2006	Valencia	No	*Columba livia*	ITS-OBT-Tg-2	A1	I2		
P37/06	2006	Alicante	No	*Columba livia*	ITS-OBT-Tg-2	C4	II2		
P52/06	2006	Alicante	No	*Columba livia*	ITS-OBT-Tg-2	C4	II2		
P54/06	2006	Alicante	No	*Columba livia*	ITS-OBT-Tg-2	C4	II5		
P88/06	2006	Valencia	No	*Columba livia*	ITS-OBT-Tg-1	A2	I4	**KP900030**	
P99/06	2006	Castellón	No	*Columba livia*	ITS-OBT-Tg-2	NOVEL C5	II8		
R5/06	2006	Valencia	Yes	*Tyto alba*	ITS-OBT-Tg-1	A1	I2	**KP900029**	**EU881913**
P5/07	2007	Valencia	No	*Columba livia*	ITS-OBT-Tg-2	C4	II4		
P7/07	2007	Valencia	No	*Columba livia*	MIXED	MIXED	II4		
P9/07	2007	Valencia	No	*Columba livia*	ITS-OBT-Tg-1	A1	I2		
R16/07	2007	Murcia	No	*Accipiter gentilis*	ITS-OBT-Tg-2	C4	II2		
R2/07	2007	Alicante	No	*Aquila fasciata*	ITS-OBT-Tg-1	A1	I2	**KP900024**	
R21/07	2007	Murcia	No	*Accipiter gentilis*	ITS-OBT-Tg-2	C4	II3		
R22/07	2007	Murcia	No	*Accipiter gentilis*	ITS-OBT-Tg-2	C4	II3		
R23/07	2007	Murcia	No	*Accipiter gentilis*	ITS-OBT-Tg-2	C4	II3		
R24/07	2007	Murcia	No	*Accipiter gentilis*	ITS-OBT-Tg-2	C4	II3		
R29/07	2007	Murcia	No	*Accipiter gentilis*	ITS-OBT-Tg-2	C4	II3		
R32/07	2007	Murcia	No	*Accipiter gentilis*	ITS-OBT-Tg-2	C4	II3		
P103/08	2008	Castellón	No	*Columba livia*	ITS-OBT-Tg-2	NOVEL C5	II6		
P17/08	2008	Valencia	No	*Columba livia*	ITS-OBT-Tg-1	A2	I4		
P19/08	2008	Valencia	No	*Streptopelia decaocto*	ITS-OBT-Tg-2	C4	II2	**KP900038**	
P29/08	2008	Castellón	No	*Columba livia*	ITS-OBT-Tg-2	NOVEL C5	II7		
P35/08	2008	Castellón	No	*Columba livia*	ITS-OBT-Tg-2	C4	II5		
P44/08	2008	Castellón	No	*Columba livia*	ITS-OBT-Tg-2	C4	II5		
P53/08	2008	Castellón	No	*Columba livia*	ITS-OBT-Tg-2	NOVEL C5	II5	**KP900040**	
P79/08	2008	Castellón	No	*Columba livia*	ITS-OBT-Tg-2	C4	II5		
P9/08	2008	Valencia	No	*Columba livia*	ITS-OBT-Tg-1	A1	I4		
R112/08	2008	Valencia	Yes	*Falco tinnunculus*	ITS-OBT-Tg-1	A1	I2	**KP900027**	
R123/08	2008	Murcia	No	*Accipiter gentilis*	ITS-OBT-Tg-2	C4	II2	**KP900035**	
R124/08	2008	Murcia	No	*Accipiter gentilis*	MIXED	A1	I4	**KP900023**	
R127/08	2008	Murcia	No	*Accipiter gentilis*	ITS-OBT-Tg-2	C4	II2		
R146/08	2008	Murcia	No	*Aquila fasciata*	ITS-OBT-Tg-1.1	A1	I4		**KP900042**
R157/08	2008	Alicante	No	*Aquila pennata*	ITS-OBT-Tg-2	C2.1	II8	**KP900032**	
R85/08	2008	Alicante	No	*Aquila fasciata*	ITS-OBT-Tg-1	A1	I2		
R86/08	2008	Alicante	No	*Aquila fasciata*	ITS-OBT-Tg-2	MIXED	II1		
R87/08	2008	Alicante	No	*Aquila fasciata*	ITS-OBT-Tg-2	C4	II3	**KP900036**	
P1/09	2009	Valencia	No	*Streptopelia decaocto*	ITS-OBT-Tg-2	C2	II7	**KP900031**	
P11/09	2009	Valencia	No	*Streptopelia decaocto*	ITS-OBT-Tg-2	C2.1	II7		
P14/09	2009	Valencia	No	*Streptopelia decaocto*	ITS-OBT-Tg-2	C2.1	II7		
P2/09	2009	Valencia	No	*Streptopelia decaocto*	ITS-OBT-Tg-2	C2.1	II7		
P3/09	2009	Valencia	No	*Streptopelia decaocto*	ITS-OBT-Tg-2	NOVEL C7	II7	**KP900034**	
P9/09	2009	Valencia	No	*Streptopelia decaocto*	ITS-OBT-Tg-2	NOVEL C7	II7		
R1/09	2009	Toledo	Yes	*Strix aluco*	ITS-OBT-Tg-1	A1	I2	**KP900028**	
R2/09	2009	Valencia	Yes	*Falco tinnunculus*	ITS-OBT-Tg-1	A1	I2		
R179/11	2011	Valencia	Yes	*Falco tinnunculus*	ITS-OBT-Tg-1	A1	I2		
R180/11	2011	Valencia	Yes	*Falco tinnunculus*	ITS-OBT-Tg-1	A1	I2		
R55/11	2011	Valencia	Yes	*Falco tinnunculus*	ITS-OBT-Tg-1	A1	I3		
R131/12	2012	Valencia	Yes	*Falco tinnunculus*	ITS-OBT-Tg-1	MIXED	I1		
R134/12	2012	Valencia	Yes	*Aquila pennata*	ITS-OBT-Tg-2	NOVEL C7	II6	**KP900033**	**KF993686**
R16/12	2012	Murcia	Yes	*Bubo bubo*	ITS-OBT-Tg-1	A1	I2	**KP900025**	**KF993696**

