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First genetic characterisation and phylogenetic analysis of Trichomonadida from *Gallus gallus domesticus* (Aves: Phasianidae) and its nematode parasite *Heterakis gallinarum* (Ascaridida: Heterakidae) in Tunisia

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Abstract: Flagellated protozoa of the order Trichomonadida infect a variety of vertebrates, including poultry such as *Gallus gallus domesticus* (Linnaeus). Several trichomonad pathogens are of significant veterinary importance due to their role in diseases that cause high mortality rates in chickens. Despite the importance of Trichomonadida in poultry health, molecular studies on these protozoa in North Africa are limited. This study addresses this gap by investigating the genetic diversity and evolutionary relationships of Trichomonadida isolated from *G. gallus domesticus* and its nematode parasite *Heterakis gallinarum* (Schrank, 1788) in Tunisia, using a multilocus molecular approach with 18S rRNA and α-actinin 1 genes. Based on both markers, all Tunisian haplotypes, which clustered with those from France, were found to belong to genotype2. 18S rRNA analysis revealed the existence of protozoans such as *Histomonas meleagridis* (Smith, 1895) and *Parahistomonas wenrichi* Lund, 1963 in coinfection with *H. gallinarum*, confirming a possible mixed infection. Additionally, when analysing caecal samples, other Trichomonadida species were identified, including *Simplicimonas* sp. and *Tetratrichomonas gallinarum* (Martin et Robertson, 1911). These findings suggest a complex protozoan community within the studied hosts. Phylogenetic analysis revealed a close relationship between *H. meleagridis* and *P. wenrichi*, as well as between *Simplicimonas* sp. and the *Monoceromonas-Tritrichomonas* group. Both *H. meleagridis* genotypes 1 and 2 exhibited a sister-group relationship with *P. wenrichi*, with strong support for a common evolutionary origin. *Tetratrichomonas gallinarum* was basal in the tree, suggesting early divergence in the Trichomonadida lineage. This study provides, for the first time, insights into the genetic diversity of trichomonadids in Tunisia. The 18S rDNA locus proved to be effective for assessing the genetic diversity of *H. meleagridis*, *P. wenrichi, T. gallinarum* and *Simplicimonas* sp. and showed a possible mixed infection. The findings provide valuable insights into the genetic characteristics of these parasites in Tunisian poultry farms and contribute to the understanding of Trichomonadida diversity, enhancing disease control and prevention efforts.

Keywords: *Trichomonadida*, genetic diversity, evolutionary relationships, 18S rDNA, α-actinin 1 gene, mixed infection.

Flagellated protozoa of the order Trichomonadida infect various vertebrates, including birds such as *Gallus gallus domesticus* (Linnaeus) (Adl et al. 2012). Several trichomonad pathogens have gained special attention because they are of veterinary importance, leading to variety of diseases that reduce productivity and result in large financial losses (Maritz et al. 2014). Among them, *Histomonas meleagridis* (Smith, 1895), a prominent pathogen within this group, triggers severe outbreaks of 'blackhead disease' or histomoniasis in chickens and turkeys, often resulting in high mortality rates.

In addition to poultry flocks, which can serve as a reservoir for trichomonad species and can infect other Galliformes through direct contact, contaminated litter with eggs of the nematode parasite *Heterakis gallinarum* (Schrank, 1788) (Ascaridida: Heterakidae) may act as a vector for *H. meleagridis* and other protozoan species (Cupo and Beckstead 2019, Daş et al. 2021, Beer et al. 2022). Other trichomonads, such as *Tritrichomonas* sp. and *Parahistomonas* sp., also impact poultry health, causing infections that range from mild to severe gastrointestinal issues (Maritz et al. 2014). Trichomonadida are a monophyletic

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Table 1. List of the samples examined of *Gallus gallus domesticus* caecal and *Heterakis gallinarum* genomic DNA (Amor et al. 2018) from different localities of Tunisia. N hosts: number of hosts; N genomic DNA: number of *H. gallinarum* genomic DNA samples.

