Sense-antisense gene overlap causes evolutionary retention of the few introns in *Giardia* genome and the implications

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24 **Abstract**

25 **Background:** It is widely accepted that the last eukaryotic common ancestor (LECA)
 26 and early eukaryotes were intron-rich and intron loss dominated subsequent evolution,
 27 thus the presence of only very few introns in some modern eukaryotes must be the
 28 consequence of massive loss. But it is striking that few eukaryotes were found to have
 29 completely lost introns. Despite extensive research, the causes of massive intron
 30 losses remain elusive, and actually the reverse question – how the few introns are
 31 retained under the pressure of loss is equally significant but was rarely studied, except
 32 that it was conjectured that the essential functions of some introns prevent their loss.
 33 The extremely few (eight) spliceosome-mediated cis-spliced introns in the relatively
 34 simple genome of *Giardia lamblia* provide an excellent opportunity to explore this
 35 question.

36 **Results:** Our investigation of the intron-containing genes and introns in *Giardia*
 37 found three types of intron distribution patterns: ancient intron in ancient gene,
 38 relatively new intron in ancient gene, and relatively new intron in relatively new gene,
 39 which can reflect to some extent the dynamic evolution of introns in *Giardia*. Not
 40 finding any special features or functional importance of these introns responsible for
 41 the retention, we noticed and experimentally verified that some intron-containing
 42 genes form sense-antisense gene pairs with functional genes on their complementary
 43 strands, and that the introns just reside in the overlapping regions.

44 **Conclusions:** In *Giardia*'s evolution, despite constant pressure of intron loss, intron
 45 gain can still occur in both ancient and newly-evolved genes, but only a few introns
 46 have been retained; the evolutionary retention of introns is most likely not due to the
 47 functional constraint of the introns themselves but the causes outside of introns, such
 48 as the constraints imposed by other genomic functional elements overlapping with the

introns. These findings can not only provide some clues to find new genomic functional elements -- in the areas overlapping with introns, but suggest that “functional constraint” of introns may not be necessarily directly associated with intron loss and gain, or that the real functions or the way of functioning of introns are probably still outside of our current knowledge.

Keywords: Evolutionary retention of introns, Gene overlap, Intron evolution, Genome evolution, *Giardia lamblia*.

Background

Spliceosomal introns are a common feature of all eukaryotic nuclear genomes, but their number and density in a genome vary dramatically among different species [1, 2], ranging from less than 0.5 intron/gene in some protists such as the Microsporidian *Encephalitozoon* species [3] and *Cyanidioschyzon merolae* [4] to over 18 per gene in *Symbiodinium minutum* [5] (even larger than those of most mammals, which is generally over eight/gene [6]). Accumulating evidence suggests that the LECA and early eukaryotes were relatively intron rich, with subsequent genome evolution dominated by intron loss, and thus those contemporary eukaryotes with remarkably few introns must have experienced massive intron loss secondarily [7-9]. But, interestingly, no eukaryotes with sequenced genomes so far have been found to have completely lost their introns except the two Microsporidia species, *Nematocida parisii* and *Nematocida* sp1[10].

Unfortunately why introns were lost, especially massively lost in some eukaryotes,

remains obscure despite extensive research [11, 12]. Obviously, the reverse question -- how introns, especially the few ones in intron-poor eukaryotes, can be retained under the pressure of loss is equally important, but it was rarely carefully studied. Although some authors thought that the reason for the retention of introns in genomes is likely due to the essential functions of these introns [13-15], this ‘functional constraint’ scenario – “only the introns with important functions can get rid of the fate of being lost” lacks evidence that the lost introns are all useless or less useful than the retained ones in any eukaryotes, and moreover, actually the functions of introns are still far from being well understood [16]. Therefore, the investigation of the evolutionary retention of intron might be helpful not only to answering the question about intron loss but also to understanding the function and evolution of introns.

