Title

Limited intra-genetic diversity in *Dientamoeba fragilis* housekeeping genes.

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Abstract

Dientamoeba fragilis is a common parasite of unsettled clinical significance. Differences in clinical outcome of intestinal parasitic infections may reflect parasite genetic diversity, and so tools to study intra-genetic diversity that could potentially reflect differences in clinical phenotypes are warranted. Here, we show that genetic analysis of three *Dientamoeba fragilis* housekeeping genes enables clear distinction between two genotypes, but that integration of housekeeping genes in multi-locus sequencing tools for *D. fragilis* may have limited epidemiological and clinical value.

Keywords

Dientamoeba, genetic diversity, actin, elongation factor, multilocus sequencing, molecular epidemiology

Highlights

Carriers of *Dientamoeba fragilis* may or may not experience symptoms → intragenetic diversity may be associated with clinical outcome →SSU rDNA analysis enables the distinction of two genotypes → analysis of two additional *D. fragilis* genes did not add further genetic resolution → analysis of *D. fragilis* housekeeping genes may have limited epidemiological value.

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1. Introduction

Dientamoeba fragilis is an intestinal parasite of unsettled clinical significance and possibly transmitted by pinworm (Johnson et al., 2004; Röser et al., 2013a; Stensvold et al., 2007a). In our laboratory 43% of approximately 22,000 faecal DNAs from patients with intestinal symptoms and analyzed by real-time PCR were positive, with a range in positive proportion from 10—70% depending on age group (Röser et al., 2013b). The parasite is common in individuals both with and without intestinal symptoms (Stensvold et al., 2009), and similar to the situation for various other intestinal parasites, identification of tools to study intra-genetic diversity that could potentially reflect differences linked to clinical outcome of infection and facilitate epidemiological studies appears relevant.

RFLP analysis of SSU rDNA PCR products enables distinction between the two genotypes currently known (genotypes 1 and 2); the sequences differ by at least 2% (Johnson and Clark, 2000; Peek et al., 2004; Stark et al., 2005). Genotyping has also been performed by SSU rDNA SNP analysis using PCR and pyrosequencing (Stensvold et al., 2007b). The value of sequencing the Internal Transcribed Spacer (ITS) region for typing studies of *D. fragilis* is limited due to intra-strain genetic heterogeneity (Windsor et al., 2006). C-profiling was developed as a means of extracting useful data from sequenced ITS clones (Bart et al., 2008), but the method has only been employed in a minor case report (Stark et al., 2009), and so little is known on its applicability and epidemiological relevance on a broader scale.

Studies of other housekeeping genes may prove useful in terms of obtaining higher resolution than can be obtained by studies of SSU rRNA genes alone, as in the case of other metamonads such as *Giardia* and *Trichomonas* (Cornelius et al., 2012; Feng and Xiao, 2011). Two *D. fragilis* genotype 1 housekeeping genes, namely actin and elongation factor 1 alpha (EF-1α) were recently sequenced (Noda et al., 2012), and this study aimed to characterize these two genes in *D. fragilis* genotype 2 and in *D. fragilis*-positive patient samples sent for parasitological analysis in our clinical microbiology laboratory.

2. Materials and methods

A total of 40 faecal DNAs were chosen randomly among those testing positive for *D. fragilis* by a *D. fragilis*-specific real-time PCR (Verweij et al., 2007) in our clinical microbiology laboratory. DNAs had been extracted directly from fresh faecal specimens from patients with gastrointestinal complaints in the absence of viral or bacterial pathogens, using the automated NucliSENS® easyMag® protocol (Andersen et al., 2013). Each DNA was submitted to single round conventional PCRs targeting actin and EF-1α genes, but also SSU rRNA genes for confirmation of the real-time PCR result and for genotyping. Primers for SSU rDNA amplification by conventional PCR and sequencing were those used by Röser et al. (2013a) (Table 1), while primers for amplification of actin and EF-1α genes were designed based on GenBank accession nos. AB468093 and AB468119, respectively. In cases where virtually complete genes (>95%) could not be obtained, primers targeting a minor fragment of the genes were used (Table 1).