Kutisova et al., 2005). Furthermore, different strains were grouped according to some characteristics, such as resistance to metronidazole, virulence and geographic origin using this technique (Vánácová et al., 1997; Snipes et al., 2000; Kaul et al., 2004; Rojas et al., 2004). Regarding *T. gallinae*, this technique supported a correlation between RAPD clusters of the parasite, the geographical distribution and the host species (Gaspar da Silva et al., 2007) and had reinforced the hypothesis that there was a clonal strain implicated in the outbreak mortality episodes along the United Kingdom (Lawson et al., 2011).

The aim of this study was to genetically characterize *T. gallinae* isolates in order to investigate the best tool for the classification of isolates into potentially pathogenic/non pathogenic for further studies. To compare the different tools available to genetically characterize the parasite we have used different techniques and targets: PCR and sequencing of the gene fragments ITS1/5.8S rRNA/ITS2 and FeHyd and RAPD analyses of genomic DNA. Variables "presence of gross lesions" and "geographic origin" were considered for statistical analysis.

2. Material and methods

2.1. Animals and sampling

Sixty isolates of *T. gallinae* obtained from the oral cavity of sixty birds from nine different species were analyzed (Table 1). Samples were obtained from different provinces of Spain and along 8 years of study (2005–2012). Fifty samples (83.3%) came from asymptomatic animals whereas ten (16.7%) were obtained from raptors which displayed oropharyngeal granulomas (Table 1). Samples from symptomatic animals were taken from clinical entries at the recovery centers (CERI, Toledo; La Granja, El Saler, Valencia; Santa Faz, Alicante and El Valle, La Alberca, Murcia). Samples from wild columbiforms came from pest control programs undertaken by the local authorities using cage traps. Other samples from raptors were obtained from chicks at the nest during scientific ringing campaigns.

Birds were sampled using sterile esophageal swabs previously moistened with sterile physiologic saline solution that was gently rubbed over the oropharyngeal cavity and crop. Samples were inoculated in 5 ml of TYM (Trypticase-Yeast-Maltose) medium supplemented with 10% of inactivated fetal calf serum (Sigma-Aldrich, St. Louis, Missouri, USA). After inoculation, cultures were maintained at 37 °C and observed daily during 15 days to assess the parasite growth.

2.2. Axenization of cultures

Axenization of cultures was carried out by means of successive passages in TYM medium supplemented with antibiotic/antimycotic solution containing Ticarcillin 0.15 μg/ml; Vancomycin 0.15 μg/ml; Ceftiofur 0.15 μg/ml; Nystatin 180 UI/ml; (Sigma-Aldrich, St. Louis, Missouri, USA). Absence of contaminants (bacteria and yeasts) was assessed by Schaedler agar with 5% sheep blood, trypticase soy agar and Saboraud dextrose agar cultures (bioMérieux, Madrid, Spain). Storage at −80 °C using 5% of DMSO (dimethyl sulphoxide, Sigma-Aldrich, St. Louis, Missouri, USA) in TYM medium was carried out to preserve the isolates for further analysis.

2.3. DNA extraction

One million trophozoites of *T. gallinae* in exponential growth phase were washed three times with PBS (pH 7.2). Cells were recovered by centrifugation at 700 × *g*, 3 min. DNA was extracted using a commercial kit (DNeasy® Tissue Kit, QIAGEN, USA) following the manufacturer's instructions. Pellets were stored at −20 °C until use.

2.4. PCR amplification of the ITS1/5.8S rRNA/ITS2 region and the Fe-hydrogenase gene

To amplify the ITS1/5.8S rRNA/ITS2 region PCR reactions were carried out using TRF1 (5′-TGCTTCAGCTCAGCGGGTCTTCC-3′) and TFR 2 (5′-CGGTAGGTGAACCTGCCGTTGG-3′) primers (Felleisen, 1997). The final reaction volume was 50 μl with 5 μl of 10× buffer without magnesium chloride ($MgCl_2$); 3 μl of 25 mM $MgCl_2$; 0.5 μl of 10 μM dNTPs; 0.5 μl of 100 μM of each primer; 2.5 UI (0.5 μl) of Taq Gold polymerase (PE Applied Biosystems, Foster City, California, USA); 5 μl of DNA; 2.5 μl of DMSO and 32.5 μl of DNase, RNase, Protease free water (Water Molecular Biology Reagent, Sigma-Aldrich, St. Louis, Missouri, USA). The reactions were developed using the following temperature profile: 9 min of initial denaturation at 94 °C to activate the enzyme followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 66 °C for 30 s and extension at 72 °C for 30 s. Finally, an extension step at 72 °C for 15 min was carried out.

