Secondary loss of a cis-spliced intron during the divergence of *Giardia intestinalis* assemblages

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**Abstract**

**Background:** *Giardia intestinalis* is a parasitic unicellular eukaryote with a highly reduced genome, in which only six cis-spliced and four trans-spliced introns have been discovered. However, we anticipate that more cis- and trans-spliced introns likely remain unidentified in genes encoding hypothetical proteins that occupy ca. 2/3 of all of the open reading frames (ORFs) in the *Giardia* genome. Consequently, comprehensive surveys of introns in ORFs for hypothetical proteins are critical for better understanding of the intron evolution in this organism.

**Results:** In this study, we identified two novel cis-spliced introns in the draft genome data of *G. intestinalis* strain WB, by surveying the conserved sequence motifs shared amongst the previously known introns. *G. intestinalis* strains can be divided into phylogenetically distinct assemblages A–H, and all the introns identified in past studies are shared among the published genome data from strains WB, DH, GS, and P15 representing assemblages A1, A2, B, and E, respectively. Nevertheless one of the two novel introns identified in this study was found to be absent in strain P15.

**Conclusion:** By considering the organismal relationship among *G. intestinalis* assemblages A1, A2, B, and E, one of the two introns identified in this study has highly likely been lost after the divergence of the assemblages. On the basis of a sequence comparison between the intron-bearing loci in WB, DH, and GS genomes and the homologous but intron-free locus in P15 genome, we propose that the loss of this particular intron was mediated by integration of the DNA fragment reverse-transcribed from mature mRNAs.

**Keywords:** Intron loss, Homologous recombination, Reduced genome, Reverse transcription

**Background**

Spliceosomal introns, which are excised from pre-mature mRNAs by RNA-protein complexes called spliceosomes [1], are one of the features exclusively found in eukaryotic genomes. However, a large variety in intron density has been found across eukaryotic genomes sequenced to date [2]. In the human genome, for example, 8.4 introns on average are annotated per gene [3], and the mean intron size is ca. 3,000 bp in length [2]. In contrast, *Giardia intestinalis*, a unicellular eukaryotic parasite belonging to the Diplomonadida (Excavata) is known to possess a highly reduced genome of only 12 Mbp in length [4]. One of the prominent natures of the *Giardia* genome is its low intron density—only 6 cis-spliced introns and 4 trans-spliced introns (split introns) have been identified prior to this study [4-12]. Henceforth here, we simply designate cis-spliced introns as ‘introns,’ and trans-spliced introns as ‘splintrons’ [8].

Most of introns/splintrons in the *Giardia* genome were identified principally as non-coding stretches intervening in open reading frames (ORFs) encoding proteins shared amongst phylogenetically diverse eukaryotes. However, the simple procedure described above may be problematic for distinguishing the coding and non-coding regions (i.e. exons and introns/splintrons) in functionally unidentified ORFs encoding *Giardia*-specific proteins. Since unidentified ORFs occupy approximately 2/3 of the ca. 9,000 ORFs encoded in the *Giardia* genome [4], a large fraction of introns/splintrons in the *Giardia* genome may have been overlooked by pioneering surveys principally based on sequence similarity.

To shed light on introns/splintrons veiled in unidentified ORFs in the genome of *G. intestinalis* strain WB, we conducted an intron survey based on the conserved...
sequence motifs in introns/splintrons, and successfully detected two novel introns in unidentified ORFs (Note that our approach is not technically applicable to survey splintrons). The two ORFs, which harbor introns in the WB genome, were identified in the genomes of *G. intestinalis* strains DH [13], GS [14] and P15 [15] as well, but one of these in the P15 genome were found to be intron-free. We propose a scenario to explain the presence/absence of the particular intron in the four *G. intestinalis* strains.