Gallus gallus domesticus		
Localities	Geographic coordinates	N hosts
Ariana	36.866N, 10.1647E	5
Monastir	35.777N, 10.8263E	5
Kasserine	35.1676N, 8.8365E	5
Tozeur	33.9197N, 8.1335E	5
Mednine	33.3549N, 10.5055E	5
Heterakis gallinarum		
Localities	Geographic coordinates	N genomic DNA
Beja	36.7417N, 9.1894E	20
Sousse	35.8301N, 10.5949E	19
Kairouan	35.6838N, 10.0871E	19
Gafsa	34.3963N, 8.7993E	19
Gabes	33.8892N, 10.0914E	19

group of flagellated anaerobic protozoa characterised by the presence of hydrogenosomes (modified mitochondria) and one or more parabasal apparatuses (Golgi complex) along with a parabasal filament (Honigberg and Brugerolle 1990, Brugerolle and Lee 2001, Kleina et al. 2004).

Traditionally, protozoan species identification relied on morphological traits, particularly the organisation and development of the cytoskeleton. However, the small size and subtle morphological variations among these taxa pose significant challenges for consistent identification. The use of molecular markers has significantly enhanced the ability to differentiate these organisms (Dufernez et al. 2007, Noda et al. 2009). Targeting several regions such as the nuclear small subunit rRNA gene (18S rDNA) and internal transcribed spacer regions (ITS) have been widely used for species identification, revealing hidden genetic diversity that is frequently missed by the morphology-based methods (Adl et al. 2005, Noël et al. 2007). Additionally, molecular studies on trichomonads have highlighted their basal position within eukaryotes, attracting greater scientific interest to elucidate eukaryotic evolution (Vanacova et al. 1997).

A multilocus molecular approach is essential for accurate pathogen identification in environmental and clinical samples (Adl et al. 2005, Hampl et al. 2007, Noël et al. 2007) and to revise the classifications of taxa within the Trichomonadidae (Delgado-Viscogliosi et al. 2000, Gerbod et al. 2002). However, there is limited research on the molecular characterisation of Trichomonadida in North African poultry. Improved understanding of the molecular epidemiology of these infections is crucial for effective disease control and prevention.

One of the biggest knowledge gaps needed to properly monitor and manage these diseases is the absence of comprehensive epidemiological and molecular data on trichomonad species from chickens in Tunisia. The use of molecular markers for genetic differentiation can aid in understanding the epidemiology, pathogenicity and potential zoonotic risks of these protozoans, while also providing insights into their evolutionary history, host adaptation and drug resistance mechanisms, all of which are essential for developing effective treatment and control strategies.

This study aims to fill this knowledge gap by investigating, for the first time, the genetic diversity and evolutionary relationships of trichomonad taxa isolated from Tunisian poultry populations through a multilocus molecular approach and phylogenetic analysis, providing valuable insights into the epidemiology of these parasites in Tunisian poultry farms and contributing to the global understanding of the diversity of the Trichomonadida.

MATERIALS AND METHODS

Sample collection

In 2022, a total of 25 samples of caecal portions of backyard chickens *Gallus gallus domesticus* (n = 25) were collected in five Tunisian localities, with five bird specimens sampled from each locality (Table 1). The localities of sampling were: Ariana, North (36.8668N; 10.1647E); Monastir, Est (35.777N; 10.8263E); Kassrine, Centre (35.1676N; 8.8365E); Tozeur, West (33.9197N; 8.1335E); Mednine, South (33.3549N; 10.5055E) (Fig. 1). Caecal samples were collected *postmortem* from birds regularly slaughtered for commercial purposes. The sampled flocks were previously diagnosed with histomonosis.

After collection, caecal samples were refrigerated at 4°C and immediately transported to the laboratory, where they were stored at -20°C. Additionally, 96 genomic DNA samples of adult *Heterakis gallinarum* nematodes found in the caecal content of chickens, collected during a previous study (Amor et al. 2018), were included in the analysis (Table 1, Fig. 1).

Fig. 1. Map of Tunisian localities where samples of *Gallus gallus domesticus* (Linnaeus) (blue triangle) and *Heterakis gallinarum* (Schrank, 1788) (genomic DNA samples – red circle; see Amor et al. 2018) were collected.

Table 2. Information on the sequences obtained from GenBank and used in the phylogenetic analysis.