Since actin and EF-1α gene sequences were available only for genotype 1 (Noda et al., 2012) and not had been characterized for genotype 2, these genes were amplified from DNA from the Bi/PA strain (kindly provided by Dr Graham Clark) and sequenced bidirectionally; sequences were submitted to GenBank (Accession nos.

KC967121-KC967122). As a control measure, the SSU rRNA gene was amplified from the Bi/PA strains as well, and the 364 bp SSU rDNA sequence obtained in the present study showed 100% identity to the Bi/PA strain sequence present in GenBank (acc. no. U37461).

Virtually complete actin and EF-1α sequences (>95% gene coverage) representing the Bi/PA strain were translated, concatenated, aligned with translated and concatenated reference sequences (Noda et al., 2012) including *D. fragilis* genotype 1 (DfA3 and DfE3C clones), and submitted to phylogenetic analysis, including distance-based (Neighbor-Joining (NJ)) and Maximum Likelihood (ML) analysis, using Molecular Evolutionary Genetics Analysis version 5 (MEGA 5) (Tamura et al., 2011); ModelTest (Posada and Crandall, 1998) was performed and the WAG + Γ model selected. Statistical support for distance-based and ML trees was evaluated using bootstrapping (1,000 replicates). Phylogenetic analysis of each individual translated gene (actin and EF-1α) was also performed; for ML analysis, the WAG + Γ model was selected for analysis of actin proteins, while the rtRev + Γ model was chosen for EF-1α proteins. Since these models are not available for NJ analysis, NJ analysis used JTT + Γ, and the gamma value (given in the ModelTest output) was 0.41 and 0.5 for the actin and EF-1α, respectively.

All data were anonymised prior to analysis, and so no personally identifiable data were included in the study.

3. Results and Discussion

Using the faecal DNA templates from patient samples, the SSU rRNA, actin, and EF-1α genes could be amplified and unambiguously sequenced in 32/40, 29/40 and 21/40 cases, respectively. As seen, EF-1α genes could be successfully amplified and sequenced in only 53% of the cases, which could be explained by the fact that Ct-

values obtained by real-time PCR (SSU rRNA gene) were significantly lower for DNAs from which EF-1α genes could be amplified and unambiguously sequenced than for the DNAs where either no amplification was obtained or where (often faint) PCR products gave rise to unclear sequence traces ($p<0.001$; Student's T-test for comparison of means (data not shown)).

Sequences were aligned and interpreted manually. One patient sample (1/32, 3%) (T14157) was found to belong to genotype 2, while the remainder of the samples (31/32, 97%) for which SSU rDNAs were available belonged to genotype 1; these data are in line with previous reports on the relative prevalence of the two genotypes (Johnson and Clark, 2000; Peek et al., 2004; Windsor et al., 2006). T14157 and Bi/PA were 100% identical across all three genes (data not shown). T14157 was from a 62 year old male with persistent intestinal symptoms, who had submitted multiple faecal samples for traditional clinical microbiology analyses with no evidence of enteric viruses, enteropathogenic bacteria or other intestinal parasites except for *Blastocystis*; this patient was the oldest patient in the study group (n=40; median age: 16.5 years [IQR 6.0—42.0]).

The two genotypes differed by 29 unambiguous SNPs scattered across the actin gene, (Supplementary Fig. 1), of which 4 were non-synonymous substitutions. Likewise, across the EF-1α gene (Supplementary Fig. 2), 25 scattered unambiguous SNPs were identified, of which 4 were non-synonymous substitutions. In comparison, SSU rRNA genes from the two genotypes differ by at least 2% and hence, the amount of genetic variation seen across the actin and EF-1α genes, which are both in the size range of 800-850 bp, is comparable to the amount of variation seen in the SSU rRNA gene, if only a little higher (about 3%).

