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Genotyping of *Histomonas meleagridis* isolates based on Internal Transcribed Spacer-1 (ITS-1) sequences

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Running title: *Histomonas meleagridis* ITS sequence analysis

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Abstract

C-profiling is a novel genotyping method for protozoan pathogens, based on PCR and sequencing of AT-rich ITS-1 sequences. It was applied to various *Histomonas meleagridis* isolates originating from outbreaks of histomoniasis in six Dutch turkey and chicken flocks. Three different *H. meleagridis* genotypes were identified. Type I and II were associated with clinical disease. In two flocks both having recovered from an outbreak of histomoniasis, a type III strain was found that was also morphologically slightly different from the type I and II isolates. C-profiling is a promising technique to differentiate between *H. meleagridis* subtypes making it useful for epidemiological studies.

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Introduction

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The flagellated protozoan *Histomonas meleagridis* (Tyzzer & Fabyan, 1920) is the cause of histomoniasis or blackhead disease in gallinaceous fowl (Smith, 1895). The disease is re-emerging in the European community after effective chemotherapeutics including arsenicals and nitroimidazoles were banned following increasing concern about drug residues in food and the identification of some compounds as potentially carcinogenic (Zenner *et al*., 2003). In turkeys Histomonas-induced mortality is high whereas in chickens disease is generally less severe (McDougald, 1987). Chicken flocks may form a reservoir for *H. meleagridis* and readily infect turkeys by either direct contact or through infected litter with eggs from *Heterakis gallinarum* acting as a vector (Graybill & Smith, 1920; Tyzzer, 1934). The possibility of wild birds as a reservoir has not been ruled out. The disease has been described in a number of species including pheasants (Lund & Chute, 1972a, b; Werney & Kinne, 2002), quail, and partridges (Lund & Chute, 1972b).

Although differences in pathogenicity of isolates have been observed (Dwyer & Honigberg, 1970), so far no subtyping of Histomonas isolates has been reported. Identification of subtypes might however prove useful in epidemiological studies, for instance evaluation of correlation of infections between flocks or species, for monitoring the efficiency of treatment, assessment of differences in virulence between strains, etc.

H. meleagridis is a trichomonad, that is morphologically (Johnson, 2004) and antigenically (Dwyer 1972a, b, 1974) closely related to *Dientamoeba fragilis*, a non-flagellated protozoan pathogenic to humans. Also the phylogenetic relation of these two trichomonads based on sequence analysis of small subunit rRNA genes is very close (Gerbod *et al*., 2001). The small subunit rRNA gene, and especially the ribosomal gene region

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containing the ITS-1/5.8S/ITS-2 has been used for extensive phylogenetic analysis of Trichomonadidae (Felleisen, 1997; Gerbod *et al*. 2001; Kleina *et al*. 2005, Cepicka *et al*. 2005; Kutisova *et al*. 2005). Its usefulness for genotyping *H. meleagridis* has not been assessed yet. In the present study ITS-1 sequences of *H. meleagridis* isolates are compared using a novel technique that we developed: C-profiling. This method is based on the fact that, due to multiple ITS-1 sequence variants in a single isolate, sequencing fluorograms are too complex for direct interpretation. But since the C-content is rather low, more simple patterns are produced by deleting in the fluorogram the peaks representing the other nucleotides (A, T and G). A single C nucleotide is then represented by multiple peaks of different heights due to small differences in size of the individual ITS-1 variants due to insertions, deletions and mutations involving C, and by the relative proportions of the different ITS-1 variants present in the PCR product of an isolate.

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Materials and Methods

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***H. meleagridis* isolates**. An isolation procedure for *H. meleagridis* as described in more detail elsewhere (Van der Heijden, *et al*. 2005) was applied to birds from several sources. Briefly, immediately after euthanasia, caeca were opened and caecal fluid or content was inoculated into Dwyer's medium and incubated at 40°C. After 48 h cultures that contained histomonads were subcultured at least once (up to 23 passages). Samples of freshly inoculated medium or (sub)cultures (with or without visible histomonads) were stored in liquid nitrogen by adding 10% dimethylsulphoxide (Merck, Germany) as a cryoprotectant, aliquoting into vials of 1 ml and freezing to -70°C under controlled conditions (approximately -1°C per min) before transfer to liquid nitrogen (-175°C).