Species	Geographical origin	Hosts	Site of infection	Accession number
Dientamoebidae sp.	Hungary	*Gallus gallus domesticus*	caecum	HG008099
	France	*Meleagris gallopavo*	caecum	HG008100
Glyptotermes fuscus	Japan	*Glyptotermes fuscus*	hindgut	AB032220
Histomonas meleagridis	Austria	Turkeys	faeces	AJ920323
	France	Turkeys	-	AF293056
	France	*M. gallopavo*	caecum	HG008093
	Austria	*G. gallus domesticus*	caecum	HG008098
	Austria	*M. gallopavo*	caecum	HG008084
	Austria	*M. gallopavo*	caecum	HG008088
	France	*M. gallopavo*	caecum	HG008085
	France	*M. gallopavo*	caecum	HG008094
	Hungary	*G. gallus domesticus*	caecum	HG008086
	France	chickens	caecum	EU647885
	France	turkeys	caecum	EU647886
	France	turkeys	caecum	EU647887
	France	*M. gallopavo*	caecum	HG008091
	France	*M. gallopavo*	caecum	HG008096
	France	*M. gallopavo*	caecum	HG008095
	France	*M. gallopavo*	caecum	HG008097
Macrotrichomonoides restis	USA	*Neotermes jouteli*	hindgut	KJ493791
Metadevescovina extranea	Australia	*Mastotermes darwilliensis*	hindgut	X87132
Monocercomonas colubrorum	Czech Republic	*Hydrosaurus pustullatus*	-	DQ174298
Parahistomonas wenrichi	France	turkeys	caecum	EU647889
	Vietnam	*G. gallus domesticus*	caecum	LK031727
	Vietnam	*G. gallus domesticus*	caecum	LK031728
	Vietnam	*G. gallus domesticus*	caecum	LK031729
Simplicimonas sp.	France	turkeys	caecum	EU647888
	Austria	*G. gallus domesticus*	caecum	HG008105
	New Guinea	*Paraectatops costalis*	intestine	KJ101559
S. similis	Czech Republic	*Uroplatus lineatus*	faeces	GQ254637
	Philippines	*Bubalus bubalis*	rectum	KC953859
Teranympha mirabilis	Japan	*Reticulitermes speratus*	intestine	AB183876
Tetratrichomonas gallinarum	Austria	turkeys	faeces	AJ920324
Tritrichomonas augusta	China	*Pelophylax nigromaculatus*	rectum	OL505402
T. foetus	USA	*Bos taurus*	prepucium	AY055799
	USA	domestic dog	faeces	AY754332
T. nonconforma	Cuba	*Anolis bartschi*	cloaca	AY055803
T. suis	Germany	*Sus scrofa domesticus*	faeces	MK801504

DNA extraction, amplification and sequencing

Genomic DNA was extracted using a modified SDS-based method (Farjallah et al. 2024). The samples were digested with SDS-proteinase K at 56 °C for 2 hours. After inactivation of proteinase K by thermal shock, proteins were precipitated using a 10 min centrifugation at 13,000 rpm. DNA was then precipitated from supernatants using 100% ethanol. The DNA was air-dried, resuspended in 100 µl TE buffer.

In order to screen samples for protozoans, the 18S rDNA (603 bp) was first targeted, using primers 18S-F/18S-R (Bilic et al. 2014). The specific primers CH-EFhF/CH-EFhR (Bilic et al. 2014) were used for the amplification of α-actinin 1 (1,160 kb) in order to reveal the possible presence of *Histomonas meleagridis* genotypes. The polymerase chain reactions (PCR) were performed in a 25 µl reaction containing 1 µl of genomic DNA, 12.5 µl 2x GoTaq Green Master Mix (Promega), 10 pmol of each primer, and ddH_2O. Negative controls were always included in PCR reactions to assess possible contamination.

The amplification reaction conditions for both α-actinin 1 and 18S rDNA were as follows: denaturation at 94 °C for 15 minutes; 40 cycles of 95 °C for 30 seconds, 53 °C for 30 seconds and 72 °C for 1 minute; followed by final elongation step at 72 °C for 10 minutes. PCR amplification was performed in a BentoLab Thermal Cycler. The amplified products were examined by gel electrophoresis (1% agarose) with the molecular weight marker HyperLadder 100 bp (Bioline Reagents Ltd., London, UK). PCR products were sequenced at Macrogen (Macrogen Inc., Seoul, Korea) using forward primers. The obtained sequences were manually checked and aligned using Unipro UGENE version 1.3 (Okonechnikov et al. 2012). Sequence alignments included reference sequences available in GenBank obtained using BLAST algorithm (Table 2, Supplementary files) (Altschul et al. 1990).