**Methods**

*In silico* detection of conserved intron sequences

*Giardia* introns/splintrons known to date bear conserved sequence motifs at the 5′ and 3′ termini, 5′-STATG-3′ and 5′-HCTRACMCVCAG-3′ (R = A or G; H = A, T, or C; M = A or C; V = A, C, or G; S = G or C), respectively. Furthermore, the two motifs may be flanked with each other within 300 bp, since all of the known introns in the *Giardia* genome range from 29 to 220 bp in length. We searched for genome segments that satisfied the above criteria in the draft genome data of *G. intestinalis* strain WB (GiardiaDB, http://www.giardiadb.org/giardiadb/).

**Cells, DNA, RNA, and reverse transcription**

*G. intestinalis* strain WB (ATCC50803) was cultivated as described previously [7]. Genomic DNA (gDNA) was extracted by cetyl trimethylammonium bromide buffer [16] from the harvested cells. Total RNA was isolated from the cells with the RNeasy Plant Mini kit (QIAGEN) following the manufacturer’s instruction. Cloning and sequencing were performed as described above.

**Detection of intron splicing**

We designed exact-match primers at the 5′ and 3′ flanking regions of intron-like sequences nominated by the *in silico* survey (see above), and performed two separate PCRs, one with total gDNA as the template (gDNA-based PCR) and the other with cDNA as the template (cDNA-based PCR). reverse transcription was performed by the 3′ ends of the mRNAs, the gene terminal region of the corresponding ORF, as seen in the previously identified *Giardia* introns, except for that found at the 3′ terminal region of rpl7A gene. In terms of intron length, both the intron in orfA (41 bp) and that in orfB (33 bp) are comparable with other *Giardia* introns (29–36 bp), except for the intron in the ORF encoding hypothetical protein GL50803_35332 (220 bp) and that in rpl7A gene (109 bp). Based on the experimentally confirmed 5′ ends of the mRNAs, the gene models for the two ORFs, as well as their intron-exon boundaries, were refined (Figure 1C and D).

**Results and discussion**

We identified two novel introns among the 11 intron-like sequences nominated by the *in silico* survey of introns in the genome data of *G. intestinalis* strain WB. For the two novel introns, the intron-free transcripts (i.e. mature mRNAs) were successfully identified in the amplicons from cDNA-based PCR (Figure 1A and B). Curiously, we detected both intron-bearing and intron-free transcripts (i.e. pre-mature and mature mRNAs) in the cDNA-based amplicons that were not clearly distinguished in size from the gDNA-based amplicons (Figure 1B), suggesting that the splicing efficiency of this particular intron is relatively low. The above conjecture about the splicing efficiency is consistent with the fact that five out of the eight introns/splintrons known to date bear conserved intron-like sequences nominated by the *in silico* survey shared across the draft genome data of *G. intestinalis* strain WB, for the two novel introns, the intron-free transcripts (i.e. mature mRNAs) were cloned into the pGEM T-easy vector (Promega) and sequenced completely. We experimentally determined the 5′ ends of the mRNAs from ORFs AACB02000068-1-10039-10248 and AACB02000001-6-305427-304747 by using the 5′ rapid amplification of cDNA ends kit (Invitrogen) following the manufacturer’s instruction. Cloning and sequencing were performed as described above.

The two introns were found in ORFs no. AACB02000068-1-10039-10248 and AACB02000001-6-305427-304747, shown in Figure 1C and D, respectively. Hereafter, we designate the ORFs AACB02000068-1-10039-10248 and AACB02000001-6-305427-304747 as orfA and orfB, respectively. Each of the novel introns locates at the 5′ terminal region of the corresponding ORF, as seen in the previously identified *Giardia* introns, except for that found at the 3′ terminal region of rpl7A gene. In terms of intron length, both the intron in orfA (41 bp) and that in orfB (33 bp) are comparable with other *Giardia* introns (29–36 bp), except for the intron in the ORF encoding hypothetical protein GL50803_35332 (220 bp) and that in rpl7A gene (109 bp). Based on the experimentally confirmed 5′ ends of the mRNAs, the gene models for the two ORFs, as well as their intron-exon boundaries, were refined (Figure 1C and D).