Birds examined. Eight turkeys with liver and caecal lesions originating from a flock (K) with acute histomoniasis were cultured for Histomonas and *H. meleagridis* was isolated from them all. Six birds with moderate to severe liver and caecal lesions were necropsied from a chicken flock (B) with acute histomoniasis and *H. meleagridis* was successfully isolated and subcultured from two. An outbreak of clinical histomoniasis occurred in turkeys on a farm (S) with both turkeys and chickens and a turkey (St) and a chicken (Sc) isolate of *H. meleagridis* were obtained. From the same flock (St') 2 months after the initial outbreak of disease, but now with no clinical histomoniasis, isolation of *H. meleagridis* was attempted from turkeys in the group that survived the disease. Culture was also attempted from four birds in a turkey flock (H) one month after an unconfirmed outbreak and from 16 birds in a chicken flock (G) with an outbreak of six-week duration. Finally, caecal contents from two SPF turkeys were inoculated into medium to serve as negative controls.

PCR. The ITS-1 region of *H.meleagridis* was amplified by PCR using a forward primer ssu2 (5'ggaatcccttgtaaatgcgt 3') based on ssu rRNA genes of *H. meleagridis* (AF293056), *D. fragilis* (U37461) and *T. vaginalis* (AY677680) and a reverse primer 5.8s1 (5'tgtgaggagccaagacatcc 3') complementary to the 5' end of the 5.8S ribosomal RNA gene of *H. meleagridis* and *D. fragilis*. The 5.8S rRNA gene sequence of these organisms as a basis for the design of the reverse primer was determined by PCR amplification of the ITS-1/5.8S/ITS-2 region using forward primer ssu2 and a reverse primer lsu1 (5'agttcagcgggtcttcctg 3'), complementary to a conserved region in the 5' end of the large subunit RNA genes of Trichomonad species, followed by cloning and sequencing.

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For PCR, DNA was isolated from 100 µL *H. meleagridis* culture (fresh or after storage in liquid nitrogen) by diluting the culture 1:2 with PBS and using a commercial DNA isolation kit (Qiamp DNA Minikit, Qiagen) according to the manufacturer's instructions.
PCR was performed using a reaction volume of 50 µL consisting of 30% DNA template, 0.3 µM of each primer, 500 µM dNTPs, 10% 10x PCR buffer (Promega), 3 mM $MgCL_2$, and 0.1 mg/mL bovine serum albumin and 0.4 mg/mL α-casein to eliminate PCR inhibitors, and 1 U of Taq polymerase (Promega). A PCR run consisted of forty cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, and elongation at 72 °C for 30 sec. Amplification products were size fractionated on 1% agarose gels (80 V, 30 minutes) and visualized by ethidium bromide staining.

Cloning and sequencing. One microliter of PCR product of a number of *H. meleagridis*-isolates was ligated in the PCR cloning pGEMTeasy vector (Promega) as described by the manufacturer. After transformation into Escherichia coli DH5α individual clones were tested in PCR using either the ssu2/lsu1 (ITS-1/5.8S/ITS-2 region) or the ssu2/5.8s1 primers (ITS-1 region). Positive clones were sequenced using an Applied Biosystems Prism 310 dye terminator-fluorescence based genetic analyzer. Initially only the ssu2 primer was used for sequencing. To confirm the results of ITS1-sequencing, PCR was repeated and sequencing performed using the 5.8s1 primer. Homology searches were done using the Blast program with default settings (at http://www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignments and C-profiles were produced using the CodonCode software (CodonCode Corporation, Dedham, USA).

Genbank submission: The 3' part of the ssu rRNA and ITS-1 region of the nine different clones of *H. meleagridis* type III was deposited in GenBank with accession numbers DQ350439 until DQ350447.

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Results

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ITS-1 sequence of *H. meleagridis*. The PCR amplified the 3'end of the ssu rRNA gene and the complete ITS-1 region of *H. meleagridis* DNA isolated from culture. The amplicon was approximately 300 bp in size. Sequence analysis of PCR products from several *H. meleagridis* isolates resulted in ITS-1 sequences with many ambiguities in sequencing fluorograms (data not shown) indicating a heterogeneous ITS-1 sequence within a single isolate or trophozoite. Using the same primers a similarly variable ITS-1 sequence with many ambiguities was also observed in *D. fragilis* but not in *T. vaginalis* (not shown).

