Gene identification and genome annotation in *Caenorhabditis briggsae* by high throughput 5' RNA end determination

Nikita Jhaveri[1$], Wouter van den Berg[1$], Byung Joon Hwang[2,3], Hans-Michael Muller[2], Paul W. Sternberg[2], Bhagwati P. Gupta[1*]

[1]Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada

[2]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, California 91125, USA.

[3]Department of Molecular Bioscience, College of Biomedical Science, Kangwon National University, Chuncheon, South Korea.

Keywords: Nematode, *C. briggsae*, Trans-splicing, Spliced leader, Operons, Paralog, Genome annotation

ORCIDs:
Bhagwati P. Gupta 0000-0001-8572-7054
Paul W. Sternberg 0000-0002-7699-0173

[*]Author for correspondence. Email: guptab@mcmaster.ca; Phone: +1-905-525-9140 x26451.
[$]Equal first authors

ABSTRACT

The nematode *Caenorhabditis briggsae* is routinely used in comparative and evolutionary studies involving its well-known cousin *C. elegans*. The *C. briggsae* genome sequence has accelerated research by facilitating the generation of new resources, tools, and functional studies of genes. While substantial progress has been made in predicting genes and start sites, experimental evidence is still lacking in many cases. Here, we report an improved annotation of the *C. briggsae* genome using the Trans-spliced Exon Coupled RNA End Determination (TEC-RED) technique. In addition to identifying 5' ends of expressed genes, the technique has enabled the discovery of operons and paralogs. Application of TEC-RED yielded 10,243 unique 5' end sequences with matches in the *C. briggsae* genome. Of these, 6,395 were found to represent 4,252 unique genes along with 362 paralogs and 52 previously unknown exons. The method also identified 493 operons, including 334 that are fully supported by tags. Additionally, two SL1-type operons were discovered. Comparisons with *C. elegans* revealed that 40% of operons are conserved. Further, we identified 73 novel operons, including 12 that entirely lack orthologs in *C. elegans*. Among other results, we found that 14 genes are trans-spliced exclusively in *C. briggsae* compared with *C. elegans*. Altogether, the data presented here serves as a rich resource to aid biological studies involving *C. briggsae*. Additionally, this work demonstrates the use of TEC-RED for the first time in a non-*elegans* nematode and suggests that it could apply to other organisms with a trans-splicing reaction from spliced leader RNA.

INTRODUCTION

Nematodes are a mainstay in fundamental biological research. While most work has been based on *C. elegans* over the last half a century since its proposed role as a model organism (Brenner, 1974), the close relative *C. briggsae* offers many of the same advantages in carrying out studies. Despite diverging roughly 20-30 million years ago (Cutter, 2008), the two species exhibit similar behavioral, developmental, and morphological processes including a hermaphroditic mode of reproduction (Gupta et al., 2007). Moreover, many of the experimental techniques and protocols developed to manipulate *C. elegans* can be adopted to *C. briggsae* with minimal to no modification (Baird & Chamberlin, 2006; Gupta et al., 2007). These features make *C. briggsae* - *C. elegans* an ideal pair for comparative and evolutionary studies.

The genome of *C. briggsae* was sequenced many years ago and revealed extensive genomic and genic conservation (Stein et al., 2003). Subsequent work reported the assembly of genomic fragments into chromosomes and improved gene predictions (Hillier et al., 2007; Ross et al., 2011). While a diverse array of techniques have been applied to improve the annotation of the *C. elegans* genome (Allen et al., 2011; Hillier et al., 2009; Hwang et al., 2004; Salehi-Ashtiani et al., 2009; Spieth & Lawson, 2006). A similar approach is lacking for *C. briggsae*. The current *C. briggsae* genome annotation is largely based on homology with the *C. elegans* genome. More analysis that uses experimental data gathered directly from *C. briggsae* is needed to improve gene identification and gene models. To this end, we used trans-spliced exon coupled RNA end determination (TEC-RED) (Hwang et al., 2004), a technique based on exploiting the phenomenon of spliced leader (SL) trans-splicing which has been observed in nematodes and several other phyla including platyhelminths, chordates and trypanosomes (Lasda & Blumenthal, 2011).

The advantage of TEC-RED compared to other genome annotation techniques like EST (Marra et al., 1998) and SAGE (Velculescu et al., 1995) is that it is capable of identifying transcripts of most expressed genes, and uniquely allows for the identification of 5' transcript start sites and alternative transcripts with different 5' ends of a gene. The approach is based on two principles: one, a short sequence from the 5' end of a transcript can be used to uniquely identify the initiation site of the transcript, and two, the 5' ends of mRNAs are spliced to common leader sequences known as spliced leader (SL) sequences. The SL trans-splicing process involves replacing the outron of a pre-mRNA with a 22 nucleotide SL sequence donated by a 100-nucleotide small ribonucleoprotein (snRNP) (Allen et al., 2011; Blumenthal, 2005). *C. elegans* and *C. briggsae* both have two types of spliced leader sequences: SL1 and SL2 (Blumenthal et al., 2015; Qian & Zhang, 2008).