The amplification of the hydrogenosomal Fe-hydrogenase gene was performed using the primers TrichhydFOR (5´-GTTTGGGATGGCCTCAGAAT-3´) and TrichhydREV (5´-AGCCGAAGATGTTGTCGAAT-3´) (Lawson et al., 2011). The Master Mix reaction was performed in 25 μl with 1 μl of forward primer, 1 μl of reverse primer (both primers at 10 μM), 12.5 μl of HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany), 2.5 μl of DNA and 8 μl of DNase, RNase, Protease free water (Water Molecular Biology Reagent, Sigma-Aldrich, St. Louis, Missouri, USA). Reactions were developed using the following temperature profile: 15 min of enzyme activation at 95 °C, 40 cycles of 30 s at 94 °C for denaturation, 1 min at 58 °C for annealing and 2 min at 72 °C for DNA elongation, followed by a final extension step of 10 min at 72 °C.

Each set of amplification contained a negative control of molecular grade water and a positive control of known *T. gallinae* DNA. All reactions were developed in a thermocycler (GeneAmp® PCR System 2700, Perkin-Elmer Applied Biosystems, USA). Results were observed in 2% (w/v) agarose gels in Tris-Acetate-EDTA (TAE) buffer stained with ethidium bromide (0.63 μg/ml) and visualized under ultraviolet light in a UV transilluminator (Syngene, Cambridge, UK).

In order to compare sequences reported by other authors, and taking into account the different nomenclature published in the literature, we have followed the ITS genotype groups described by Martínez-Herrero et al. (2014). New sequences of the Fe-hydrogenase gene were named consecutively considering previous reports (Lawson et al., 2011, Chi et al., 2013, Ganas et al., 2014).

2.5. Analysis of sequences and phylogenetic trees

The PCR amplification products were purified using a commercial kit (MinElute PCR Purification Kit; QIAGEN, Valencia, California, USA) following the manufacturer's instructions and submitted for sequencing to "Sistemas Genómicos, S. A." (Paterna, Valencia, Spain). The reaction was developed in an automatic sequencer 3730XL DNA Analyzer (Applied Biosystems, Foster City, California, USA) with the ABI PRISM® BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). All the chromatograms of the ITS1/5.8S rRNA/ITS2 region and Fe-hydrogenase gene were manually checked in both directions (forward and reverse) and assembled using Lasergene SeqMan software version 7.0.0 (DNASTAR, Madison, Wisconsin, USA).

To construct the phylogenetic trees, representative sequences of each genotype were employed. Codon positions included were 1st + 2nd + 3rd + Noncoding. All the positions containing gaps and missing data were eliminated. There were a total of 241 positions in the final dataset for ITS1/5.8S rRNA/ITS2 region and 549 positions for the Fe-hydrogenase gene. Multiple alignments of the sequences were done employing Clustal W algorithm of MEGA software version 6.06 (http://www.megasoftware.net, Tamura et al., 2013). The average Jukes-Cantor distance was 0.067 for ITS1/5.8S rRNA/ITS2 region and