Giardia lamblia is a parasitic protozoan belonging to Diplomonadida (Excavata). It has a very minimalistic genome, compact in structure and content [17], and only eight spliceosomal introns were found in its genome [17-22]. Thus it can be speculated that this organism must have undergone massive intron loss, with very few left in the genome. Therefore, this organism may provide an excellent opportunity for exploring how the few introns were retained. In the present work, by investigating the intron-containing genes and the few introns of *Giardia*, besides finding the distribution patterns that can reflect the dynamic evolution of intron in *Giardia*, we observed and experimentally confirmed an interesting phenomenon that sense-antisense (SAS) gene overlaps appear in the areas of some introns, and thus “overlap constraint” might be at least one of the causes for preventing introns from

94 being lost, though it is uncertain whether the other retained introns also overlap with
 95 any unknown genomic functional elements yet. The implications of these findings for
 96 intron evolution and function are discussed.

97

98 **Results**

99 **Characteristics of the intron-containing genes and their introns in *Giardia*** 100 **genomes**

101 In the genome database of *Giardia*, GiardiaDB, four of the eight *Giardia*
 102 intron-containing genes are annotated to code proteins with sequence similarity to
 103 known proteins, and the other four to code hypothetical proteins. Our investigation
 104 (mainly by sequence comparative analysis) indicated that: 1) the former four
 105 intron-containing genes are all common eukaryotic-conserved genes, which are most
 106 likely vertically inherited from the LECA and thus are very ‘ancient’, while the latter
 107 four are all *Giardia*-specific genes (not found in other eukaryotes including other
 108 Excavata species), which thus most likely emerged after the divergence of *Giardia*
 109 from other Excavata and thus are ‘relatively new’ genes compared with the ‘ancient’
 110 ones above; 2) the introns in the three (GL50803-15604, GL50803-15124,
 111 GL50803-17244) of the four ancient genes are eukaryotic-conserved (Additional file
 112 1), and thus they are ‘ancient’ introns in ancient genes, while the intron in the other
 113 one ancient gene (GL50803-27266) is a *Giardia*-specific intron (not found in other
 114 eukaryotes including other Excavata species), and thus this intron most likely
 115 emerged after the divergence of *Giardia* from other Excavata and is a ‘relatively new’

116 intron in an ancient gene; 3) all four *Giardia*-specific ('relatively new')
 117 intron-containing genes (GL50803-37070, GL50803-35332, GL50803-15525 and
 118 GL50803- 86945), which account for only about 0.6 percent of all the ~700
 119 *Giardia*-specific protein-coding genes in the genome[17], each contain an
 120 *Giardia*-specific intron (not found in other eukaryotes including other Excavata
 121 species), and thus the four introns all are 'relatively new' introns in 'relatively new'
 122 genes (Table 1).

123 These observations suggest that: 1) while *Giardia* massively lost its introns, new
 124 introns also arose in both the ancient and relatively newly evolved genes; 2) the
 125 pressure of intron loss seems to constantly exist in the whole evolutionary process of
 126 *Giardia*, but only a few of both the ancient and newly-emerged introns have been
 127 retained in the genome.

128 To find the reason why these few introns can be retained in *Giardia* genome under
 129 strong pressure of loss, we investigated the characteristics of these introns in many
 130 aspects. It had been shown that seven of the eight *Giardia* introns are bounded by
 131 canonical GT-AG splice signals, only one, the [2Fe-2S] ferredoxin (GL50803-27266)
 132 intron, has a noncanonical splice signal CT-AG [19]. The sizes of the eight introns are
 133 all small (most of them are less than 40 bp long and are not the multiple of three) and
 134 do not have any conserved sequence motifs. Our further analysis predicted no special
 135 secondary structures that would be able to form in these introns. Besides, our survey
 136 also showed that there were not any reports about alternative splicing of the two
 137 introns in genes GL50803-15525 and GL50803-86945 [18, 22]. Taken together, these

138 results suggest that the retention of the few introns seems to be neither due to the
139 structural features nor necessarily due to the functional importance of these introns.

140 Interestingly, on the complementary strands, we found that two intron-containing
141 genes, GL50803-17244 (ribosomal protein L7a gene) and GL50803-37070 (a
142 “hypothetical protein” gene), each have an antisense gene, GL50803-20429 and
143 GL50803-28204, which are just annotated as “hypothetical protein” and “unspecified
144 product” in the genome database, respectively. That is, the two intron-containing
145 genes and their antisense gene form SAS gene pairs. We thought this phenomenon
146 might be related to the intron retention. Nevertheless, the two anti-sense genes need to
147 be further verified, and the details of the overlaps with their sense genes also need to
148 be analyzed in detail.