Genetic distances based on Kimura 2-parameter model were calculated within and between the observed taxa using Mega X version 10.2.5 (Kumar et al. 2018). Comprehensive examination of polymorphism and divergence in sequence datasets was made using DnaSP, namely number of polymorphic sites, average nucleotide difference (k), haplotype diversity (Hd), nucleotide diversity (Pi) (Librado and Rozas 2009). PartitionFinder version 2.1 (Lanfear et al. 2017) was used to identify the best-fit nucleotide substitution model for each genetic marker. RAxML (Randomised Axelerated Maximum Likelihood) version 8 (Stamatakis 2006) was used to create maximum likelihood (ML) phylogenetic trees. Using bootstrapping, the phylogenetic trees' robustness was evaluated using 2,000 pseudoreplicates. *Teranympha mirabilis* Koidzumi, 1917 was added as an outgroup (AB183876).

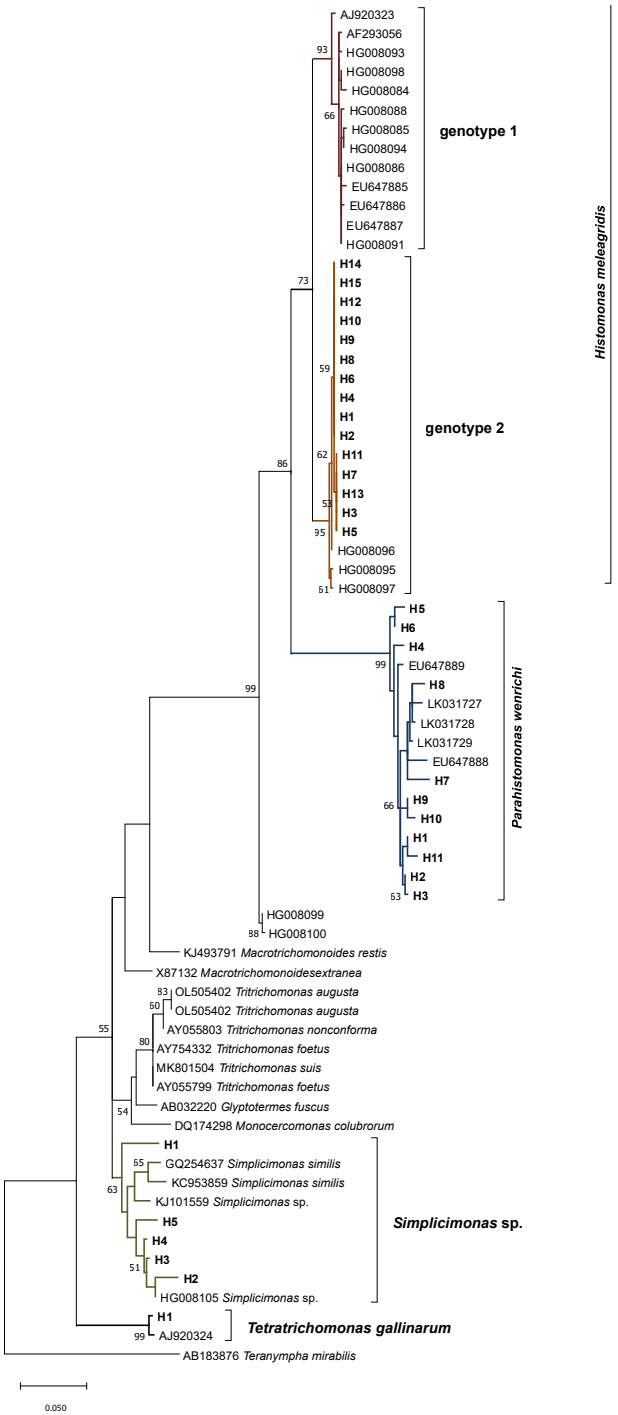

Fig. 2. Maximum Likelihood tree of trichomonad species from Tunisian samples based on 18S rDNA sequences. Terminal nodes within the trichomonad clades (highlighted in bold) represent the studied populations. Support values for each node are the bootstrap value (BS) of ML. Only nodal support values > 50% are shown. Studied populations are highlighted in bold

RESULTS

18S rDNA dataset analysis

Upon examination, all caecal samples from suspected cases exhibited typical gross lesions, including thickened caecal walls and the formation of caseous cores. A total of 121 18S rRNA gene sequences from 25 caecal samples and genomic DNA of 96 *Heterakis gallinarum* nematodes were obtained by PCR amplification and sequencing. A careful analysis of the chromatogram data verified that there were no double peaks, ensuring that all chromatogram showed a single, unique peak every time. Since the 18S rDNA primers can generate amplicons from different protozoan species, the specificity of the obtained sequences was assessed using the BLAST algorithm.