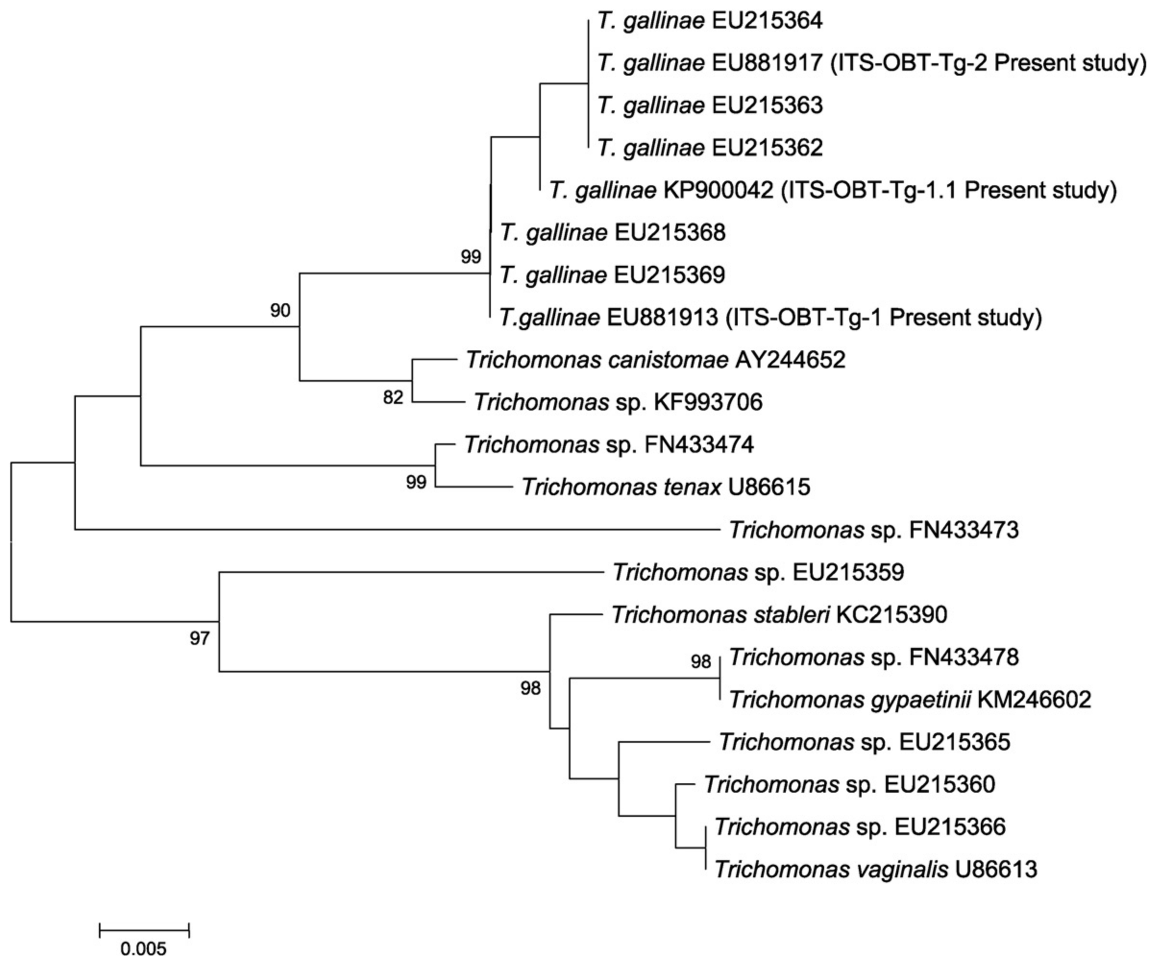

Fig. 1. Phylogenetic tree using the region ITS1/5.8S rRNA/ITS2 of *Trichomonas* sp. by Neighbor Joining method. Bootstrap values lower than 70 are not shown (n = 2000 replicates). Sequences from other authors are indicated with GenBank accession number and organism name. "Present study" is added for the sequences reported in this paper.

0.058 for Fe-hydrogenase gene. Evolutionary distances were computed with Maximum Composite Likelihood method for Neighbor-Joining tree (Figs. 1 and 2). Bootstrap of 2000 replicates was employed to estimate trees reliability. Each sequence was compared with the available ITS1/5.8S rRNA/ITS2 region and Fe-hydrogenase gene sequences available at NCBI GenBank using the BLAST search algorithm.

2.6. Random amplified polymorphic DNA and clustering

Sixty axenic isolates were used for the RAPD analysis. DNA was amplified using three random primers (OPD3, GTCGCCGTCA; OPD5, TGAGCGGACA; OPD8, GTGTGCCCCA) previously employed with *T. gallinae* by other authors (Gaspar da Silva et al., 2007, Lawson et al., 2011). The reaction was carried out in a final volume of 25 μl which contained 2.5 μl of 10× buffer without $MgCl_2$; 2.5 μl of 25 mM $MgCl_2$; 0.5 μl of 100 μM primer; 0.35 μl of 5 U/μl AmpliTaq Gold polymerase (PE Applied Biosystems, Foster City, California, USA); 2.5 μl DNA and 2.5 μl of DMSO. The volume was completed with DNAses and RNAses free water (Water Molecular Biology Reagent, Sigma-Aldrich, St. Louis, Missouri, USA). The temperature profile was as follows: initial step at 94 °C for 5 min for enzyme activation, followed by 40 cycles of 1 min at 94 °C for denaturation, 1 min at 36 °C and 2 min at 72 °C. A final extension step of 15 min at 72 °C was carried out. Agarose gels (1.5% w/v) were stained with ethidium bromide (0.63 μg/ml) and observed in a UV transilluminator (Syngene, Cambridge, UK).

To construct the RAPD tree, presence or absence of individual bands observed in the gels were included in a binary matrix. Each band was coded as "1" if it was present and "0" if it was absent in each sample. Only bands that were identified in three different amplifications were included. The record of the bands was done by the same person using *in silico* analysis (CLIQS 1D Pro software, Totallab, Nonlinear Dynamics, Newcastle, UK). The resultant matrix was analyzed using FreeTree software (http://www.natur.cuni.cz, Hampl et al., 2001) and the genetic distances were calculated using Nei and Li's similarity coefficient (Cepicka et al., 2005). A dendrogram was generated using an Unweighted Paired Grouping Method with Arithmetic Averages (UPGMA) algorithm, with a bootstrap value of 2000 replicates. The obtained dendrogram was edited using MEGA version 6.06 software.

2.7. Statistic analysis

The relationships between the presence or absence of clinical signs and the genetic analysis (sequence of the ITS region, sequence of the Fe-hydrogenase gene and cluster of RAPD) were assessed using a binary logistic regression model. IBM SPSS Statistics 20.0 software was used for the estimation of odds ratios (OR) employing 95% confidence intervals (CI). The distribution of RAPD clusters according to geographical origin and host species was assessed in the same way. When the number of isolates in each stratum (i.e. species or location) was low ($n < 5$) the statistical analysis was not performed.

2.8. Sequences

New sequences and sequences found in hosts not described previously were submitted to GenBank database with accession numbers KP900023-42.