The strains of *G. intestinalis* can be divided into 9 assemblages (A1, A2, B–H) in light of their sequence diversity [4,17,18]. All of the introns/splintrons identified in past and present studies, except for that in orfB (see below), were found to be shared across the draft genome data of the four *G. intestinalis* strains WB, DH, P15, and GS, which represent assemblages A1, A2, B, and E, respectively. Unlike other introns/splintrons, the orfB intron may not be ubiquitously present in *G.
intestinalis strains (Additional file 1: Figure S1A and B)—an intron-bearing orfB was found in the GS genome (ORF ACGJ01002919-1) and in the DH genome (ORF ACGJ01000002-2), but we identified only the intron-free homologue in the P15 genome (ORF ACVC01000101-5). Thus, we propose that (i) the common ancestor of the four assemblages possessed an intron-containing orfB, and (ii) orfB in assemblage E lost the corresponding intron after the split of assemblages A1/A2 and E (Figure 2) if the corresponding locus of the published P15 genome is not derived from misassembling. To depict more detailed features, we identified only the intron-free homologue in the P15 genome (ORF AHGT01000002-2) but we further identified the intron-contained homologue in the GS genome (ORF ACGJ01002919-1), indicating that assemblages A1/A2 are the closest relatives to each other, and A1/A2 complex is more closely related to E than B [19,20]. Thus, we propose that (i) the common ancestor of the four assemblages possessed an intron-containing orfB, and (ii) orfB in assemblage E lost the corresponding intron after the split of assemblages A1/A2 and E (Figure 2) if the corresponding locus of the published P15 genome is not derived from misassembling. To depict more detailed features of the intron in orfB, the genome data from G. intestinalis strains representing other assemblages are indispensable.

There are three major models to explain how eukaryotic genomes lost spliceosomal introns: (i) ‘de-intronization’ by mutations, (ii) non-homologous end joining (NHEJ) repair of double strand break (DSB) in an intron sequence, and (iii) homologous recombination of the cDNA e.g., [21-23; see also Additional file 2: Figure S2A-C]. The first model assumes the conversion of an intron sequence to an exon sequence by nucleotide substitutions, which results in extension of the corresponding ORF (Additional file 2: Figure S2A). Nevertheless, the length of orfB was found to be uniform among WB, DH, GS, and P15 genomes (Figure 2; see also Additional file 2: Figure S2A-C).
also Additional file 1: Figure S1B), suggesting that the loss of orfB intron cannot be rationalized by deintronization. The second model demands ‘microhomology’ pairing between 5′ and 3′ splice sites to anchor the upstream and downstream exons, which are split by DSB in the intron, during NHEJ repair [22; see also Additional file 2: Figure S2B]. Importantly, both 5′ and 3′ splice sites need to be 5′-AG/GT-3′ (the slash indicates the boundary between intron and exon) in the second intron loss model (Additional file 2: Figure S2B). As the key assumption does not fit to the splice sites of the orfB introns (Figure 2), suggesting that the intron has not been eliminated from the P15 genome by NHEJ repair. The last model invokes integration of a reverse-transcribed mRNA (i.e. cDNA; intron-free) into the original, intron-containing locus through homologous recombination (Additional file 2: Figure S2C). We regard that the homologous recombination of the cDNA, which can eliminate the entire intron sequence but does not require sequence conservation at the 5′ and 3′ splice sites, is more appropriate to explain the loss of orfB intron in the P15 genome than the two models described above, (Figure 2). It is intriguing to point out the presence of putative reverse transcriptase genes in the genome data of the four G. intestinalis assemblages [e.g., Genbank/EMBL/DDB] accession nos. AF434198 (WB), AHGT01000152 (DH), EES99684 (GS), and EFO60876 (P15)], although reverse transcription activity of these encoded proteins has yet to be experimentally confirmed in G. intestinalis cells [24].

Conclusion
In this study, we found two novel cis-spliced introns and their punctate distribution in the genomes of G. intestinalis assemblies. Together with the recently found trans-spliced introns, the data presented here suggest that the intron evolution in this organism is more complex than we previously thought.