C-profiling. In total 80 *H. meleagridis isolates* were tested in PCR, resulting in 68 positive results of which 46 PCR products were C-profiled. This resulted in three different C-profiles (Figure 1). Type I and type II profiles were more alike than the type III profile. This type III was found in three unrelated isolates from both chickens and turkeys. At the time of isolation it was observed upon microscopic examination of these cultures that these unicellular organisms had a morphology that was distinct from *H. meleagridis*: i.e. slightly larger in size and with a more granular appearance and no visible flagellum (Figure 2). Like *H. meleagridis*, this organism also ingested rice particles. However, it was not possible to subculture these protozoa in Dwyer's medium or to re-start culture from cryopreserved isolates stored in liquid nitrogen. Phylogenetic analysis of the sequence of the 150 bp at the 3' end of ssu rRNA of the type III strains showed that this organism is closely related to *H. meleagridis* and *D. fragilis* and less related to other trichomonads.

C-profiling proved highly reproducible and gave consistent results for a particular sample or isolate (data not shown). Also subculturing of type I or type II strains (the organism with a type III C-profile could not be subcultured) did not result in changes in the C-profile.

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For each of the three C-profiles, the DNA-products of an isolate were cloned to analyze the sequence variability within the ITS-1 region. The ITS-1 region of nine or 10 individual clones per isolate was fully sequenced (Table 1) and proved extremely AT rich (between 90 and 94%). The ITS-1 length of type I or II clones was on average 78 bp, while ITS-1 of type III clones was on average 93 bp long. Some clones had identical sequences to others. For the 19 type I or II clones in total 11 different sequences were found, while for the 10 type III clones nine different sequences were found. The type I clone sequences were similar to type II clone sequences with differences due to base substitutions and inserts or deletions of one or two bases. Initially, sequencing was done with a single primer (ssu2). To confirm the sequencing results PCR was repeated followed by sequencing using reverse primer 5.8s1. A few clones proved repeatedly PCR-negative and could not be sequenced using primer 5.8s1.

A ten-fold limiting dilution from a type I and a type II strain of *H. meleagridis* was made. From each dilution step DNA was isolated, tested in PCR and the positive PCR products C-profiled. The C-profiles were consistent, indicating that the ITS-1 variation is intragenomic.

The 68 PCR positive isolates came from various sources. For birds that were culture-negative only primary samples were available (medium inoculated with caecal contents), whereas for culture-positive birds in addition samples from various passages after subculturing were stored in liquid nitrogen. DNA was isolated either directly from these samples, or from culture media after successfully restarting culture. Within a flock (and in several samples from a single bird) we never observed a mixed infection with type I and type II strains (table 2). Type I was detected in all eight birds of a single turkey flock (K) that suffered from an acute outbreak of histomoniasis, while type II strains were found in three flocks (B, G, and S). These were all chicken flocks, although in flock S, with both turkey and

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Table 1: *Sequence alignment of the ITS-1 regions of 29 H. meleagridis type I, II, or III clones.*¶ ... [1]

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chicken, the type II strain was isolated from the turkeys. Type III strains were isolated from single birds in three flocks (B, H and St'). Two of these flocks (B and St') had suffered from an outbreak of histomoniasis several weeks before sampling. SPF turkeys, serving as negative controls, gave negative PCR results.