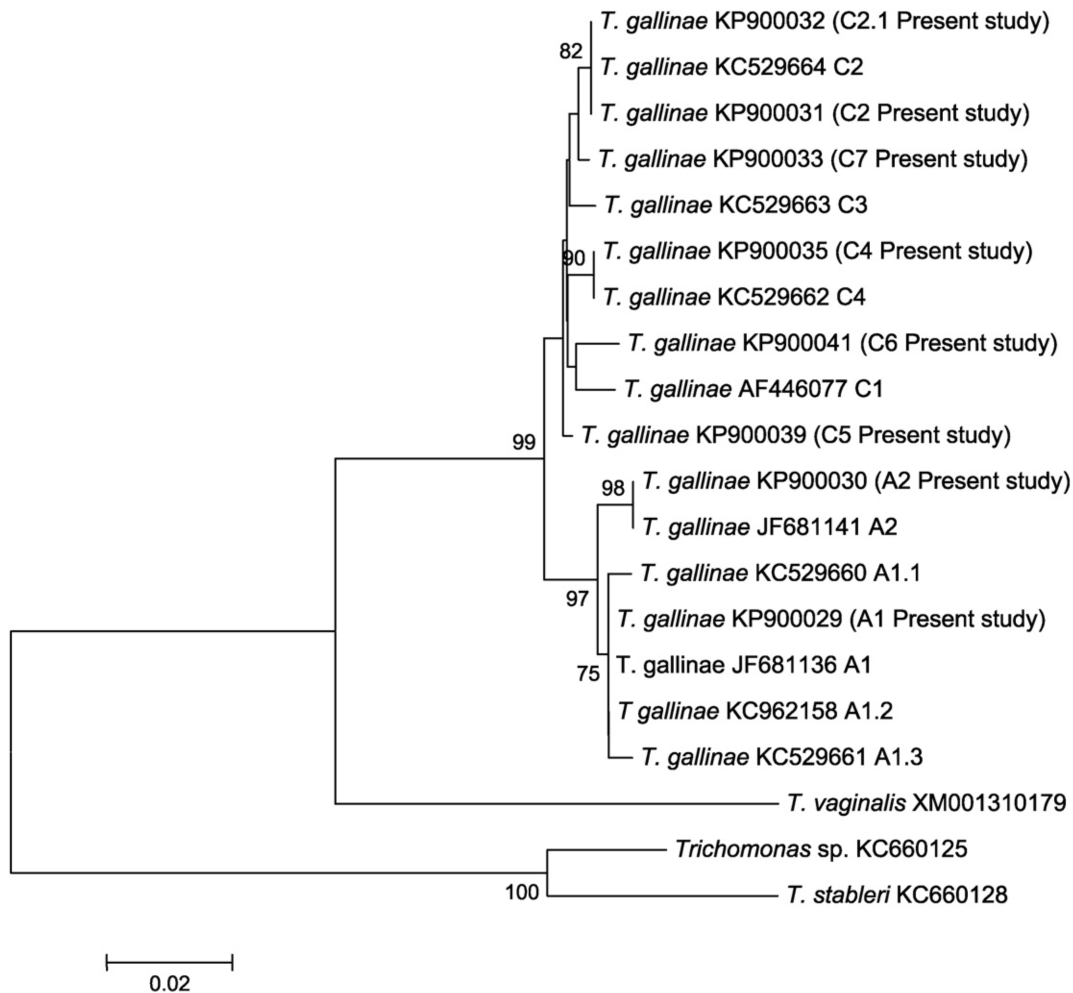

Fig. 2. Phylogenetic tree using the Fe-hydrogenase gene of *Trichomonas* sp. by Tamura-Nei method. Bootstrap values lower than 70 are not shown (n = 2000 replicates). Sequences from other authors are indicated with GenBank accession number and organism name. "Present study" is added for the sequences reported in this paper.

3. Results

3.1. Isolates

All the isolates of *T. gallinae* (n = 60) were genetically characterized by RAPD analysis and sequencing of the ITS1/5.8S rRNA/ITS2 region and Fe-hydrogenase gene (Table 1). Isolates were obtained from nine different species belonging to four different orders: Columbiformes, Accipitriformes, Strigiformes and Falconiformes. At the end of the study trichomonad isolates were recovered from 25 rock pigeons (*Columba livia*) (41.7%), 10 Northern goshawks (*Accipiter gentilis*) (16.7%), seven Eurasian collare d-doves (*Streptopelia decaocto*) (11.7%), six European kestrels (*Falco tinnunculus*) (10%), seven Bonelli's eagles (*Aquila fasciata*) (11.7%), two booted eagles (*Aquila pennata*) (3.3%), one tawny owl (*Strix aluco*) (1.7%), one eagle owl (*Bubo bubo*) (1.7%) and one barn owl (*Tyto alba*) (1.7%). In regard to the presence of clinical signs of oral trichomonosis, 16.7% of the samples (n = 10) were isolated from animals with macroscopic lesions whereas 83.3% (n = 50) had no clinical signs at the moment of sampling.

3.2. Sequence analysis of the ITS1/5.8S rRNA/ITS2 region

The obtained sequences were equivalent in nucleotide sequence and coverage to the most common *T. gallinae* genotype groups found in other parts of the world, ITS-OBT-Tg-1 and ITS-OBT-Tg-2 (Martínez-Herrero et al., 2014) (Table 1) (Fig. 1), except one isolate (R146/08) which was named ITS-OBT-Tg-1.1 (GenBank accession No. KP900042). This new genetic variant had one substitution compared to ITS-OBT-Tg-1 group (99% BLAST identity, E-value 3e-167) and it was isolated from a chick of a booted eagle, which did not show macroscopic lesions at the moment of sampling. In addition, two isolates (R124/08 and P7/07) displayed a mixed pattern at the ITS1/5.8S rRNA/ITS2 region.