Additional files

Additional file 1: Figure S1. Amino acid sequences of ORFs AACB02000006-1-10039-10248 (orfA) and AACB02000001-6-305427-30747 (orfB) in strain WS (assemblage A1) and the homologous regions in the ORF AHGT01000002-2-45140-45820 in strain DH (assemblage A2), the ORF ACVC01000101-5-40937-40224 in strain P15 (assemblage E), and the ORF ACVC01000107-3-18771-18962, respectively. B orfA. Amino acid residues and nucleotides identical among the three sequences are shaded in black background. Asterisks indicate stop codons. The inserted position and nucleotide sequences of orfA intron are presented in the balloon. ORF nos. of orfA homologues in strains WB, DH, GS, and P15 are AACB02000006-1-10039-10248, AHGT01000008-5-18630-18839, ACVC01000193-2-4190-4396, and ACVC01000007-5-18771-18962, respectively. C orfB. The details of this figure are the same as described in A. ORF nos. of orfB homologues in strains WB, DH, GS, and P15 are AACB02000001-6-305427-30747, AHGT01000002-2-45140-45820, ACVC010002919-1-19018-19737, and ACVC01000101-5-40937-40224, respectively. The intron sequences, which are a part of Figure 2, are not provided here.

Additional file 2: Figure S2. Proposed models for intron loss. A. Deintronization by substitutions. An intron sequence (red) is changed to an exon sequence (light blue) by nucleotide substitutions (asterisks), resulting in extension of exon sequence. B. Non-homologous end joining repair of double strand break in intron sequence. In this model, ‘microhomology’ pairing between 5′ and 3′ splice sites anchors the upstream and downstream exons, which are split by double strand break. Subsequently, the broken strands are repaired, resulting in elimination of the entire intron sequence. C. Homologous recombination between an intron-containing gDNA and the corresponding intron-free cDNA (dark blue). This model assumes that the cDNA fragment, which is reverse-transcribed from a mature mRNA bearing no intron, is recombined into the corresponding intron-containing locus in the genome, resulting in elimination of the entire intron sequence.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
RK determined the sequences and analyzed the data. YI and TH provided research materials. RK, YI, and TH prepared the manuscript. All the authors read and approved the final manuscript.
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References
1. Wahl MC, Will CL, Lührmann R: The spliceosome: design principles of a dynamic RNP machine. Cell 2009, 136:701–718.
2. Keeling PJ, Slamovits CH: Causes and effects of nuclear genome reduction. Curr Opin Genet Dev 2005, 15:560–608.
3. Rodríguez-Trelles F, Tarrio R, Ayala FJ: Origins and evolution of splicing introns. Annu Rev Genet 2006, 40:47–76.
4. Morrison HG, McArthur AG, Gillin FD, Aley SB, Adam RD, Olsen GJ, Best AA, Carde WZ, Chen F, Cicignino MJ, Davids BJ, Dawson SC, Brnendorf HG, Hesl AB, Holder ME, Huse SM, Kim UU, Lasek-Nesselquist E, Manning G, Nigam A, Nixon JE, Palm D, Passamaneck NV, Pabzhu A, Reich CI, Reiner DS, Samuelson J, Svard SG, Sogin ML: Genomic minimalism in the early diverging intestinal parasite Giardia lamblia. Science 2007, 317:1921–1926.
5. Nixon JE, Wang A, Morrison HG, McArthur AG, Sogin ML, Loftus BJ, Samuelson J: A spliceosomal intron in Giardia lamblia. Proc Natl Acad Sci U S A 2002, 99:3701–3705.
6. Russell AG, Shutt TE, Watkins RF, Gray MW: An ancient spliceosomal intron in the ribosomal protein L7a gene (Rpl7a) of Giardia lamblia. BMC Evol Biol 2005, 5:45.