149 **Verification of the antisense genes**

150 The strand-specific RT-PCR of the two antisense genes, GL50803-28204 and
151 GL50803-20429, generated two products with the expected lengths of 172 and 288 bp,
152 respectively. The sequencing further confirmed that the two products just seem to be
153 transcribed from the opposite direction of the two sense (intron-containing) genes,
154 GL50803-37070 and GL50803-17244, respectively, and the two introns are just
155 located within the two overlapping regions of the two SAS gene pairs, respectively
156 (Figure 1).

157 The RACE of the two antisense genes generated a 1232 bp product for
158 GL50803-28204 and a 1177 bp product for GL50803-20429. After sequencing and
159 comparing with their corresponding genomic DNA sequences in the GiardiaDB

160 database, we found the two antisense genes contain no introns, especially in the
161 regions corresponding to the two introns of the sense genes. But the software ORF
162 Finder predicted that the largest ORFs of the two antisense genes are only 264 bp for
163 GL50803-28204 and 363 bp for GL50803-20429, and moreover, no proteins
164 homologous to the putative proteins coded by the two largest ORFs could be found in
165 other organisms including *Giardia*'s close relative *Spironucleus* in GenBank.
166 Therefore, the two antisense genes have transcriptional activity and are most likely
167 *Giardia*-specific non-coding genes.

168 Discussion

169 To investigate the reasons for the evolutionary retention of the few introns in the
170 eukaryotes having undergone massive intron loss, we chose the extremely intron-poor
171 eukaryote *Giardia* as the study object. When investigating the characteristics
172 (including distribution patterns) of the eight intron-containing genes and their
173 corresponding introns in *Giardia* genome, we found that in spite of the massive loss
174 of introns, intron gain also occurred in both *Giardia*'s ancient and relatively
175 newly-evolved genes; it turns out that the selective pressure of intron loss seems to
176 constantly exist in the whole evolutionary course of *Giardia*, but a few of both the
177 ancient and the newly-emerged introns have been retained in modern *Giardia*.

178 To explore how these few introns can be retained under the constant strong pressure
179 of loss. First, we investigated whether there exist some features in these few
180 intron-containing genes and the introns probably responsible for the retention, but
181 failed to find any special regularities or unusualnesses in many aspects such as

182 splicing signal, intron size and secondary structure, and alternative splicing. This
183 suggests that there are neither any special structural features nor necessarily any
184 functional importance of introns responsible for the intron retention. This is consistent
185 with the fact that so many introns, at least part of which definitely possesses important
186 functions, have been lost in intron-poor eukaryotes like *Giardia*. Thus, the reasons for
187 the retention might lie outside the intron-containing genes and the introns themselves.

188 Interestingly, we noticed that on the complementary strands of two of the eight
189 intron-containing genes, GL50803-37070 and GL50803-17244, there exist
190 correspondingly two anti-sense genes, GL50803-28204 and GL50803-20429, though
191 they are just annotated as “product unknown” in GiardiaDB. By strand-specific
192 RT-PCR, RACE and sequencing, we got the transcripts and sequences of the two
193 genes and found they both have no introns. Thus the two anti-sense genes have been
194 verified to be really genes that are actively transcribed. And actually the anti-sense
195 gene GL50803-20429 has been reported to be a mRNA gene being expressed during
196 excystation and encystation, and in trophozoites but not cysts [23]. As for the other
197 anti-sense gene GL50803-28204, it has a quite short putative ORF but has no
198 homologs in other organisms including *Spironucleus*. Although the corresponding
199 DNA sequence regions in the four other *G. lamblia* isolates (DH, P15, GS and GS-B)
200 with genomic data exhibit significant similarities ($\geq 83.9\%$ similarity) to those of the
201 two anti-sense genes, there are not any annotations and transcriptome information
202 about those regions. Besides, the total RNA were processed using Poly(A)
203 Polymerase to add a poly(A) tail at the 3'ends before we performed rapid