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Discussion

Although a PCR for specific detection of *H. meleagridis* based on amplification of part of the 18S rRNA gene has been described (Huber *et al*. 2005), so far no subtyping method for *H. meleagridis* has been published. Subtyping of *H. meleagridis* could be useful, e.g. for epidemiological and pathogenesis studies, as well as for assessment of treatment efficiency and virulence differences between isolates. Variations in virulence between isolates have been examined with the aid of experimental inoculations, however it remained unclear whether these could be attributed to a strain effect (Dwyer and Honigberg, 1970). It is generally accepted that there are no differences between Histomonas strains from chickens and turkeys, and in the present study using C-profiling we found a type II *H. meleagridis* in both turkeys and chickens. Differences within the ITS regions have been frequently used as markers for subtyping in various organisms (Chilton and Gasser, 2001; Olivier *et al*., 2001) because these genes have no function in the mature ribosome and therefore show more genetic variation than the coding regions. In the present work we describe a novel method, C-profiling, for subtyping of *H. meleagridis* based on DNA-sequences of the ITS-1 region, rather than the ssu or lsu rRNA gene regions, that are very conserved among trichomonads. The sequences of type I and type II clones were rather similar, but still clearly distinct C-profiles were obtained. Our assumption is that the relative proportions of different ITS-1 sequences in the genome of the *H. meleagridis* genotypes are probably very conserved.

The sequencing primer (ssu2) has a complete match with several *Tetratrichomonas gallinarum* strains (AY245111 until AY245121, AY245143 until AY245146, AY247746, AY247747, AY247749), but partial sequences in the18S-region of the *H. meleagridis* isolates differed from sequences of corresponding regions in *T. gallinarum* (data not shown), and

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matched with *H. meleagridis* sequences (AF293056 or AJ920323). Therefore, it is unlikely that *T. gallinarum* interfered in our study.

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Using C-profiling, consistent results were obtained for *H. meleagridis* strains, also after subculturing, storage below 0°C, restarting and further subculturing. From these observations in combination with the limiting dilution experiments it was concluded that the ITS-1 variation observed in *H. meleagridis* was intragenomic (Wörheide *et al.* 2004) and not due to the presence of a mixture of *H. meleagridis* types in a single specimen.

In the limited number of outbreaks included in the present study we were able to find three different C-profiles. Possibly more C-profiles would be identified in a larger survey including isolates from other countries. In clinical outbreaks a type I or a type II strain was detected. In one case (flock G) a type II strain was also found six weeks after initial clinical disease. Culturing *H. meleagridis* from this flock proved difficult because of contamination of the cultures with an unidentified yeast (although *H. meleagridis* trophozoites were identified based on morphology). In three unrelated flocks a third C-profile (type III) was found. In the turkey flocks H and St' this type III strain was detected in birds from a group of turkeys that survived a clinical outbreak of histomoniasis. This profile was also encountered in a single bird from chicken flock B suffering from an acute outbreak of histomoniasis, while two other birds in this flock were infected with *H. meleagridis* type II. In all these cases isolation of *H. meleagridis* was attempted. Upon microscopic examination an amoeboid organism was seen that was morphologically similar, but not identical to *H. meleagridis*. It was slightly larger, with a more granular appearance, and had no flagellum. Although the organism, like *H. meleagridis*, ingested rice particles, it proved impossible to grow or subculture the organism in Dwyer's medium. Morphologically, the organism is different from *Tetratrichomonas gallinarum, Trichomonas gallinae,* or *Histomonas wenrichii* (Honigberg & Kuldova, 1969). Sequence analysis of the ssu rRNA gene revealed a close phylogenetic relationship between

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this organism and the human pathogen *D. fragilis* and with *Tritrichomonas foetus*, which is the common ancestor of *D. fragilis* and *H. meleagridis* (Gerbod *et al*, 2001) at a slightly larger distance. The sequence homology with *T. gallinarum* was less. Although it is well known that *D. fragilis* and *H. meleagridis* are closely related (Gerbod *et al*, 2001; Johnson *et al*, 2004) it is remarkable that the ITS-1 sequence of this organism compares better with *D. fragilis* than with *H. meleagridis* type I and II. It is hypothesized that this might be an amoeboid species in turkeys and chickens that has not been identified earlier because it is morphologically similar (but not identical) to *H. meleagridis*. At present it is not known whether this species also occurs in chicken or turkey flocks without a history of histomoniasis or whether it occurs in other species of birds.

ITS-1 C-profiling can be considered a fingerprint technique for *H. meleagridis* for easy identification of sub-strains. The technique proved highly reproducible and easy to perform and interpret. At present only two (or three) different *H. meleagridis* C-profiles have been found and for epidemiological studies a larger number of subtypes would be desirable. It would be useful if additional markers could be found in the ITS-2 part of the rRNA gene of *H. meleagridis*.