7. Kamikawa R, Inagaki Y, Tokoro M, Roger AJ, Hashimoto T: Split introns in the genome of Giardia intestinalis are excised by spliceosome-mediated trans-splicing. Curr Biol 2011, 21:311–315.
8. Kamikawa R, Inagaki Y, Roger AJ, Hashimoto T: Splintrons in Giardia intestinalis: spliceosomal introns in a split form. Commun Integr Biol 2011, 4:454–456.
9. Nageshan RK, Roy N, Hesl AB, Tatu U: Post-transcriptional repair of a split heat shock protein 90 gene by mRNA trans-splicing. J Biol Chem 2011, 286:7116–7122.
10. Kamikawa R, Inagaki Y, Hashimoto T: A novel spliceosome-mediated trans-splicing can change our view on genome complexity of the divergent eukaryote Giardia intestinalis. Biophys Rev 2012, 4:193–197.
11. Roy SW, Hudson AJ, Joseph J, Yee J, Russell AG: Numerous fragmented spliceosomal introns, AT-AC splicing, and an unusual dynein gene expression pathway in Giardia lamblia. Mol Biol Evol 2012, 29:43–49.
12. Franzén O, Jerström-Hultqvist J, Einassion E, Ankarklev J, Ferrilla M, Andersson B, Svärd SG: Transcriptome profiling of Giardia intestinalis using strand-specific RNA-Seq. PLoS Comput Biol 2013, 9:e1003000.
13. Adam RD, Dahlstrom EW, Martens CA, Bruno DP, Barbian KD, Rickles SM, Hernandez MM, Narla NP, Patel RB, Porcella SF, Nash TE: Genome sequencing of Giardia lamblia genotypes A2 and B isolates (DH and GS) and comparative analysis with the genomes of genotypes A1 and E (WB and Pig). Genome Biol Evol 2013, 5:2408–2511.
14. Franzén O, Jerström-Hultqvist J, Castro E, Sherwood E, Ankarklev J, Reiner DS, Palm D, Andersson JO, Andersson B, Svärd SG: Draft genome sequencing of Giardia intestinalis assemblage B isolate GS: Is human giardiasis caused by two different species? PLoS Pathog 2009, 5:e1000350.
15. Jerström-Hultqvist J, Franzén O, Ankarklev J, Xu F, Nohynkova E, Andersson JO, Svärd SG, Andersson B: Genome analysis and comparative genomics of a Giardia intestinalis assemblage E isolate. BMC Genomics 2010, 11:543.
16. Kamikawa R, Nishimura H, Sako Y: Analysis of the mitochondrial genome, transcripts, and electron transport activity in the dinoflagellate Alexandrium catenella (Gonyaulacales, Dinophyceae). Phycol Res 2009, 57:1–11.
17. Lasek-Nesselquist E, Welch DM, Sogin ML: The identification of a new Giardia duodenalis assemblage in marine vertebrates and a preliminary analysis of G. duodenalis population biology in marine systems. Int J Parasitol 2010, 40:1063–1074.
18. Nash TE, Keister DL: Differences in excretory-secretory products and surface antigens among 19 isolates of Giardia. J Infect Dis 1988, 152:1166–1171.
19. Monis PT, Andrews RH, Mayhofer C, Ely PL: Molecular systematics of the parasitic protozoan Giardia intestinalis. Mol Biol Evol 1999, 16:1135–1144.
20. Monis PT, Caccio SM, Thompson RCA: Variation in Giardia: towards a taxonomic revision of the genus. Trends Parasitol 2009, 25:93–100.
21. Roy SW, Gilbert W: The evolution of spliceosomal introns: patterns, puzzles and progress. Nat Rev Genet 2006, 7:211–221.
22. Farlow A, Meduri E, Schlötterer C: DNA double-strand break repair and the evolution of intron density. Trends Genet 2011, 27:1–6.
23. Cohen NE, Shen R, Carmel L: The role of reverse transcriptase in intron gain and loss mechanisms. Mol Biol Evol 2012, 29:179–186.
24. Burke WD, Malik HS, Rich SM, Eckbush TH: Ancient lineages of non-LTR retrotransposons in the primitive eukaryote, Giardia lamblia. Mol Biol Evol 2003, 19:610–630.

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