As reported in other studies (Sansano-Maestre et al., 2009; Martínez-Herrero et al., 2014), isolates of the group ITS-OBT-Tg-1 (n = 17, 28.3%) were significantly associated with macroscopic lesions in the sampled birds (OR = 47.3; $IC_{95\%}$ = 5.2–426.4), and have a sequence identical to the *T. gallinae* isolates responsible for the last mortality reports of birds in Europe and the USA (GenBank accession No. HG008050, EU215369, KC215387, GQ150752, Gerhold et al., 2008; Robinson et al., 2010; Chi et al., 2013; Ganas et al., 2014; Girard et al., 2014b). However, most of the isolates circulating in the area of study (n = 40, 66.7%) displayed genotype ITS-OBT-Tg-2, which was not associated with the presence of oral lesions in birds and showed the same sequence than previously reported in isolates from non-sick animals (GenBank accession No. U86614, EU215363-64, FN433475, EU881912, Felleisen, 1997; Gerhold et al., 2008; Grabensteiner et al., 2010).

3.3. Sequence analysis of the Fe-hydrogenase gene

As expected, sequences of the Fe-hydrogenase gene showed a greater variability than the ITS1/5.8S rRNA/ITS2 region (Fig. 2). We have found eight different sequences of this gene, four of them are new reports in GenBank database (Table 1). As it was described by Chi et al. (2013), FeHyd genotype A was associated with ITS-OBT-Tg-1 group

whereas genotype C was associated with ITS-OBT-Tg-2 group. Subtype A1 was identified in 26.7% of the isolates (n = 16) whereas subtype A2 was a minority, found only in 5% of the isolates (n = 3). Both genotypes (A1, A2) were isolated more frequently in raptors than in columbiforms, mainly in the orders Falconiformes and Strigiformes. In our study, FeHyd type C sequences showed more variability, two of the subtypes (C2 and C4) were previously described in the literature and four were new sequences (novel subtypes C5, C6 and C7 and novel variant C2.1). Subtype C4 was the most frequently identified (38.3%, n = 23), and it was predominant in the orders Columbiformes and Accipitriformes. Both orders also harbored subtypes C2 and the novel variant C2.1 (6.7%, n = 4) and C7 (5%, n = 3). The novel subtype C5 was obtained in five isolates from Rock pigeons and one isolate from a Bonelli's eagle. The C6 novel subtype was isolated from a booted eagle. Finally, mixed patterns were obtained from a rock pigeon, a Bonelli's eagle and an European kestrel.