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Table 1: *Sequence alignment of the ITS-1 regions of 29 H. meleagridis type I, II, or III clones.*

type/clone	sequence 10 20 30 40 50 60 70 80 90
I/1	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATTGAATACAAAATTAATTTTCAATCATTAATTAAA--
I/3, I/4, I/8[a], II/10[a]	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATTAAATACAAAATTAATTTTCAATCATTAATTAAA--
I/5, I/7, II/1, II/2	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATAATATACAAAATTAATTTTCAATCATTAATTAAA--
I/9[a]	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATAATATACAAAATTAATTTTCAATCTTAAAATTTA--
I/6	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAA-TTAAATACAAAATTAATTTTCAATCATTAATTAAA--
I/2	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATTATATACAAAATTAATTTTCAATCATTAATTAAA--
II/6	GTTTAGTAAATAAAATCTAAACAATAATTTTAAAAATAAAAAAATTAAATACAAAATTAATTTTCAATCATTAATTAAA--
II/7	GTTTAGTAAATAATATTTAAACAATAATTTTAAAAATAAAAAA-TAATATACAAAATTAATTTTCAATCATTAATTAAA--
II/4, II/8[a], II/9[a]	GTTTCGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATAATATACAAAATTAATTTTCAATCATTAATTAAA--
II/3	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATTAAATACAAAATTAATTTTCAATCTTAAAATTTAAA
II/5	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATAATATACAAAATTAATTTTCAATCTTAAAATTTAAA
III/1	GTTTCGTTTTTTAAATAAAAAAAAAA-CAATAATTTTTTT---AAATTAAATAAAAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTCAA
III/2, III/4	GTTTAGTTTTTTATTTAAAAAAAAA--CAATAATTTTTTT-ATAAAATTTCA--CAATTTATACAAAATTCAAAATCATTAAATTTCAAAAATTCAA
III/3	GTTTAGTTTTTTATTTAAAAAAAA---CAATAATTTTTTTTATGAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTCAA
III/5	GTTTAGTTTTTTATTTTAAAAAAAAA-CAATAATTTTTTT-ATAAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTCAA
III/6	GTTTAGTTTTTTATTTAAAAAAAAAAC-AATAATTTTTTTTATAAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTAA-
III/7	GTTTAGTTTTTTATTTAAAAAAAAA--CAATAATTTTTTTTATAAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTCAA
III/8	GTTTCGTTTTTTATTTAAAAAAAAA--CAATAATTTTTTT--AAAATTAAATAAAAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTAAA
III/9	GTTTAGTTTTTTATTTAAAAAAAAAAACAATAATTTTTTTTATAAAATTTAG--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTAA-
III/10	GTTTAGTTTTTTATTTAAAAAAAAAAACAATAATTTTTTTTATAAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTAAA

[a]: results based on sequencing using only primer ssu2.

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Table 2: *Results of C-profiling on H.* meleagridis *field strains from various sources.*

Flock	Species	n	PCR+	# of C-profiles	C-profiling type I	type II	type III
K	turkey	8	8	25	25	0	0
B	chicken	6	3	11	0	10	1
St	turkey	1	1	2	0	2	0
Sc	chicken	1	0	nd			
St'	turkey	1	1	2	0	0	2
H	turkey	4	1	1	0	0	1
G	turkey	16	8	5	0	5	0
SPF	turkey	2	0	nd			

n = number of bids examined in PCR
PCR+ = number of PCR-positive birds
# of C-profiles = total number of profiles produced.

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Figure legends

Fig. 1. *C-profiling of H. meleagridis ITS-1 and 5.8 S region: examples of C-profiles of two isolates per type I, II or III.*

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Fig. 2. *Dixenic cultures: Histomonas meleagridis (B) and an unicellular organism with slighty different morphology (A). Bar is 30 µm.*