We recovered well over 120,000 5' end tags from sequencing reactions representing 10,243 unique ones (7,234 for SL1; 3,009 for SL2) with matches in the *C. briggsae* genome. The tags were analyzed using WormBase release WS276 and it was found that more than 60% could be aligned to exons curated in WormBase (*www.wormbase.org*). Most of the tags were found to have unique hits in the genome and identified a total of 4,252 genes. The remainder identified 52 novel exons and 362 paralog genes. The novel exons could either represent previously unknown genes or new exons of existing genes. The paralogs define 133 sets of two or more genes. Of these sets, 21 were confirmed as exact matches with known paralogs in WormBase. The rest could potentially be new paralogous pairs that need further validation. While the majority of the genes discovered by tags confirmed 5' ends of genes listed in WormBase, there are many for which 5' ends indicated by tags differ from current gene models, suggesting the need to revise existing annotations.

A comparison of the splicing pattern of *C. briggsae* genes with *C. elegans* revealed some changes. Specifically, 14 genes are spliced to leader sequences in *C. briggsae* but their *C. elegans* orthologs lack such splicing information. We also investigated the presence of operons. It was reported earlier that 96% of *C. elegans* operons are conserved in *C. briggsae* based on collinearity (Stein et al., 2003). Our analysis revealed a total of 1,199 operons including 493 for which splicing identities of two or more genes are reported in this study. Of these operons, 334 are fully supported by tags. Comparison of the latter with *C. elegans* revealed that 39% are conserved, the largest of which is composed of seven genes. Another 21% of tag-supported operons in our dataset are novel, i.e. consisting of divergent genes as well as genes whose *C. elegans* orthologs are not reported in operons. The remaining 40% are termed partially conserved since gene sets do not fully correspond to any of the operons in *C. elegans*. Lastly, two SL1-type operons have been identified.

Overall, the results presented in this paper have substantially improved the annotation of the *C. briggsae* genome by identifying the 5' ends of a large number of genes as well as discovering many novel genes, operons and paralogs. The findings serve as a platform to facilitate comparative and evolutionary studies involving nematodes as well as other organisms.

MATERIALS AND METHODS

Generation of tags

The detailed protocol to obtain *C. elegans* tags was described earlier (Hwang et al., 2004). We followed the same steps for *C. briggsae*. Briefly, the steps of TEC-RED involved purification of poly(A) RNA from the wild type *AF16* mixed stage animals, RT-PCR to generate cDNA, amplification of cDNAs using biotin-attached primers homologous to SL1 and SL2 sequences that carry mismatches to create *Bpm*I restriction enzyme site (see Supplementary Tables 1-3), digestion of amplified cDNAs using *Bpm*I to produce short fragments (termed “5’ tags”), ligation of tags to adaptor DNA sequences, and sequential ligation of DNA to create concatenated products. The ligated DNA pieces are finally cloned into a vector and sequenced. Sequencing was carried out at The University of British Columbia (Vancouver) facility.

5' Tag Sequence Analysis and exon identification

A set of custom Perl scripts were used to analyze the tags and genes. A flowchart is provided in Supplementary figure 1. Briefly, tags were collected and assigned a unique tag ID. Tag locations in the genome were determined by comparing the tag sequence to WS176 and WS276 genome files, where orientation and chromosome location for each tag was noted. Subsequently the splice sequence for each tag was obtained by finding the first 7 bases upstream at each location where the tag matched on the genome.

We used the criteria described in Hwang et al. (Hwang et al., 2004) to identify tag matches to exonic regions. These included ‘same orientation of the tag as that of the corresponding exon’, ‘distance to the first ATG’, ‘a minimum distance to the nearest in-frame stop codon’ and ‘presence of a splice acceptor sequence following the tag’. The latter was scored on how well they fit the consensus splice site TTTTCAG (Blumenthal & Steward, 1997). In cases where tags had multiple matches, we applied stricter splice acceptor site criteria. Perfect consensus sequence was given the highest weight. Sites having mismatches were assigned lower weights with priority given to bases that were most conserved in the splicing consensus sequence. While this approach resulted in most tags identifying unique exons, a small number still showed multiple matches and were used to analyze potential paralogs (see below).

Each tag was used to find the nearest ATG of an open reading frame (ORF), i.e, the proposed start of a coding sequence (CDS). This ATG location was compared to known coordinates of start sites of nearest exons as annotated in WS176 and WS276 genome annotation (gff3) files. This was done using coordinates of annotated CDS. Two broad categories of exon matches were identified based on tags that had unique matches: one, where the 5' end corresponded to the start of a known exon (first exon: 1a, internal exon: 1b) and two, matches for which the 5' end differed from a nearest exon (Figure 1). Depending on the distance between the 5' end and the exon, the second category of matches were further divided into two sub-categories. These consisted of exons that were either within 20 bp from the 5' end ('minor misprediction') or further away ('major misprediction'). The major misprediction sub-category also includes matches where 5' ends were more than 3 kb away and may define brand new exons of existing genes as well as potentially new, previously unknown genes.