204 amplification of their cDNA 3'ends, thus from the experiment we still did not know
205 whether the transcripts of GL50803-28204 are polyadenylated or not, namely, mRNA
206 or not. Therefore, we can only conjecture that the two anti-sense genes are either
207 non-protein-coding genes or *Giardia*-specific protein-coding genes. Considering that
208 many identified non-coding RNAs in *Giardia* overlap with protein-coding genes on
209 the antisense strands [24], the antisense gene might also be noncoding RNA gene. But
210 there is still no tangible evidence for what the two genes are despite our lots of
211 experimental efforts (not shown) to identify them. Whatever the antisense genes code
212 for, our work showed that they are functional genes and form SAS gene overlap with
213 their intron-containing sense genes, and that the introns just reside in the overlap
214 regions. Considering that the gene sequence mutation (especially deletion) cannot
215 occur randomly, the antisense genes must have imposed the restriction of variation
216 (especially deletion) on the introns of the sense genes in the overlapping areas, and
217 thus such a kind of SAS gene overlap must have prevented the introns from being
218 lost.

219 As for the other six introns, we did not find any ORFs on their corresponding
220 complementary strands. Although we also experimentally examined whether their
221 complementary strands (especially the areas overlapping with these introns) are
222 transcribed, no transcripts were found (Additional file 2). Nevertheless, it is uncertain
223 whether the corresponding complementary strands of these introns are resided by
224 some unknown genomic functional elements which are not transcribed at all. If this is
225 true, these introns are also retained by the same cause as the former two ones.

226 Certainly, it is also possible that the six introns are retained by other unknown reasons.
 227 We have also analyzed the intron regions of many intron-poor eukaryotes including
 228 *Microsporidia*, *Trichomonas*, *Spironucleus*, but unfortunately did not find such
 229 sense-antisense gene overlaps as in *Giardia* (data not shown). But considering some
 230 genes overlap with the UTRs of the adjacent genes (as found in *Antonospora locustae*
 231 and *Encephalitozoon cuniculi* [25]), we can not obtain the UTRs information of those
 232 genes in current database, thus many of the overlaps might be able to be found out.
 233 More importantly, some introns might overlap with unknown genomic functional
 234 elements including non-coding and non-transcribed ones, since there are so huge
 235 remaining component of eukaryotic genomes, much of which was traditionally
 236 regarded as "junk" and is still undetermined. This might be the important cause for
 237 that few SAS gene overlaps in intron regions that can be identified at present.

238 Theoretically, overlapping with any genomic functional elements on either the
 239 same strand or the complementary one (namely, either same-strand overlapping or
 240 different-strand overlapping) can result in intron retention, as long as the introns are
 241 just in the overlapping areas. Therefore, since such overlapping structures are widely
 242 distributed in eukaryotes [26], it can be expected that quite a number of introns in
 243 diverse eukaryotes may also be retained due to this kind of "overlap constraint". We
 244 believe that more and more examples might be able to be found in diverse eukaryotes
 245 in the future. Conversely, such an intron retention phenomenon probably can provide
 246 a valuable clue to find new genomic functional elements – in the overlapping area
 247 with introns.

248 **Conclusions**

249 In conclusion, by investigating the extremely intron-poor eukaryote *Giardia*, we
 250 have revealed some interesting findings about the dynamic evolution of introns in the
 251 intron-poor eukaryotes: the pressure of intron loss may constantly exist in these
 252 eukaryotes, but new introns can still arise either in ancient genes or new-evolved
 253 genes, but only a few introns can be retained in the genome; the retention of the few
 254 introns is not caused by special features or functional constraint of the introns
 255 themselves but due to the reasons outside of the introns, and “overlap constraint”
 256 imposed by other genomic functional elements overlapping with the introns is at least
 257 an important one of the causes. First, our findings not only support the “intron-rich
 258 ancestor” theory, but also can explain why few eukaryotes were found to be
 259 completely intronless. Second, our finding may conversely provide a clue to find new
 260 genomic functional elements (which was probably traditionally regarded as "junk"
 261 and is still undetermined) in such kinds of overlap regions. Most importantly, our
 262 work implicates that “functional constraint” of introns is not necessarily directly
 263 associated with intron loss and gain, or that the real functions or the way of
 264 functioning of introns are probably still outside of our current knowledge. Therefore
 265 our work may be able to shed some new lights on the research of evolution and
 266 function of introns and genomes.

