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1 Title

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

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24 Abstract

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

31

32 Keywords

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

34

35 Highlights

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

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47 Manuscript

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49 **1. Introduction**

50

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

59

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

68

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

75

## 76 **2. Materials and methods**

77

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

88

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

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

95

96 Virtually complete actin and EF-1 $\alpha$  sequences (>95% gene coverage) representing the Bi/PA strain were  
97 translated, concatenated, aligned with translated and concatenated reference sequences (Noda et al., 2012)  
98 including *D. fragilis* genotype 1 (DfA3 and DfE3C clones), and submitted to phylogenetic analysis, including  
99 distance-based (Neighbor-Joining (NJ)) and Maximum Likelihood (ML) analysis, using Molecular Evolutionary  
100 Genetics Analysis version 5 (MEGA 5) (Tamura et al., 2011); ModelTest (Posada and Crandall, 1998) was  
101 performed and the WAG +  $\Gamma$  model selected. Statistical support for distance-based and ML trees was evaluated  
102 using bootstrapping (1,000 replicates). Phylogenetic analysis of each individual translated gene (actin and EF-  
103 1 $\alpha$ ) was also performed; for ML analysis, the WAG +  $\Gamma$  model was selected for analysis of actin proteins, while  
104 the rtRev +  $\Gamma$  model was chosen for EF-1 $\alpha$  proteins. Since these models are not available for NJ analysis, NJ  
105 analysis used JTT +  $\Gamma$ , and the gamma value (given in the ModelTest output) was 0.41 and 0.5 for the actin and  
106 EF-1 $\alpha$ , respectively.

107

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

109

### 110 **3. Results and Discussion**

111

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

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

119

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

128

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

135

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

139

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

145

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

148

149 The data show that analysis of intra-genetic diversity in house-keeping genes may have limited epidemiological  
150 and clinical usefulness in studies of *D. fragilis* in humans. However, pigs and gorillas have been identified as  
151 natural hosts of *D. fragilis* (Cacciò et al., 2012; Lankester et al., 2010; Stark et al., 2008), and while SSU rDNA  
152 data point towards the probability that pigs are natural hosts of genotype 1 (Cacciò et al., 2012), it remains to  
153 be seen whether analysis of non-SSU rRNA genes in isolates from non-human hosts identify intra-genetic  
154 variation, thereby enabling studies of transmission and further exploration of zoonotic potential.

155 As yet, *D. fragilis* genome sequences have not been published, but steadily decreasing costs related to genome  
156 sequencing using high-throughput platforms and identification of ways to obtain genomic data from small  
157 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-1 $\alpha$  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).

174

175 Supplementary Figure 2: Alignment of EF-1 $\alpha$  gene sequences for genotype 1 (DfE3C clone; AB468119) and  
176 genotype 2 (Bi/PA strain; KC967122).

177

178 Supplementary Figure 3: Phylogenetic analysis of translated EF-1 $\alpha$  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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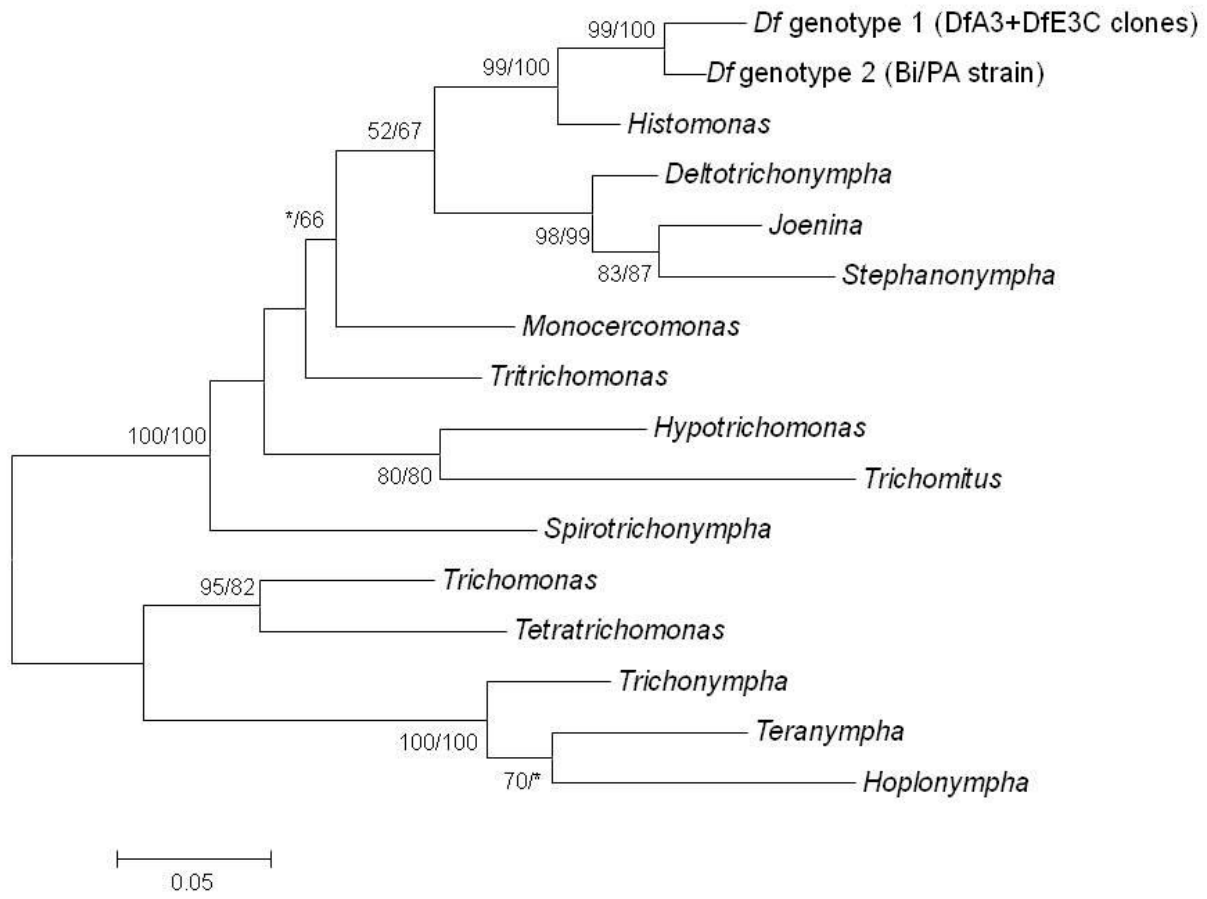
266 Table 1. Primers used in the study (see text for details).