Manual curation of genes

We found that 75 tag-matched genomic regions in the WS276 gff3 file had no known genes/exons within 3kb downstream of the matched ATG. The surrounding chromosomal regions of these matches were searched manually in the WormBase genome browser for presence of annotated exons. Of the 75, 21 were false positives due to incorrect script calls. Two were excluded from analysis because the genes are not assigned to any chromosomes. The remaining 52 matches may represent novel exons.

Analysis of intergenic regions and operons

The intergenic regions (IGRs) were determined based on the distance from the end of the 3' UTR of the upstream gene to the 5' CDS start of the nearest downstream gene. Graphs were generated using Graphpad Prism 7.0 and Microsoft Excel. Genes having IGR >5000 bp (257) were excluded from the analysis. For pairs of genes where the second gene is located within the first gene, IGR length is calculated as a negative value.

To identify genes that could be present in operons, all genes trans-spliced with SL2 or SL1/SL2 and present downstream of an SL1-spliced gene were categorized into a single operon model along with the upstream SL1 spliced gene. If the splicing of the first upstream gene was unknown, the operon models were termed 'non-tag supported' whereas those models in which the identity of the first upstream gene was known were termed 'tag supported'. We compared the 'tag supported' operon models to those in *C. elegans* (Wormbase) to determine how well operons are conserved. Based on the conservation of genes, the identified operons were classified into Exact match, Partial match, and Novel.

We examined the enrichment of germline genes in *C. briggsae* high confidence operons. For this, *C. elegans* orthologs were identified and searched for association with germline function (Wang et al., 2009). The significance of overlap was tested by the hypergeometric probability test. Next, to identify processes related to genes in operons, we carried out Gene Ontology (GO) (Ashburner et al., 2000) for all operon genes. We also conducted a similar analysis for genes present in *C. elegans* operons. This information was retrieved from the Allen et al. (Allen et al., 2011) data set.

Paralog analysis

Tags that had multiple hits in the genome were used to generate a list of predicted paralogs, which were then compared with those annotated in WormBase. This allowed us to classify the predicted paralogs into three categories: Exact match, Partial match, or No match.

Uniquely spliced C. briggsae genes

To identify genes that are uniquely spliced in *C. briggsae*, we used the *C. elegans* orthologs to compare with data reported previously by two groups that together constitute the most complete collection of genes trans-spliced in *C. elegans* (Allen et al., 2011; Tourasse et al., 2017). Initial comparisons with Allen et al. dataset revealed 198 genes that are present only in our analysis. The number was further reduced to 14 genes when compared with Tourasse et al. study (Supplementary data file 3).

RESULTS

Overview of the TEC-RED method in *C. briggsae*

To implement the TEC-RED approach to identify transcripts, we first isolated *C. briggsae* mRNAs containing an SL1 or SL2 sequence at their 5′ ends. A total of 121,189 5′ tags (91,733 for mRNA with an SL1 and 29,456 for mRNA with an SL2 spliced leader sequence) were recovered from DNA sequencing reactions. These tags represent almost fifteen thousand different sequences, of which 10,400 (71%) are for SL1 and 4,278 (29%) for SL2 sites. More than two-thirds of all tags found matches in the genome (10,243, 70%), of which 46% are unique, i.e., matching only once and others matching multiple times (Table 1). The proportions were similar for both spliced leader categories, demonstrating no bias in the experimental protocol. The remaining 4,434 tags (30%) had no match, likely due to reasons such as sequencing errors, gaps in the genome sequence, and incorrect sequence assembly.

Exon validations and predictions in *C. briggsae* based on 5' tag matches

After filtering the matches (see Methods), 62.5% of all tags (6,395 of 10,243) were retained for further analysis. Next, we determined the locations of these tags relative to annotated exons in Wormbase. Most of the tags (6,192, 96.8%) matched uniquely to one exon, with a small number having multiple matches (203, 3.2%) (Table 2, Supplementary data file 1). For both SL1 and SL2 tags, roughly 80% of the matches correspond to known 5' ends of annotated genes (Category 1a), providing support to existing gene models in Wormbase. Less than one percent of the tags matched to internal exons (Category 1b), suggesting an alternate 5' end of the corresponding genes. The remaining tags identified start sites that differed from current Wormbase gene models and were categorized as mispredicted genes. In most of these cases (roughly three-quarters of all mispredictions) the nearest exon was more than 20 bp away. This leads us to suggest that, particularly in these cases, existing gene models may need to be revised. These exons may define new 5' ends of known genes as well as novel, previously unidentified genes. More experiments are needed to investigate these possibilities. As expected, both types of tags, i.e., with unique and multiple hits have a roughly similar distribution of categories (Figure 2, Supplementary data file 1).