No strain-unique SNPs were detected across any of the two genes among the genotype 1 samples. However, there were several positions in each sequence exhibiting consistent allelic heterozygosity, although difficult to discern in some of the trace files, and representing synonymous substitutions only.

Phylogenetic analysis of concatenated actin and EF-1α proteins using translated sequence data and reference sequences from the alignment given by (Noda et al., 2012)) consolidated the existence of two genotypes clustering with maximum bootstrap support, and sharing a most recent common ancestor with *Histomonas* (Figure 1); individual trees produced for each translated gene consolidated these phylogenetic inferences (Supplementary Fig. 3).

Although the study is limited by the fact that *D. fragilis* from healthy individuals was not included, the present data suggest a high degree of conservation in *D. fragilis* housekeeping genes.

The data show that analysis of intra-genetic diversity in house-keeping genes may have limited epidemiological and clinical usefulness in studies of *D. fragilis* in humans. However, pigs and gorillas have been identified as natural hosts of *D. fragilis* (Cacciò et al., 2012; Lankester et al., 2010; Stark et al., 2008), and while SSU rDNA data point towards the probability that pigs are natural hosts of genotype 1 (Cacciò et al., 2012), it remains to be seen whether analysis of non-SSU rRNA genes in isolates from non-human hosts identify intra-genetic variation, thereby enabling studies of transmission and further exploration of zoonotic potential.
As yet, *D. fragilis* genome sequences have not been published, but steadily decreasing costs related to genome sequencing using high-throughput platforms and identification of ways to obtain genomic data from small amounts of DNA should prompt the initiative of complete sequencing of mitochondrial or even nuclear

158 genomes in future efforts to screen isolates from symptomatic and asymptomatic carriers for genetic variation.
159 Finally, the prevalence and clinical significance of genotype 2 should be studied and compared to genotype 1.
160
161 Acknowledgements
162
163 Lis Lykke Wassmann is thanked for excellent laboratory assistance.
164
165 Figure legends
166
167 Figure 1. Phylogenetic analysis of translated and concatenated actin and EF-1α sequences representing the
168 Bi/PA and the DfA3 strains along with reference organisms from the publication by (Noda et al., 2012); ML tree
169 is shown with the support values in the order ML/NJ . Values less than 50% with both methods are either not
170 shown or marked by an asterisk. *Df* = *D. fragilis*.
171
172 Supplementary Figure 1: Alignment of actin gene sequences for genotype 1 (DfA3 clone; AB468093) and
173 genotype 2 (Bi/PA strain; KC967121).
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175 Supplementary Figure 2: Alignment of EF-1α gene sequences for genotype 1 (DfE3C clone; AB468119) and
176 genotype 2 (Bi/PA strain; KC967122).
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178 Supplementary Figure 3: Phylogenetic analysis of translated EF-1α and actin gene sequences. The ML tree is
179 shown with the support values in the order ML/NJ. Values less than 50% with both methods are either not
180 shown or marked by an asterisk. *Df* = *D. fragilis.*

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266 Table 1. Primers used in the study (see text for details).

Gene	**Primers**	**Reference**
SSU rRNA (18S)	DFpn_1f 5'-GCC AAG GAA GCA CAC TAT GG-3' DFpn_364r	(Röser et al., 2013a)

	5’-GTA AGT TTC GCG CCT GCT-3’	
Actin	DF_ACTIN_3f 5’-CCA CAC ATT CTA CAA CGA ATT AC-3’ DF_ACTIN_157F 5’-GTT CTT TCA CTT TAC TCA TCA GGT C-3’ DF_ACTIN_291R 5’-GAC CAG CAA GGT TGA GTC TC-3’ DF_ACTIN_843r 5’-TGG ACC AGC TTC ATT GTA TTC-3’	Present study
EF-1α	DF_EF_1f 5’-CTC ACT TTG GAA GTT CGA ATC-3’ DF_EF_265F 5’-TCA AAG GCT CGT TAT GAT GAA ATC-3’ DF_EF_364R 5’-GAA ACC TGA GAT TGG AAC AAA C-3’ DF_EF_ 836r 5’-CTG TGT GGC AAT CGA AAA C-3’	Present study