267 **Methods**

268 **Database and bioinformatics methods**

269 The template sequences for designing primers of *Giardia* genes were downloaded

from GiardiaDB (December 1, 2017 release) [27]. The software ORF Finder were used to predict the ORFs of the RACE products (see blow), then the predicted ORFs were used as queries to search their homologs with Blastp against the NCBI non-redundant protein sequences (nr) database. The program RNAfold web server was used to analyze the secondary structure of introns. The sequences of the four genes and their coding proteins, 2Fe-2S ferredoxin, 26S proteasome non-ATPase regulatory subunit 4, Dynein light chain, and Ribosomal Protein L7A from other organisms were identified and collected by Blastp searching against GenBank with *Giardia*'s corresponding sequences as queries. Protein alignments were generated with ClustalX 2 applying default alignment parameters. The introns in the genes were determined by comparing cDNA and gene sequences. The other four intron-containing genes with annotated as hypothetical protein also identified by sequence comparative analysis to determine whether they are *Giardia*-specific or not.

***Giardia* cultures**

The cell line of WB isolate (assemblage A), namely WB clone C6 (ATCC 50803), was used in the study. Its cultures were routinely grown in filter-sterilized TYI-S-33 medium supplemented with bovine bile in glass screw cap tubes at 37 °C and were sub-cultured every 3 to 4 days.

RNA extraction

Giardia total RNA was extracted and treated to remove any contaminated genomic DNA by RNAPrep Pure Cell/Bacteria Kit (TIANGEN) using about 5×10^6 *Giardia* trophozoites that were harvested by ice-slush incubation and centrifuged at 6000g for

292 5 min according to the manufacturer's instructions. The quality and quantity of the
293 RNA preparation were assessed with agarose gel electrophoresis and the absorption at
294 260 and 280nm.

295 **Strand-specific RT-PCR**

296 First-strand cDNAs of the two antisense genes, GL50803-28204 and
297 GL50803-20429, were synthesized from 500ng DNase-I treated total RNA per
298 reaction at 54°C 30 min, 99°C 5 min, and 5°C 5 min with the specific antisense
299 primer 4C and 9C respectively instead of the reverse primers in the kit, and then
300 amplified with specific primers pairs of 4A/4S and 9A/9S by using RNA PCR Kit
301 (AMV) Ver.3.0 (Takara, Japan). The PCR conditions were as follows: 94°C for 30 s,
302 followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45s. The PCR
303 products were purified using the Wizard SV Gel and PCR Clean-Up System kit
304 (Qiagen, Germany), and cloned into pMD-19T vectors from 35 ng purified PCR
305 products using TaKaRa pMD-19T Vector Cloning Kit (TaKaRa, Japan) according to
306 the manufacturer's instructions. Then, the ligation products were transformed into
307 DH5α Chemically Competent *E. coli*. Colony PCR with vector-specific primers
308 provided in the kit was adopted to select colonies. These selected colonies were
309 sequenced using vector-specific forward and reverse primers by Sangon Biology
310 Company (Shanghai, China).

311 **Rapid amplification of cDNA ends**

312 The total RNA were processed using Poly(A) Polymerase(TaKaRa, Japan) to add a
313 poly(A) tail at the 3'ends of the RNA before performing rapid amplification of their

314 cDNA 3'ends. We experimentally determined the 3'ends by using nested PCR primer
315 (3R4O/3R4I and 3R9O/3R9I) according to the RNA PCR Kit (AMV) Ver.3.0 (Takara,
316 Japan). 5'-RACE was performed by using a SMARTer RACE 5'/3'Kit (TaKaRa, Japan)
317 with 500ng total RNA as the template and the gene-specific 5'-RACE primers 5R4
318 and 5R9 for the two antisense genes, GL50803-28204 and GL50803-20429,
319 according to the manufacturer's instructions. Both the 3'-RACE and 5'-RACE primers
320 (Additional file 3) were designed based on the transcripts from the Strand-specific
321 RT-PCR. The RACE-PCR products were analyzed by agarose gel electrophoresis and
322 sequenced as described above.

323 **List of abbreviations**

324 LECA: last eukaryotic common ancestor.

325 SAS: sense-antisense.

326 RACE: rapid amplification of cDNA ends.

327 **Declarations**

328 **Ethics approval and consent to participate**

329 Not applicable

330 **Consent for publication**

331 Not applicable

332 **Availability of data and materials**

333 All data generated or analysed during this study are included in this published
334 article and its supplementary information files.