Identification of genes based on tag matches

Next, we compiled a list of *C. briggsae* genes consisting of tag-identified exons. Excluding potential paralogous pairs and cases where 5' ends did not match with any of the exons of known genes, a total of 4,252 unique genes were recovered by SL1 and SL2 tags (Supplementary data file 2). Almost two-thirds of the genes (65%) are spliced with SL1 (Table 3; Supplementary data file 2) and 18% with SL2. Another 18% of exons matched with both SL1 and SL2 tags (SL1/SL2), suggesting the genes are part of hybrid operons (Allen et al., 2011).

The genomic locations of genes revealed roughly even distribution on chromosomes except for V and X. Gene count was highest on V and lowest on X. However, the trend was different for gene density with III being the densest chromosome and X the sparsest (Supplementary table 4) Whether the uneven distribution is by chance or a characteristic of trans-spliced genes in *C. briggsae* remains to be seen. A tiny fraction of genes (0.1%) is located in unmapped genomic fragments.

Almost 95% of the curated genes identified by tags (4,025 of 4,252) are associated with unique tag sequences, i.e., 5' ends matched to just one exon, providing support for the presence of a single transcript for these genes (Table 4). In the majority of cases (82%, 3,290 of 4,025), the tag-identified 5' ends matched with a known first exon (category 1a tags). Less than one percent of the tags identify 5' ends that match with internal exons (category 1b). The remaining genes (18%) consist of exons belonging to minor and major misprediction categories.

The rest of the genes (5%, 227 of 4,252) identified by tags consist of those that produce multiple transcripts (Table 5). In 84% of these cases, at least one 5' end identified by tags matched with the first exon (category 1a). Five of the genes were alternatively spliced using internal exons as the 5' start site (category 1b). Most of the genes consisted of at least one major mispredicted exon, suggesting that genes with multiple splice variants require further validation.

The identification of *C. briggsae* genes prompted us to examine evolutionary changes in trans-splicing. A comparison with *C. elegans* studies (Allen et al., 2011; Tourasse et al., 2017) revealed 14 genes that appear to be uniquely spliced to leader sequences in *C. briggsae* but not in *C. elegans* (Supplementary data file 3).

Validations of TEC-RED-identified transcripts

We took three different approaches to validate subsets of TEC-RED predictions with the goal of demonstrating the usefulness of the technique in improving gene identification and gene models.

One approach involved comparing different categories of tag-identified exons between two WormBase releases. As described above, a significant number of exons are categorized as minor and major mispredictions (22%, 943 of 4,252; see Tables 4 and 5). We hypothesized that mispredicted exons may be confirmed with improvements in genome annotation. To test this hypothesis, 1a category of transcripts were compared with those reported in an old WormBase release (WS176). The analysis involved SL1 spliced transcripts belonging to category 1a (2,143) (Table 4). As expected, a vast majority of the genes (74%, 1583) are in category 1a in both releases, providing support for these gene models (Figure 3A, 3D, Supplementary data file 4). The next two largest categories consist of genes that are mispredicted (11.7%, 240 genes) and newly predicted, i.e., absent in WS176 (13.2%, 286 genes). Few genes (0.5%, 11) have start sites that correctly match with internal 5' ends of internal exons. The rest (0.5%, 14 genes) could not be uniquely placed into a single category since these had multiple tag matches in the older annotation. Roughly similar results were obtained by analyzing 1a category of SL2 spliced and SL1/SL2 spliced genes (Table 4; Figure 3B,3C,3D; Supplementary data file 4). Altogether, 845 annotation improvements are supported by our analysis. The demonstrated improvements in gene identification and genome annotation as observed in WS276 prove the accuracy of our 5' start site determination method. Overall, the 5' tag analysis serves as a rich resource to improve the *C. briggsae* genome annotation.

The second type of validation focused on a subset of the major misprediction category of genes whose 5' ends mapped more than 3 kb away from nearest exons. Most of these (94%, 49 of 52) are in intergenic regions (Supplementary data file 5). 37% (19 of 52) of the exons were supported by RNA sequencing reads (Wormbase), providing proof of accuracy to our method (Supplementary figure 2). These novel exons are likely to either belong to nearby existing genes or define brand new genes.

The last set of validations consisted of comparisons with *C. elegans* gene models. In this case category 1b of single and multiple transcripts (Tables 4 and 5 respectively) were manually examined. The results showed that 38% of newly discovered 5' ends (6 single transcript and 2 multiple transcripts) are supported by *C. elegans* orthologs (Supplementary figure 3, Supplementary data file 6), providing further support to our analysis. We took a similar approach to analyze a subset of transcripts in the major mispredictions category. Of the 10% of such predictions that were tested, 34% (17 of 50) are supported by WormBase *C. elegans* gene models. With this success rate, another 115 of the remaining single transcript genes of the major misprediction category are likely to be validated.