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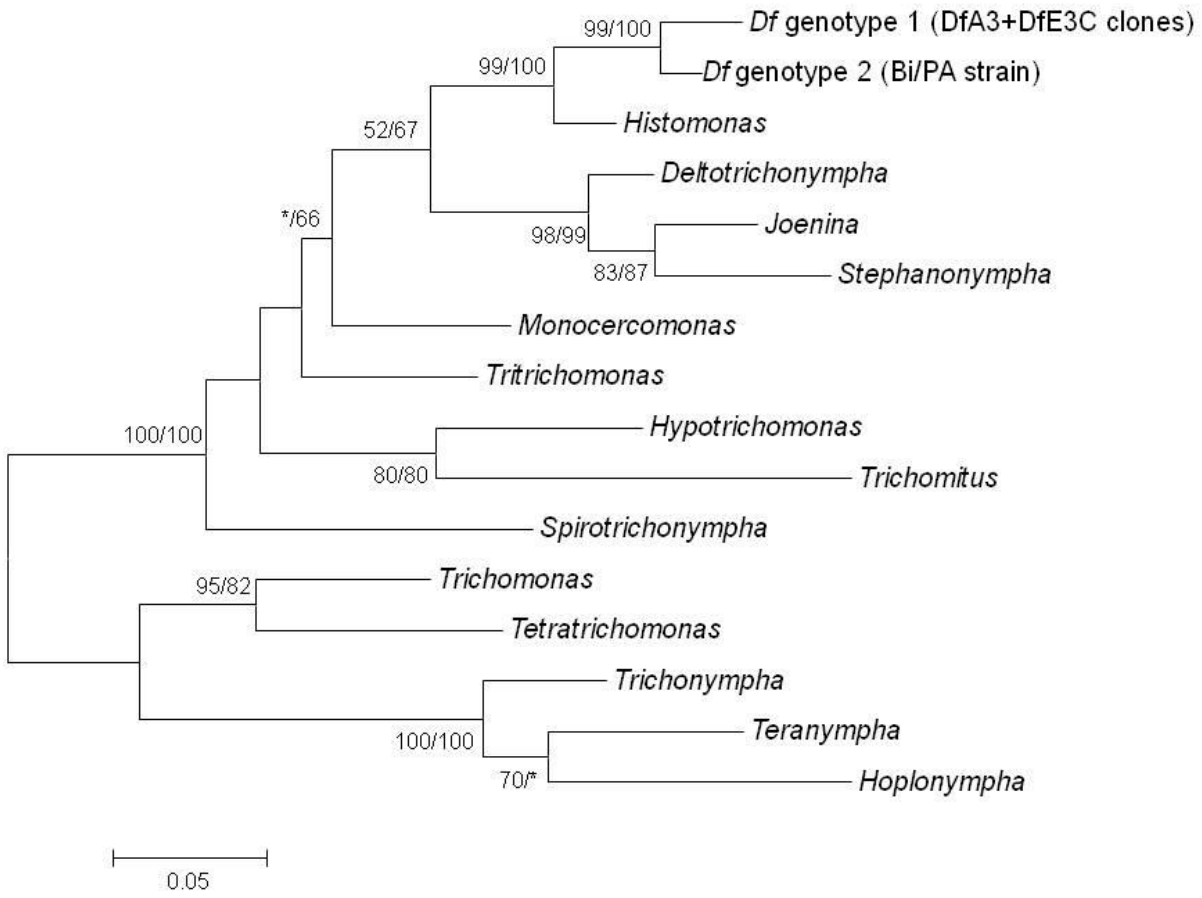