Consecutively, reference sequences from several Trichomonadida available in GenBank were added to the 18S DNA dataset. The 25 sequences, from caecal samples, were identified as: *Histomonas meleagridis* (96–99% identity, HG008095; n = 14); *Parahistomonas wenrichi* (Lund, 1963) (94–97% identity, LK031729; n = 5); *Simplicimonas* sp. (97–98% identity, HG008105; n = 5); *Tetratrichomonas gallinarum* (Martin et Robertson, 1911) (98% identity, AJ920324; n = 1). *Histomonas meleagridis* sequences generated 15 haplotypes, *P. wenrichi* 11, *Simplicimonas* 5 and *T. gallinarum* one haplotype (Table 3).

Sixty-two sequences obtained from the 96 *H. gallinarum* genomic DNA shared 96–99% identity with *H. meleagridis* (HG008095) whereas 34 sequences shared 94–98% identity with *P. wenrichi* (LK031729). The haplotypes obtained were deposited in GenBank under the accession numbers PQ682583-PQ682614. *Histomonas meleagridis* and *P. wenrichi* were isolated in all studied localities from both caecal samples and *H. gallinarum* genomic DNA. The other species were only found in caecal samples: *Simplicimonas* sp. in Kasserine (n = 3) and Tozeur (n = 2), *T. gallinarum* in Ariana (Table 3).

Multiple alignment of *H. meleagridis* sequences (n = 31) showed 25 mutations of which 23 were polymorphic and the average nucleotide difference was k = 9.172. *Parahistomonas wenrichi* sequences differed by 26 mutations of which 16 were polymorphic and k = 6.075. *Simplicimonas* sp. dataset included 9 sequences, but it showed 84 mutations defining 74 polymorphic sites with k = 22.900. Haplotype diversity (Hd) ranged from 0.875 in the case of *H. meleagridis* to 1 for *Simplicimonas* sp. for *T. gallinarum*, Hd was 1, but this clade contained only two sequences. Nucleotide diversity (Pi) values varied from 0.64% (*H. meleagridis*) to 4.9% (*Simplicimonas* sp.) (Table 4).

The maximum likelihood phylogenetic tree topology (ML) showed several highly supported clades. *Histomonas meleagridis* sequences were distributed in two subclades identified previously by Bilic et al. (2014) as genotype 1 and genotype 2, with all 15 *H. meleagridis* haplotypes of the current study clustering within the genotype 2 subclade, clustered with French sequences (HG008095, HG008096 and HG008097) (Fig. 2).

All *P. wenrichi* appeared as a highly supported monophyletic clade including the 11 haplotypes of the current study. *Simplicimonas* sp. haplotypes were grouped within a highly supported clade, including *Simplicimonas similis* Čepička, Hampl et Kulda, 2010 and previously published sequences of *Simplicimonas* sp. The last clade with a basal position within the phylogenetic tree included two specimens identified as *T. gallinarum* (Fig. 2).

Table 3. Species of Trichomonadida observed in this study, with their geographical origin, type of sample and GenBank accession numbers. N – number of obtained 18S rRNA gene sequences.

Species	Geographical origin	Hosts	Type of sample	N	Haplotypes	Accession number
Histomonas meleagridis	all studied localities	*Gallus gallus domesticus* *Heterakis gallinarum*	caecal samples nematode genomic DNA	14 62	H1–H15	PQ682583–PQ682597
Parahistomonas wenrichi	all studied localities	*Gallus gallus domesticus* *Heterakis gallinarum*	caecal samples nematode genomic DNA	5 34	H1–H11	PQ682598–PQ682608
Simplicimonas sp.	Kasserine Tozeur	*Gallus gallus domesticus*	caecal samples	5	H1–H5	PQ682609–PQ682613
Tetratrichomonas gallinarum	Ariana	*Gallus gallus domesticus*	caecal samples	1	H1	PQ682614

The genetic distance between clades ranged from 11.3% (*H. meleagridis – P. wenrichi*) to 21.8% (*T. gallinarum – P. wenrichi*) (Table 5). The distance within clades ranged from 0.58% (*T. gallinarum*) to 3.41% (*Simplicimonas* sp.). The genetic distance between genotype 1 and genotype 2 of *H. meleagridis* was 4%.