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Table 1: *Sequence alignment of the ITS-1 regions of 29 H. meleagridis type I, II, or III clones.*

type/clone	sequence 10 20 30 40 50 60 70 80 90
I/1	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATTGAATACAAAATTAATTTTCAATCATTAATTAAA--
I/3, I/4, I/8[a], II/10[a]	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATTAAATACAAAATTAATTTTCAATCATTAATTAAA--
I/5, I/7, II/1, II/2	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATAATATACAAAATTAATTTTCAATCATTAATTAAA--
I/9[a]	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATAATATACAAAATTAATTTTCAATCTTAAAATTTA--
I/6	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAA-TTAAATACAAAATTAATTTTCAATCATTAATTAAA--
I/2	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATTATATACAAAATTAATTTTCAATCATTAATTAAA--
II/6	GTTTAGTAAATAAAATCTAAACAATAATTTTAAAAATAAAAAAATTAAATACAAAATTAATTTTCAATCATTAATTAAA--
II/7	GTTTAGTAAATAATATTTAAACAATAATTTTAAAAATAAAAAA-TAATATACAAAATTAATTTTCAATCATTAATTAAA--
II/4, II/8[a], II/9[a]	GTTTCGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATAATATACAAAATTAATTTTCAATCATTAATTAAA--
II/3	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATTAAATACAAAATTAATTTTCAATCTTAAAATTTAAA
II/5	GTTTAGTAAATAAAATTTAAACAATAATTTTAAAAATAAAAAAATAATATACAAAATTAATTTTCAATCTTAAAATTTAAA
III/1	GTTTCGTTTTTTAAATAAAAAAAAAA-CAATAATTTTTTT---AAATTAAATAAAAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTCAA
III/2, III/4	GTTTAGTTTTTTATTTAAAAAAAAA--CAATAATTTTTTT-ATAAAATTTCA--CAATTTATACAAAATTCAAAATCATTAAATTTCAAAAATTCAA
III/3	GTTTAGTTTTTTATTTAAAAAAAA---CAATAATTTTTTTTATGAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTCAA
III/5	GTTTAGTTTTTTATTTTAAAAAAAAA-CAATAATTTTTTT-ATAAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTCAA
III/6	GTTTAGTTTTTTATTTAAAAAAAAAAC-AATAATTTTTTTTATAAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTAA-
III/7	GTTTAGTTTTTTATTTAAAAAAAAA--CAATAATTTTTTTTATAAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTCAA
III/8	GTTTCGTTTTTTATTTAAAAAAAAA--CAATAATTTTTTT--AAAATTAAATAAAAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTAAA
III/9	GTTTAGTTTTTTATTTAAAAAAAAAAACAATAATTTTTTTTATAAAATTTAG--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTAA-
III/10	GTTTAGTTTTTTATTTAAAAAAAAAAACAATAATTTTTTTTATAAAATTTAA--CAATTTATACAAAATTCAAAATCAATAAATTTCAAAAATTAAA

[a]: results based on sequencing using only primer ssu2.

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Table 2: *Results of C-profiling on H.* meleagridis *field strains from various sources.*

Flock	Species	n	PCR+	# of C-profiles	C-profiling type I	type II	type III
K	turkey	8	8	25	25	0	0
B	chicken	6	3	11	0	10	1
St	turkey	1	1	2	0	2	0

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Sc	chicken	1	0	nd			
St'	turkey	1	1	2	0	0	2
H	turkey	4	1	1	0	0	1
G	turkey	16	8	5	0	5	0
SPF	turkey	2	0	nd			

n = number of bids examined in PCR
PCR+ = number of PCR-positive birds
# of C-profiles = total number of profiles produced.