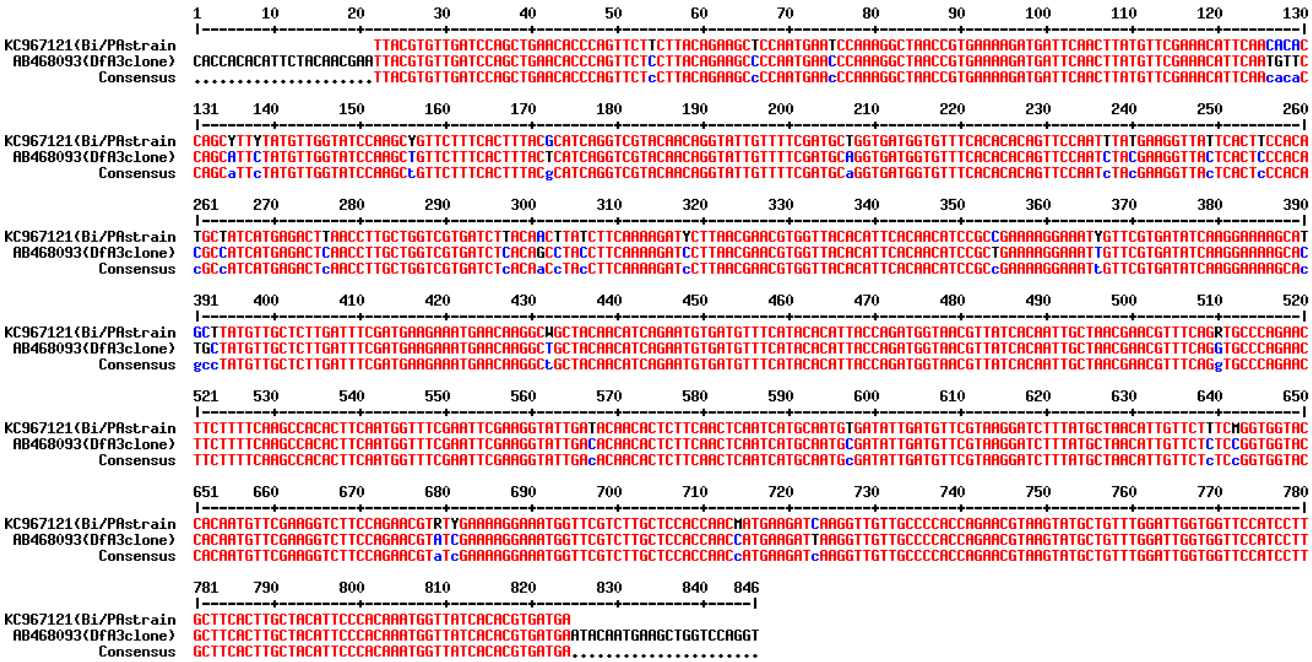
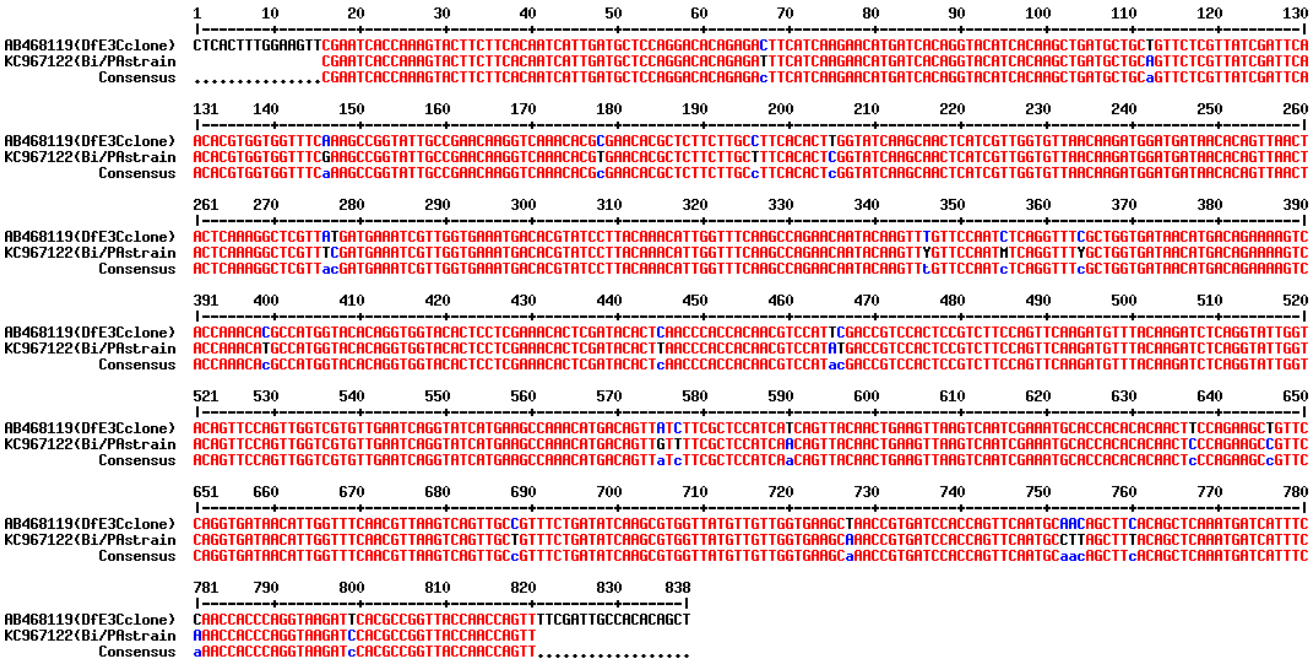