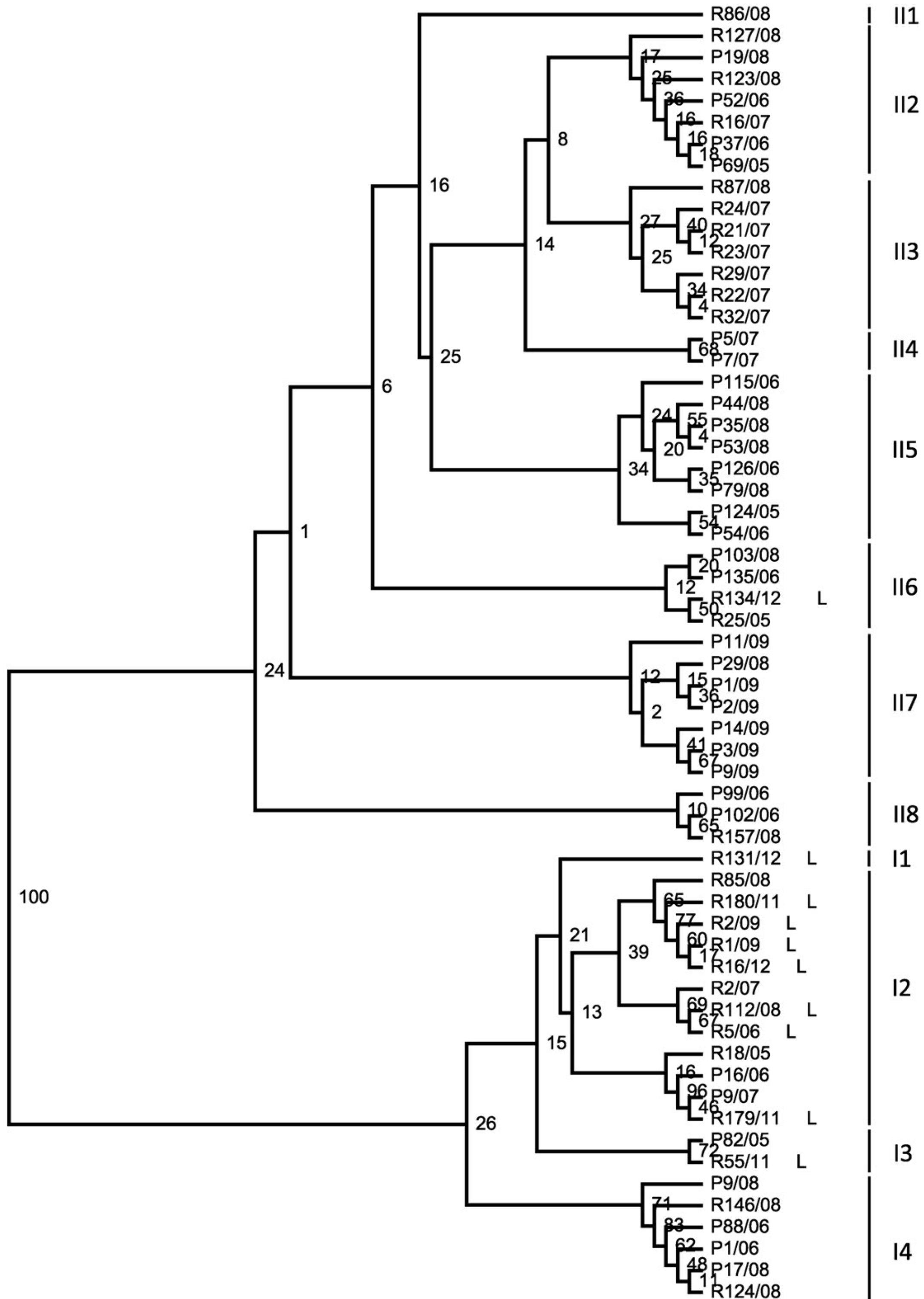

Fig. 3. Dendrogram of *T. gallinae* isolates based on the RAPD data using UPGMA method. The distance matrix was calculated using the coefficient of Nei and Li on the basis of 800 bands resulting from the combination of OPD3, 5 and 8 primers. Bootstrap values are obtained using 2000 replicates. Isolates obtained from lesions are marked with an “L”.

Regarding the presence of lesions, most of the animals which displayed macroscopic granulomas harbored subtype A1 (n = 10). One bird with lesion compatible with trichomonosis was typed as C7 and another has a mixed genotype. Thus, in our geographic area, there is an association between FeHyd subtype A1 and the presence of macroscopic lesions in birds (OR = 21; $IC_{95\%}$ = 3.7–117.8).

3.4. Random amplified polymorphic DNA

The band profiles displayed by each replicate were manually examined and verified by three independent amplifications for each OPD primer. As a result of the amplification a total of 800 bands were taken into account to analyze the intra-specific variability among *T. gallinae* isolates. OPD3 showed less variability between the isolates and displayed 241 bands, while OPD8 showed a total of 290 bands for the analysis. OPD5 amplified a total of 269 bands for the study. A dendrogram was constructed using the UPGMA method taking into consideration all the bands displayed from each isolate in three independent amplifications. The results obtained after the combination of the three primers is shown in Fig. 3. Two major groups were observed supported by the highest value of bootstrap (100), the largest one (cluster II) included most of the isolates belonging to genotype group ITS-OBT-Tg-2 (n = 39) whereas the smallest one (cluster I) included most of the isolates belonging to genotype group ITS-OBT-Tg-1 (n = 21). Cluster II could be sub-divided into eight different sub-clusters, while cluster I only deployed four groups. The new variant of the ITS-OBT-Tg-1 genotype was located in the minor cluster with most of the ITS-OBT-Tg-1 strains. Strains with a mixed pattern of ITS1/5.8S rRNA/ITS2 sequence (n = 2) appeared one in each group.

Most of the strains isolated from animals with clinical signs clustered together within cluster I (n = 9). In fact, we found association between sub-cluster I2 and presence of trichomonosis characteristic lesions (OR = 21 $IC_{95\%}$ = 4.1–108). In this case there was no relationship with avian species, geographical origin or date of sampling. One sample isolated from a booted eagle with lesions (R134/12) was grouped in cluster II. We would like to point out that within cluster I, which contains most of the isolates with lesions, sub-cluster I4 grouped isolates obtained from non-sick animals.

4. Discussion

Our results showed a high heterogeneity among the isolates of *T. gallinae* circulating in wild birds when several techniques were used for the genetic study. The analysis of ITS1/5.8S rRNA/ITS2 sequence displayed the most frequent genotypes found in previous studies carried out in the same area, ITS-OBT-Tg-1 and ITS-OBT-Tg-2, (Sansano-Maestre et al., 2009; Martínez-Herrero et al., 2014), but also a new variant of the ITS-OBT-Tg-1 genotype has been identified for the first time (GenBank accession No. KP900042). Sequence group ITS-OBT-Tg-1 was the main genotype identified by other authors in Europe (Lawson et al., 2011; Chi et al., 2013) and in the USA (Gerhold et al., 2008; Girard et al., 2014b). In fact, this was the only genotype isolated by authors such as Gaspar da Silva et al. (2007) in Mauritius, Ecco et al. (2012) in Brazil and Ganas et al. (2014) in Austria and Slovenia. However, in our study this genotype has lower prevalence in wild birds than genotype ITS-OBT-Tg-2. This difference could be due to the fact that most of the authors carried out the genetic identification using samples obtained from animals with clinical signs or during outbreaks of the disease. In our study, genotype ITS-OBT-Tg-1 was not always detected in animals with lesions. Also, 16% of the birds infected with ITS-OBT-Tg-1 did not displayed macroscopic lesions. Other factors such as the immune response of the animals or the presence of pathogenic determinants in the isolates have not been studied. The type of host (columbiforms *vs.* raptors) could not be analyzed either, since we did not find columbiforms with lesions in this study.