335 **Competing interests**

336 The authors declare that they have no competing interests.

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341 **Authors' contributions**

342 J.W. designed and supervised this study. M.X performed genetic characterization
343 work. M.X., B.C., Q.Y., J.S., and Z.L. analyzed the data. M.X. and J.W. wrote the
344 manuscript. All authors read and approved the final manuscript.

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424

425 **Table 1. The integrated information of the eight spliceosome-mediated cis-spliced**
426 **introns and their host genes and complementary strands.**

Intron-containing gene			Intron		Complementary strand
Gene ID	Product	Age	Age	Size (bp)	
GL50803-27266	2Fe-2S ferredoxin	ancient	relatively new	35	No ORF, no transcripts detected
GL50803-15604	26S proteasome non-ATPase regulatory subunit 4		ancient	29	No ORF, no transcripts detected
GL50803-15124	Dynein light chain			32	No ORF, no transcripts detected
GL50803-17244	Ribosomal Protein L7A			109	Antisense gene with transcripts
GL50803-37070	Hypothetical protein	relatively new	relatively new	41	Antisense gene with transcripts
GL50803-35332	Hypothetical protein			220	No ORF, no transcripts detected
GL50803-15525	Hypothetical protein			33	No ORF, no transcripts detected
GL50803-86945	Hypothetical protein			36	No ORF, no transcripts detected

427

428 Figure Captions

429 **Fig 1. Results of strand-specific RT-PCR and sequencing of the two antisense**
430 **genes, and the schematic diagram of this two SAS gene pairs.**

431 **A.** Lane 1, Strand-specific RT-PCR product of the GL50803-20429; Lane 4, the Strand-specific
432 RT-PCR product of the GL50803-28204; Lane 2 and lane 3, negative controls (with no RTase)
433 corresponding to lane 1 and lane 4, respectively; M, molecular markers. **B.** Nucleotide sequence of
434 GL50803-28204 gene acquired by Strand-specific RT-PCR and sequencing. The locations of the
435 primers are underlined. **C.** Nucleotide sequence of GL50803-20429 gene acquired by Strand-
436 specific RT-PCR and sequencing. **D.** Schematic diagram of the two SAS gene pairs. The sequence
437 lengths of GL50803-28204 and GL50803-20429 are according to the Strand-specific RT-PCR
438 products, and the lengths of GL50803-37070 and GL50803-17244 are based on the GiardiaDB

439 database. Arrow represents the orientation of transcription; and the dashed box and solid lines

440 represent introns and exons, respectively.

441

442 **Additional files**

443 **Additional file 1:** The conservative analysis of Giardia's introns among diverse

444 eukaryotes

445 **Additional file 2:** Results of strand-specific RT-PCR of the complementary areas of

446 the other six introns of *G. lamblia*.

447 **Additional file 3:** The primers designed for the strand-specific RT-PCR and RACE of

448 the complementary areas of the eight introns of *G. lamblia*.

A **B****GL50803-28204:**

ATGCCGATAAAGATAAAGGACGCGCGAAAG
 ACGCTCTTGGGGGAGATGCGTGCAACGGGA
 GCGTGCTTCTCTGTCTTTCTGCGTGTTAGACG
 TCGGGTGGAACGAGCAATGAAACATACGCCA
 TCTCTGGATTTTTTTCTTTGGCGGCTTTATGG
CAGGCTACCGATTACC

C**GL50803-20429:**

AAAGGTGGGCTTGTCCTCTGGGTTCACGTCGGTGAAGCAGACGCTGGTCGTCTTCTTC
 AGATGGACGAGCTTGCCCAGATCGCCCTTAGTGCGAACGATGGCGTACGGGACGCCC
 ATCTTGTGACAGAGTGTGGGAAGCCAAAGTACGAGCTGTGGGTCA GTTGTCAGGTGA
 ACAGCGAAGTCCACCCGCTGACAACACACAACCCGCAATCAGAGGTGTGTGCGGTCA
 CGGACGGCTCCTCGCGCATAAGAACATACTTCAAGGGGGTTCGACATCATTGCAATCA

D**GL50803-37070:** **GL50803-28204:** **GL50803-17244:** **GL50803-20429:**