99/100
Df genotype 1 (DfA3+DfE3C clones)
99/100
Df genotype 2 (Bi/PA strain)
Histomonas
52/67
Deltotrichonympha
*/66
98/99
Joenina
83/87
Stephanonympha
Monocercomonas
Tritrichomonas
100/100
Hypotrichomonas
80/80
Trichomitus
Spirotrichonympha
95/82
Trichomonas
Tetratrichomonas
Trichonympha
100/100
Teranympha
70/*
Hoplonympha
0.05

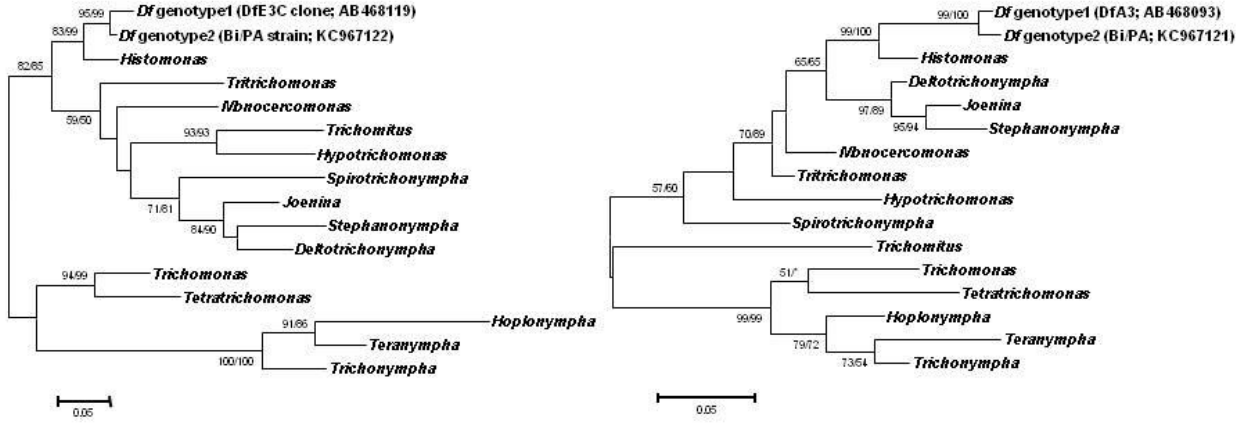
Fig 1



Suppl Fig 1



Suppl Fig 2



Ef1α

Actin

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280 Suppl Fig 3