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-1 $\alpha$	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

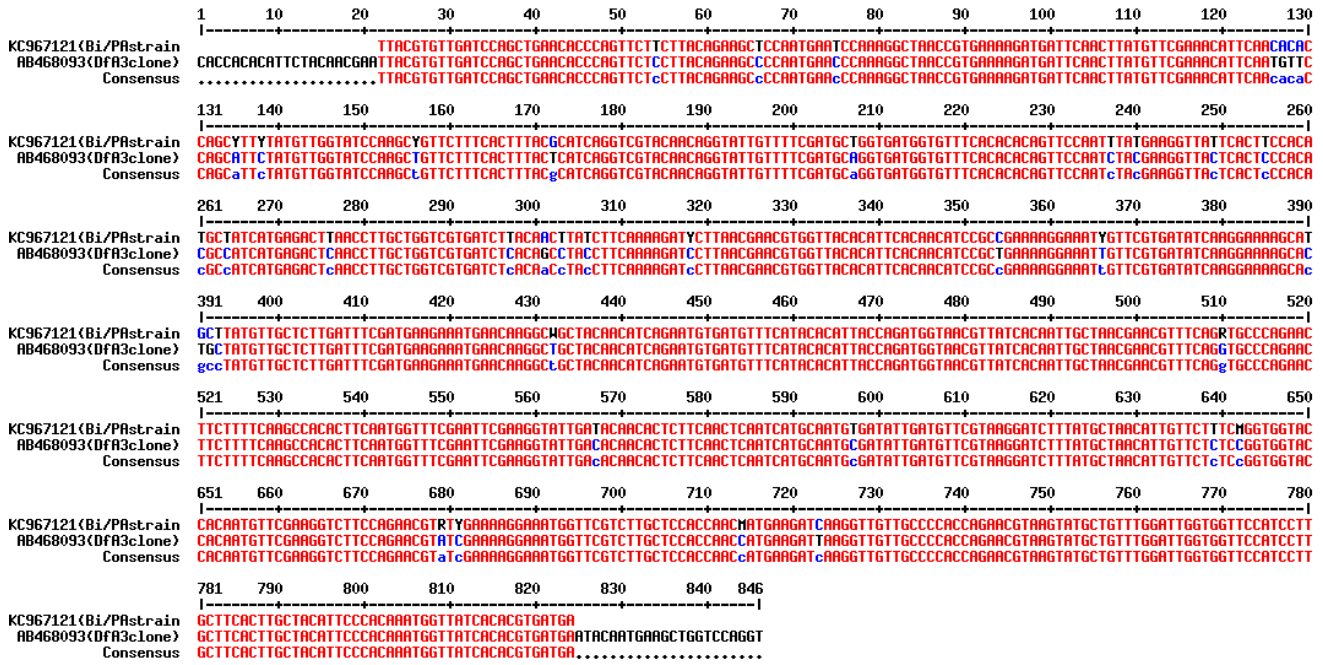
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270 Fig 1

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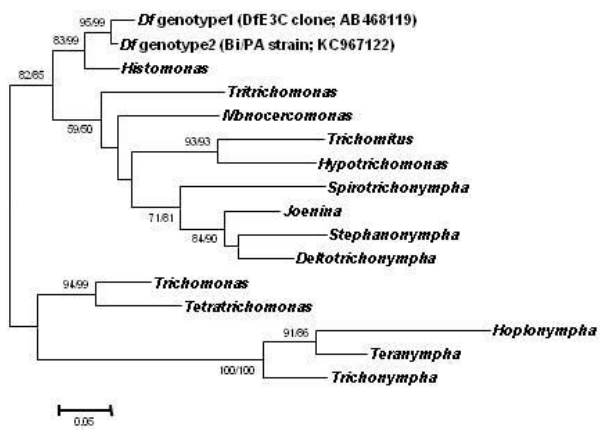
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Suppl Fig 1

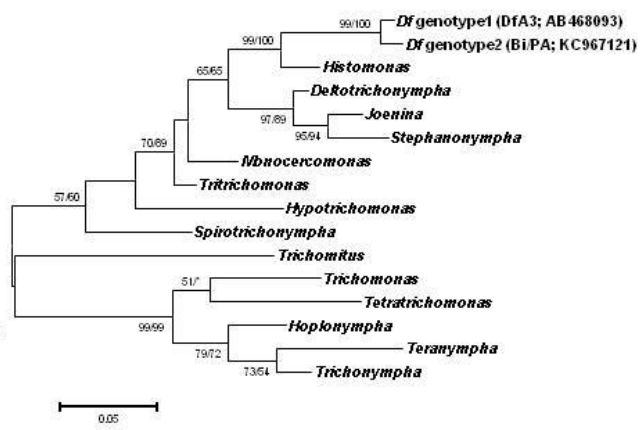
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*E1α*



*Actin*

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280 Suppl Fig 3