Discovery of operons

The identification of genes based on unique tag matches in *C. briggsae* allowed us to search for operons. In *C. elegans* it has been shown that the first gene in an operon is SL1 spliced (Conrad et al., 1991) whereas downstream genes are spliced either with SL2, SL2 variants or both SL1 and SL2 (Blumenthal, 2005). Thus, global analysis of trans splicing in *C. briggsae* should reveal all operons and operon genes. Moreover, genes that are both SL1 and SL2 spliced should reveal 'hybrid' operons.

Our data suggests the existence of a maximum of 1,199 *C. briggsae* operons (Table 6, Supplementary data file 7). These include 334 operons that are fully supported by tags, i.e., we were able to determine the splicing pattern of every gene, with operons ranging from 2 to 7 genes (Table 6). The remaining 865 operons (ranging between 2 to 6 genes) are categorized as 'Predicted operons' since the splicing identity of the first gene in these cases remains to be determined. Out of this set, the predicted operons that contain 3 or more genes (159) (Table 6) are large enough to be labeled as bona fide operons. Added together with the 334 fully supported operons, this allows us to report at least 493 operons in *C. briggsae* with sufficient certainty.

In *C. elegans*, operon genes tend to be very closely spaced, typically having less than 1 kb of intercistronic region (ICR) (Allen et al., 2011; Blumenthal et al., 2015). To examine whether the same is true in *C. briggsae*, ICR was calculated and found to have a similar distribution pattern as in *C. elegans* (Figure 4). A vast majority of the genes have ICRs of less than 200 bp (78%).

The above results suggested that the distance to the nearest upstream gene ('intergenic region' or IGR) of SL2-spliced genes will be smaller compared to those spliced with SL1 and SL1/SL2. To examine this possibility, we performed genome-wide analysis of intergenic distances for SL1, SL2 and SL1/SL2 spliced genes. The results showed that SL2-spliced genes have a median distance of roughly 180 bp. The medians of SL1 and SL1/SL2 spliced genes are 4,631 bp and 1,242 bp, respectively (Figure 5A). Furthermore, as we would expect, genes with larger IGRs are more likely spliced with SL1 than SL2 or SL1/SL2 (Figure 5B, Supplementary data file 8).

Tag-supported operons

We focused on the tag-supported operons to investigate the extent of conservation with *C. elegans*. The analysis of orthologs helped define three distinct categories (Supplementary data file 7). The two largest categories are termed 'exact match' and 'partial match' operons (40%, 38% respectively, 78% in total). Exact match operons consist entirely of *C. elegans* orthologs, whereas in partial match operons only some of the genes are conserved. The remaining one-fifth of operons define a third category, termed 'novel' (73). While a majority of these (61, 18%) consist of conserved genes whose orthologs are not present in *C. elegans* operons, others (12, 4%) consist of divergent, *C. briggsae*-specific genes.

Further examination of the *C. briggsae* operons revealed the largest cluster (CBROPX0001) consisting of 7 genes, 6 of which (CBG25571, CBG03062, CBG25572, CBG03061, CBG03060, CBG03059) are conserved in *C. elegans* and are part of the orthologous operon CEOP2496. The 5th gene in CBROPX0001 (CBG25573) does not appear to have a *C. elegans* ortholog. Syntenic alignments revealed that CBG25573 is conserved in *C. brenneri*, suggesting that the gene may have been lost in the *C. elegans* lineage (Supplementary figure 4). Another interesting observation relates to *rpb-6*, the first gene in CEOP2496. While we did not recover a tag for *Cbr-rpb-6* (CBG03063), based on the distance from its neighbor CBG25571 (195 bp), it is possibly part of *C. briggsae* operon CBROPX0001 (Figure 6). More experiments are needed to confirm if *Cbr-rpb-6* is the eighth gene in CBROPX0001.

Many other operons were manually updated. For example, CBROP0002 and CBROPX0002 were split based on homology information in *C. elegans*, resulting in four different operons: CBROP0002A (CBG02635, CBG02634), CBROP0002B (CBG02633, CBG02632), CBROP0132 (CBG01778, CBG31146, CBG01779), and CBROP0133 (CBG01783, CBG01784). In a different case, CBROPX0007 is predicted to consist of four genes (CBG03212, CBG03213, CBG03214, and CBG03215) (Supplementary Figure 5). The *C. elegans* orthologs of these genes constitute two distinct operons (CEOP2396 and CEOP2749) (Figure 7). While the ICR between CBG03213 and CBG03214 is larger than 2 kb, all downstream genes in CBROPX0007 are either SL2 or SL1/SL2 spliced. Further experiments are needed to validate the structure of CBROPX0007. Table 7 lists the updated numbers of operons in each category.