α-actinin 1 dataset analysis

Due to the specificity of the CH-EFhF/CH-EFhR primers, the α-actinin 1 sequence analysis revealed two patterns corresponding to the previously observed 18S rDNA genotype 1 and genotype 2 of *H. meleagridis*. No genetic variability was detected among the 76 Tunisian sequences analysed, all of which grouped within the genotype 2 and with 100% identity with the reference sequences HG008107 and ON960042. The obtained sequences were deposited in GenBank under accession numbers PV548923–PV548926.

DISCUSSION

The aim of this study was to assess for the first time the genetic diversity of Trichomonadida in Tunisia by examining two nuclear markers, the 18S rDNA and α-actinin1. One set of samples included 25 caecal samples collected from five Tunisian localities whereas the other one includes 96 *Heterakis gallinarum* genomic DNA from five localities previously studied for the molecular characterisation of the nematode *H. gallinarum*. Based on the 18S rDNA dataset analysis, the Tunisian samples were split into four main clades.

The first clade was identified as *Histomonas meleagridis*, originating from both the caecal samples and the genomic DNA of *H. gallinarum*. When including reference sequences from GenBank, *H. meleagridis* clade was divided into two distinct clusters previously identified as genotype 1 and genotype 2 (Bilic et al. 2014). The use of the term 'genotype' was justified by Bilic et al (2014) based on the observed genetic distances relatively high ranging between 2.0% and 4.4%. Based on both markers, all Tunisian haplotypes, which clustered with the French ones, were found to belong to genotype 2, with a genetic distance of 4% (18S rDNA) between the two genotypes.

However, Bilic et al. (2014) found that in European countries and Azerbaijan, genotype 1 had a higher prevalence than genotype 2. The different patterns of genetic structure of *H. meleagridis* observed by Bilic et al. (2014) could be explained by the geographic distance (isolation-by-distance). Populations close to each other are often more similar genetically, while distant populations are often more divergent (Sexton et al. 2014, Bontrager and Angert 2018, Goudarzi et al. 2019). Host genotype and diversity can, also, influence the evolution of parasites, leading to the development of distinct parasite lineages (Ekroth et al. 2021).

In fact, the fitness of parasites is severely affected by variations in the host immune response, which is determined by the host genetic background (Tavalire et al. 2016). Some parasite genotypes may infect specific host genotypes but not others, and similarly, hosts may exhibit varying levels of susceptibility to particular parasite genotypes (Barribeau et al. 2014). Finally, human activities can influence the pattern of genetic structure, leading to the spread of the same haplotype within a region, or to the formation of divergent populations when host trade is limited (Mweu et al. 2012).

Despite the observed low genetic variation, the analysis of the coding α-actinin1 gene supported the existence of two different genotypes within *H. meleagridis*. The observed differences in genetic variation across the studied markers could be attributed, on the one hand, to the varying level of conservation, with a stronger conservation in the protein-coding regions. On the other hand, the repetitive nature of the 18S rDNA region may be the source of micro-variation even within the same genome. Moreover, several studies have reported micro-variations within partial 18S rDNA regions of *H. meleagridis* when examining PCR clones (Gerbod et al. 2001, Mantini et al. 2009). Since these studies did not use single-cell clones, the observed variations could potentially be attributed to mixed infections or amplification errors.

Interestingly, 18S rDNA analysis revealed the existence of protozoans such as *H. meleagridis* and *Parahistomonas wenrichi* in coinfection with *H. gallinarum*, confirming

Table 4. Standard population genetic statistics of the 18S rDNA sequences of trichomonad species from Tunisian samples. PS – number of polymorphic sites; k – average number of nucleotide differences; Hd – haplotype diversity; Pi – nucleotide diversity.

Clade	Number of sequences	Total number of mutations	PS	k	Hd	Pi
Histomonas meleagridis	31	25	23	9.172	0.875	0.64%
Parahistomonas wenrichi	16	26	20	6.075	0.92	1.14%
Simplicimonas sp.	9	84	74	22.9	1	4.97%
Tetratrichomonas gallinarum	2	2	2	2	1	0.38%

Table 5. Genetic distances based on Kimura 2-parameter (K2P) within trichomonad species from Tunisian samples 18S rDNA sequences.