Much more variability was found in the Fe-hydrogenase gene analysis. The isolates investigated in our study displayed up to eight different types of sequences in this gene. Four of them were previously described by other authors whereas four of them were novel reports, all related to ITS1/5.8 rRNA/ITS2 genotype C. Among all the subtypes within this group, C4 was the predominant in our study. This subtype was also the main sequence obtained within the isolates from the UK. However, Girard et al. (2014b) only identified subtype C1 in the USA, which could indicate certain differences in the FeHyd genotype related to the geographic region. This hypothesis is reinforced attending to the FeHyd sequences of the pathogenic isolates. Our results showed that all the FeHyd variants found in the isolates genotyped as ITS-OBT-Tg-1 belonged to the subgroups A1 and A2, being A1 the most frequent. These results are similar to those described by other authors in Europe (Lawson et al., 2011, Chi et al., 2013, Ganas et al., 2014). In the USA, Girard et al. (2014b) found that the strains of *T. gallinae* obtained belonged mostly, although not only, to the FeHyd subtype A2. Regarding to the pathogenicity of the isolates both variants of FeHyd, A1 and A2, were involved in clinical episodes of oral trichomonosis, although A1 predominated in mortality outbreaks in Europe (Chi et al., 2013; Ganas et al., 2014) whereas A2 was most frequent in mortality events in the USA (Girard et al., 2014b). These results suggest that FeHyd sequences are more useful to investigate the origin of the outbreaks (host and/or geographic origin) than to determine the pathogenicity of the isolates.

Our results showed that there was a good correlation between the ITS sequencing and RAPD analysis, although the last technique has a higher discrimination power and defines more precisely the pathogenic character of the isolates. Both tools established two major different groups of the isolates of *T. gallinae*: cluster II obtained in the RAPD analysis grouped most of the isolates showing genotype ITS-OBT-Tg-2, while cluster I grouped most of the isolates with sequence ITS-OBT-Tg-1 (mostly pathogenic). Only one isolate (n = 1/10) obtained from an animal with lesions was not grouped with the rest in the RAPD analysis, which suggests that mixed infections could be present and one of the genotypes predominate over the other during sub-culturing (Sansano-Maestre et al., 2009; Grabensteiner et al., 2010; Chi et al., 2013; Girard et al., 2014b; Martínez-Herrero et al., 2014). As we found a strong correlation between the sub-cluster I2 and the presence of oropharyngeal lesions, the RAPD technique has allowed us a greater precision to classify the isolates of *T. gallinae* associated with lesions, since this sub-group was present in different host species, several years of sampling and distinct geographical origin. This classification could facilitate the realization of further studies using potentially pathogenic isolates of the parasite.

Lawson et al. (2011) did not find high variability in the RAPD analysis in the isolates of *T. gallinae* from the UK; however, we found a high variation among the isolates in our area of study. This could be partially explained by the low number of isolates studied by Lawson et al. (2011) employing RADP analysis (n = 14). Using a higher number of isolates (n = 60) and more variable in origin (species, lesions, geographic origin), we found relationships between some clusters and host species and geographic areas. As examples, we can mention samples taken from goshawks in the same breeding territories (Murcia) that grouped in sub-clusters II2 and II3 although in different years, samples from Eurasian collared-doves that were all taken in Valencia and grouped in sub-cluster II7 and samples classified in the sub-cluster II5 from domestic pigeons from Castellón. Gaspar da Silva et al. (2007) suggested that interspecific transmission between bird species and subsequent selection of strains in a determinate place could explain the geographical distribution of the RAPD patterns of the parasites found in their study. We think that this explanation is valid in our area of study, despite it is a continental area that differs from the insular environment studied by Gaspar da Silva et al. (2007). Thus, local selection of strains could be done by resident host birds of a determinate area which could be

infected by direct (feeding the offspring) or by indirect transmission (through water or food).

The RAPD technique offers advantages for the genetic characterization of isolates, comparing the polymorphism of independent genomic regions instead of focusing on the variation of only one locus (Fraga et al., 2002) and it has been successfully used in the characterization of trichomonads (Cepicka et al., 2005; Kutisova et al., 2005). Although the RAPD technique is useful it also has limitations. The first one is that it is not able to discriminate mixed infections which has been observed frequently by sequencing of the ITS1/5.8S rRNA/ITS2 in birds (Sansano-Maestre et al., 2009; Grabesteiner et al., 2010; Chi et al., 2013; Ganas et al., 2014, Girard et al., 2014b, Martínez-Herrero et al., 2014). This fact could explain the location of some strains that were genotyped as mixed ITS. Only cloning could avoid this limitation, but cloning of the isolates could also be a source of loss of information because not all the cells from one isolate can be cloned and analyzed. Other disadvantages of the technique are the impossibility of using DNA extracted from tissues or the need to use axenic cultures (Lawson et al., 2011).

Avian trichomonosis is considered nowadays a worldwide emergent disease that can influence negatively the population dynamics of some species of wild birds (Robinson et al., 2010). This study presents data from a high number of isolates fully characterized using three different molecular tools, obtained from different bird species, including raptors and columbiforms, as well as symptomatic and asymptomatic cases of avian trichomonosis. The obtained data could have great value regarding the epidemiology and control of the disease and could be useful for the planning of bird conservation programs. However, other approaches such as the influence of the immune status of the host or the conditions in the oropharynx of the birds should be studied as well.

5. Conclusion

We have examined the utility of three techniques in the genetic classification of potentially pathogenic isolates of *T. gallinae* using as a reference the capability to induce lesions in the oropharyngeal cavity of birds. Most of the potentially pathogenic isolates obtained in this study fulfill the following criteria: isolated from lesion + genotype ITS-OBT-Tg-1 + RAPD sub-clade I2. In order to classify the isolates according to their pathogenicity and use them for further studies, and also to save time and money, it could be useful to follow the proposed consecutive genetic analysis. Fe-hydrogenase genotype could also be employed, but more related to the hosts and the geographic origin of the sample.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.rvsc.2016.05.016.

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