We also analyzed partially conserved operons in some detail. While all of these contain *C. elegans* orthologs, their structures are not conserved. Specifically, the number of genes or some of the orthologs in corresponding operons differ between the two species (Supplementary data file 7). Of the 127 such operons, 83 contain two or more conserved genes including 58 (70% of 83) with less than 1 kb ICR between every gene. One such operon (CBROPX0003) consists of five genes (Figure 8). While the *C. elegans* operon CEOP1484 contains orthologs of all of these, CEOP1484 encompasses three additional genes.

Our tag searches identified 73 novel operons (Supplementary data file 7). A majority of these (59, 81%) consist of a mix of conserved genes and those that lack orthologs in *C. elegans*. It is important to point out that none of the conserved genes are part of *C. elegans* operons. The other 12 (19%) operons consist entirely of genes that lack orthology in *C. elegans*. In seven of these cases, ICRs are less than 1 kb, providing further support to the operon structures (Table 8).

Predicted (Non-tag supported) operons

We report 865 predicted operons (Supplementary data file 7). While the downstream genes in these cases are spliced either with SL2 or SL1/SL2, the splicing status of the upstream gene is unknown. Most, if not all, of these are predicted to be genuine operons, especially those that are larger, i.e., consist of more than 2 genes. A comparison of 159 operons containing three or more genes with *C. elegans* revealed that 26 (16%) are fully conserved. A couple of examples include CBROPX0206 (5 genes) (Figure 9A,C) and CBROPX0207 (5 genes) (Figure 9D). The corresponding *C. elegans* operons are CEOP4500 (6 genes) (Figure 9B,C) and CEOP5248 (7 genes) (Figure 9E). Comparison of genes in CBROPX0206 and CEOP4500 revealed that these share four orthologs. We also observed two additional differences between CBROPX0206 and CEOP4500. One, the order of genes has changed and, two, CBROPX0206 includes CBG26297 which appears to lack a *C. elegans* ortholog (Figure 9C). Given that CBG06240 and CBG36241 are immediately upstream of CBROPX0206 and their orthologs are part of CEOP4500, it is possible that the *C. briggsae* operon could be extended to include both these genes. However, the two genes were not identified by TEC-RED tags and have therefore not been included in our operon model. The second example, CBROPX0207, contains five genes, all of which have orthologs in CEOP5248. However, the *C. elegans* operon contains two additional genes (ZK856.16 and ZK856.19) which are not conserved in *C. briggsae*.

SL1-type operons

We also found two operons in *C. briggsae* that contain two adjacent SL1-spliced genes. SL1-type operons have previously been described in *C. elegans* (Williams et al., 1999). SL1-spliced genes in such operons are positioned directly adjacent to one another, with no space between them. One of the SL1-type operons consists of two genes: CBROP0134 (CBG16825, *Cbr-vha-11*/CBG16826. Its *C. elegans* ortholog, CEOP4638, also consists of 2 genes. Another SL1-type operon identified by our study is CBROPX0001. Its *C.elegans* ortholog is CEOP2496. Interestingly, CBROPX0001 and CEOP2496 consist of more than 2 genes. While the first two genes in CEOP2496 (*rpb-6* and *dohh-1*) are spliced exclusively with SL1 (defined as SL1 operon), the remaining downstream genes are spliced with SL2 or SL1/SL2.

There is also a potential SL1-type operon consisting of CBG03984 and CBG03983. These two genes have a single base pair IGR (Figure 10). Interestingly, the *C. elegans* orthologs, F23C8.6 and F23C8.5 (SL1 and SL1/SL2 spliced, respectively) are in an operon, CEOP1044, with an ICR of more than 400 bp (Allen et al., 2011). More work is needed to determine whether the *C. briggsae* genes are indeed part of an SL1-type operon.

C. briggsae operons show enrichment of germline genes and highly expressed growth genes

Studies in *C. elegans* and *P. pacificus* have reported that germline genes are overrepresented in operons (Reinke & Cutter, 2009; Sinha et al., 2014). We did a gene-association study in *C. briggsae* to examine a similar possibility. The results revealed a significant enrichment of germline genes in high confidence operons ($p < 7.40E-98$) (Supplementary data file 9).

In addition to investigating germline genes, we performed GO term analysis of operon genes and found enrichment of terms associated with metabolic and biosynthesis processes. The pattern of enrichment was similar to what was observed with a *C. elegans* operon dataset (Supplementary data file 9). We also observed enrichment of growth-related genes, as found in *C. elegans*, specifically, female gamete generation (GO:0007292), embryo development ending in birth or egg hatching (GO:0009792), reproduction (GO:0000003) and embryo development (GO:0009790) (Zaslaver et al., 2011). It is important to point out that while GO terms are similar in both species, *C. briggsae* operon genes associated with specific processes are not always the orthologs of *C. elegans* gene sets. We conclude that functions of operon genes are conserved even if specific genes are not.