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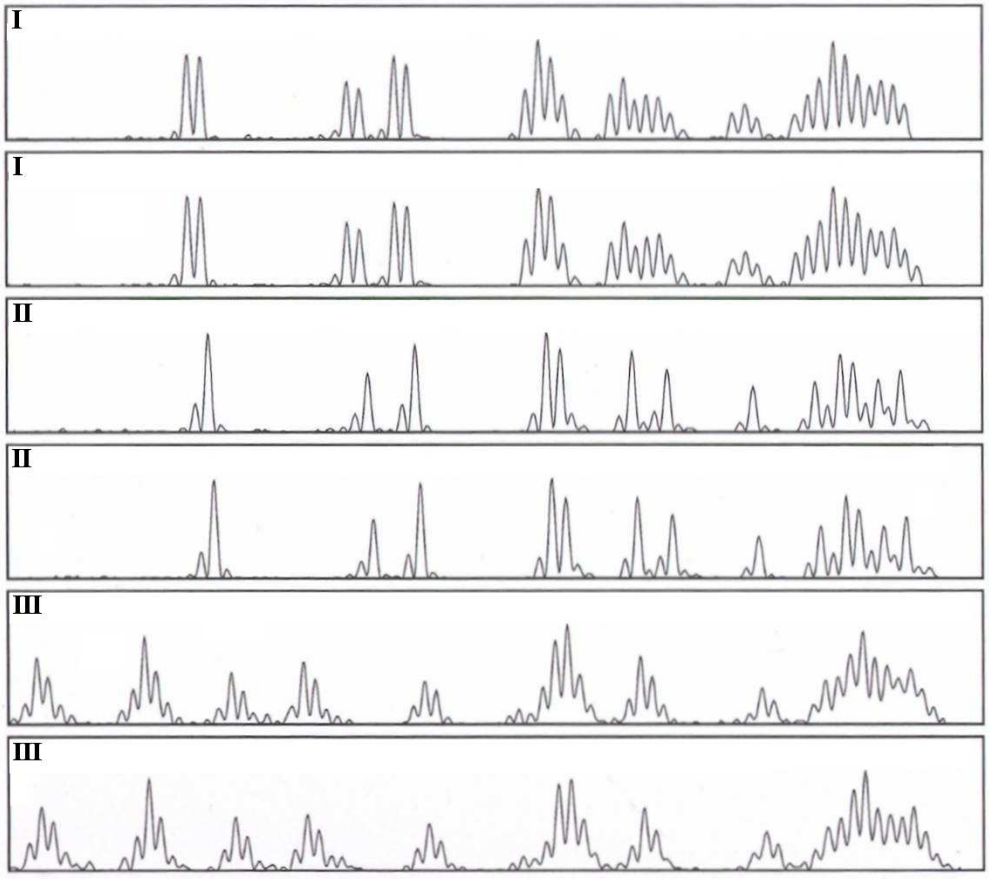

Fig. 1. C-profiling of H. meleagridis ITS-1 and 5.8 S region: examples of C-profiles of two isolates per type I, II or III.
194x172mm (150 x 150 DPI)

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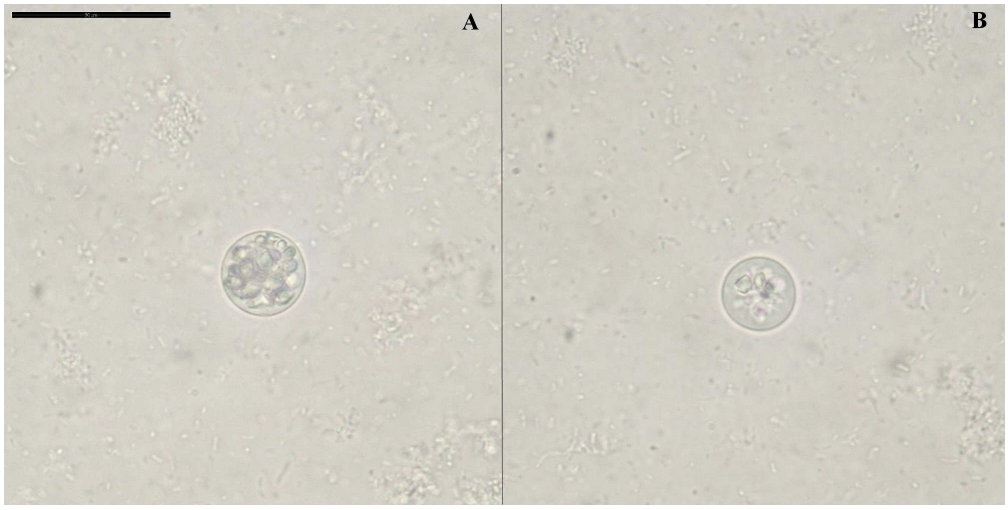

Fig. 2. Dixenic cultures: Histomonas meleagridis (B) and an unicellular organism with slighty different morphology (A). Bar is 30 µm.
705x352mm (72 x 72 DPI)

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