	Histomonas meleagridis	***Parahistomonas wenrichi***	***Simplicimonas* sp.**
Histomonas meleagridis			
Parahistomonas wenrichi	0.1133		
Simplicimonas sp.	0.1623	0.1890	
Tetratrichomonas gallinarum	0.1913	0.2176	0.1180

a possible mixed infection (Mantini et al. 2009, Bilic et al. 2014). In addition, when analysing caecal samples, other Trichomonadida were identified, such as *Simplicimonas* sp. and *Tetratrichomonas gallinarum*. These findings suggest a complex protozoan community within the studied hosts. Previous studies have indeed identified the presence of *Trichomonas gallinae* (Rivolta, 1878), *T. gallinarum*, *Blastocystis* sp., *Simplicimonas* sp., *P. wenrichi*, *Tritrichomonas* sp., and *Dientamoeba* sp. in the poultry gut, alongside *H. meleagridis* (Kemp and Reid 1965, Stenzel and Boreham 1996, Lollis et al. 2011, Bilic et al. 2014, Nguyen et al. 2015). This underscores the importance of their specific characterisation, particularly in instances of concomitant infection.

Bilic et al. (2014) acknowledged the broad specificity of the designed primers 18S-F/18S-R allowing the detection of several trichomonadida taxa. Here a multilocus approach has shown to be more accurate to resolve species diversity and to avoid mixed infection complications. In the case of *Simplicimonas* sp., species identification was unsuccessful due to the observed high genetic variation and the lack of 18S rDNA matching sequences in databases.

The phylogenetic tree analysis revealed a close relationship between *H. meleagridis* and *P. wenrichi*, as well as between *Simplicimonas* sp. and the *Monoceromonas*-*Tritrichomonas* group. In the obtained maximum likelihood (ML) phylogenetic tree, both *H. meleagridis* genotypes 1 and 2 exhibited a sister-group relationship with *P. wenrichi*, indicating a common evolutionary origin. This clustering is strongly supported by a bootstrap value of 86%, reinforcing previous phylogenetic inferences that suggested the grouping of *Parahistomonas* Lund, 1963 and *Histomonas* Tyzzer, 1920 (Gerbod et al. 2001, Mantini et al. 2009).

As indicated by the present phylogenetic analysis, the genera *Histomonas* and *Parahistomonas* seem to share a common ancestor and, as previously mentioned, both are found in the caecum of galliform birds, including chickens, turkeys, quail, partridges and pheasants, infecting the same hosts and environmental niche (Malewitz et al. 1958, Lund and Chute 1972, Wernery and Kinne 2002, Esquenet et al. 2003, Mantini et al. 2009).

These close phylogenetic and morphological relationships suggest that speciation leading to these taxa likely occurred recently in birds infected with their common ancestor. Indeed, as highlighted by Mantini et al. (2009) two independent colonisation events of the avian caecum by *Histomonas* and *Parahistomonas* over evolutionary time seem improbable, given their close relationship.

Histomonas and *Parahistomonas* represent a remarkable example of parallel adaptation to the same host following speciation among trichomonads. Note that *T. gallinarum* occupied a basal position in the phylogenetic tree, suggesting that it diverged earlier in the evolutionary history of these trichomonadid species. This phylogenetic topology was concordant with phylogenetic studies on parabasalid microorganisms (Gerbod et al. 2001, Mantini et al. 2009, Cepicka et al. 2010, Malik et al. 2011, Noda et al. 2012). However, further studies based on multilocus approach and more comprehensive taxon sampling are needed to resolve the reported uncertainties and to better understand the evolutionary history of these protozoans.

In conclusion, this study is the first investigation of the diversity of Trichomonadida in Tunisia. The 18S rDNA locus proved to be effective in assessing the genetic diversity of *H. meleagridis*, *P. wenrichi*, *T. gallinarum* and *Simplicimonas* sp., and showed possible mixed infections. Further studies should focus on the relationships between these protozoan taxa, the biological significance of each group or genotype, their epidemiological roles and pathogenic effects. Moreover, animal surveys in endemic areas are essential to improve the evaluation of specific discrimination between other molecular markers.

Author contributions. SF: conceptualisation, methodology, formal analysis, writing—original draft preparation, review and editing, supervision, PM: writing—original draft preparation, review and editing, supervision, ANA: funding acquisition, NA: formal analysis, investigation. All authors have read and agreed to the published version of the manuscript.

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