Identification of paralogs

As described above, not all tags could be uniquely matched to the genome. A total of 158 tags each identified multiple tags, adding up to a total of 362 genes. We reasoned that these represent potential paralogs. Further analysis suggested that the genes belong to 133 sets, roughly two-thirds (63%, 84) of which are on the same chromosome (Supplementary data file 10). The sets of genes fall into three distinct categories. Category 1 consists of paralog sets that fully match with WormBase annotation (21 paralogous sets, 42 genes). The other two categories showed either partial matches, i.e., WormBase reports larger sets of paralogs than those identified by our analysis (Category 2: 66 paralogous sets, 174 genes) or no match at all (Category 3: 46 paralogous sets, 146 genes). It is worth mentioning that half of the Category 3 genes have no paralogous information available, whereas the remaining ones have paralogs in WormBase but these differ from our analysis. To further validate paralogous relationships of Category 3 genes, we determined their intergenic distances. Studies in humans and other higher eukaryotes have revealed that intergenic distances between paralogous genes are smaller than random gene pairs on the same chromosome (Ibn-Salem et al., 2017). Our IGR analysis revealed that the distances in five cases are less than 10 kb (Supplementary table 5), substantially less than the average distance between a random pair of genes on the same chromosome (5.58 +/- 0.89 Mb in *C. elegans*) (Lee & Sonnhammer, 2003). Two of these pairs showed significant sequence homology in BLAST matches.

DISCUSSION

This paper reports the use of the TEC-RED technique in *C. briggsae* to improve genome annotation. We recovered 10,243 unique 5' end tags with matches in the genome, of which 6,395 correspond to SL1 and SL2 spliced exons and provide support to the existence of 4,252 unique trans-spliced genes. Another 362 genes have been identified as paralogs including 42 whose paralogous relationship is supported by Wormbase annotation.

In *C. elegans* 84% of all genes are spliced to leader sequences (Tourasse et al., 2017). If the percentage is comparable in *C. briggsae*, then our work has resulted in the identification of roughly one-quarter of all trans-spliced genes in this species. Further analysis revealed that two-thirds of all *C. briggsae* genes are spliced with SL1 and the remaining split evenly between SL2 and SL1/SL2 hybrid sequences (65% SL1, 18% SL2 and 18% SL1/SL2). Assuming that our TEC-RED method was unbiased in regard to the recovery of SL1 and SL2 spliced transcript tags, the proportion of spliced genes in *C. briggsae* differs from *C. elegans* as reported in the Allen et al. (Allen et al., 2011) study (82% SL1, 12% SL2 and 8% SL1/SL2). Interestingly, 14 genes were found to be spliced to leader sequences only in *C. briggsae* and not in *C. elegans*. More work is needed to determine if trans-splicing of these genes has indeed diverged between the two species.

Our analysis revealed that most of the genes identified by unique tag matches are represented by a single transcript (94.8%) and very few (5.2%) by multiple transcripts. Studies in *C. elegans* have reported roughly 18% of genes giving rise to multiple isoforms (Spieth et al., 2014; F. Wang et al., 2010), although this number is predicted to be as high as 25% (Ramani et al., 2011; Zahler, 2012). Considering this, along with the fact that our experiments captured only a partial set of all spliced genes, the actual proportion of genes with multiple transcripts in *C. briggsae* is likely to be much higher. Among other things, it was found that 77.8% of genes in our study have 5' start sites that match with those annotated by Wormbase. The remaining ones were considered mispredictions, most of which are major mispredictions (15.7%) as 5' start sites in these cases map anywhere between 20 bp to 3 kb away from known locations. We also found 52 new, previously unreported exons that map more than 3 kb upstream to the nearest exon of existing genes, and potentially include some that define the 5' start site of new genes.

Several approaches were taken to validate tag-based gene models. One of these involved comparing results with those obtained using an older gff release. The findings revealed that a total of 845 genes for which 5' ends were correctly annotated in WS276 were mispredicted or absent in the older version and demonstrate that our data can help improve start sites of many of the *C. briggsae* genes. Another approach involved comparing 5' ends of some of the genes with those of *C. elegans* orthologs. Of the 21 alternate start sites and 50 major mispredicted start sites analyzed, 38% and 34%, respectively, are supported by *C. elegans* transcripts. Finally, we examined the 52 newly discovered exons and found that 37% of these are supported by RNA-seq data in WormBase. To conclude, the above three validations provide significant support to the TEC-RED method for identification of expressed transcripts in *C. briggsae*.

The identification of genes spliced with leader sequences in *C. briggsae* allowed us to curate operons and study their conservation. Even though the operon-based organization of genes in *C. elegans* and *C. briggsae* is similar to those found in bacteria and archaea, work in *C. elegans* has shown that worm operons have no ancestral relationship with prokaryotes and appear to have evolved independently within the nematode phylum (Blumenthal, 2004; Qian & Zhang, 2008). We identified a total of 1,199 operons, of which 28% consist entirely of tag-supported genes. Of the remaining operons with partial tag support, 159 contain 3 or more genes. Combined with the fully tag supported operons, this totals to 493 operons in *C. briggsae* with a high degree of confidence. Comparison of tag-supported operons with *C. elegans* revealed that 134 (40%) are conserved, with the remainder being partially conserved (127, 39%) and novel (73, 21%). A subset of novel operons (17%) consists entirely of genes that lack *C. elegans* orthologs. Along with the above-mentioned operons, we also uncovered two conserved SL1-type operons. Together, these data demonstrate that while many of the operons are conserved, there are substantial differences between the two species. The findings represent the first comprehensive analysis of operons in *C. briggsae*.

In conclusion, the TEC-RED study described here has significantly improved the annotation of the *C. briggsae* genome by validating existing gene models, refining start sites of many genes, identifying novel gene exons, alternate transcripts, and provide a comprehensive analysis of operons and paralogous gene sets. These improvements to the genome annotation are expected to strengthen *C. briggsae* as a model for comparative and evolutionary studies.

ACKNOWLEDGEMENTS

We thank Wormbase for assistance with some aspects of data analysis, Mary Ann Allen and Tom Blumenthal for discussions on *C. elegans* operons, and members of the Gupta lab for feedback on the manuscript. This work was supported by grants to BPG (NSERC Discovery) and PWS (U24-HG002223). PWS was an Investigator with the HHMI, which partially supported this work.

List of references

Allen, M. A., Hillier, L. D. W., Waterston, R. H., & Blumenthal, T. (2011). A global analysis of C. elegans trans-splicing. *Genome Research*, *21*(2), 255–264. https://doi.org/10.1101/gr.113811.110

Ashburner, M., Ball, C., Blake, J., Botstein, D., Butler, H., Cherry, J. M., Davis, A., Dolinski, K., Dwight, S., Eppig, J., Harris, M., Hill, D., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J., Richardson, J., Ringwald, M., Rubin, G., & Sherlock, G. (2000). Gene Ontology: tool for the unification of biology. *Nature Genetics*, *25*(may), 25–29. https://doi.org/10.1038/75556

Baird, S. E., & Chamberlin, H. M. (2006). Caenorhabditis briggsae methods. *WormBook□: The Online Review of C. Elegans Biology*, 1–9. https://doi.org/10.1895/wormbook.1.128.1

Blumenthal, T. (2004). Operons in eukaryotes. *Briefings in Functional Genomics & Proteomics*, *3*(3), 199–211. https://doi.org/10.1093/bfgp/3.3.199

Blumenthal, T. (2005). Trans-splicing and operons. *WormBook□: The Online Review of C. Elegans Biology*, 1–9. https://doi.org/10.1895/wormbook.1.5.1

Blumenthal, T., Davis, P., & Garrido-Lecca, A. (2015). Operon and non-operon gene clusters in the C. elegans genome. *WormBook□: The Online Review of C. Elegans Biology*, 1–20. https://doi.org/10.1895/wormbook.1.175.1

Blumenthal, T., & Steward, K. (1997). RNA Processing and Gene Structure. In *C. elegans II edition*. Cold Spring Harbor Laboratory Press. https://www.ncbi.nlm.nih.gov/books/NBK19975/

Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics*, *77*(MAY), 71–94. https://doi.org/10.1002/cbic.200300625

Conrad, R., Thomas, J., Spieth, J., & Blumenthal, T. (1991). Insertion of part of an intron into the 5’ untranslated region of a Caenorhabditis elegans gene converts it into a trans-spliced gene. *Molecular and Cellular Biology*, *11*(4), 1921–1926. https://doi.org/10.1128/mcb.11.4.1921-1926.1991

Cutter, A. D. (2008). Divergence times in Caenorhabditis and Drosophila inferred from direct estimates of the neutral mutation rate. *Molecular Biology and Evolution*, *25*(4), 778–786. https://doi.org/10.1093/molbev/msn024

Gupta, B. P., Johnsen, R., & Chen, N. (2007). Genomics and biology of the nematode

Caenorhabditis briggsae. *WormBook□: The Online Review of C. Elegans Biology*, 1–16. https://doi.org/10.1895/wormbook.1.136.1

Hillier, L. D. W., Miller, R. D., Baird, S. E., Chinwalla, A., Fulton, L. A., Koboldt, D. C., & Waterston, R. H. (2007). Comparison of C. elegans and C. briggsae genome sequences reveals extensive conservation of chromosome organization and synteny. *PLoS Biology*, *5*(7), 1603–1616. https://doi.org/10.1371/journal.pbio.0050167

Hillier, L. W., Reinke, V., Green, P., Hirst, M., Marra, M. A., & Waterston, R. H. (2009). Massively parallel sequencing of the polyadenylated transcriptome of C. elegans. *Genome Research*, *19*(4), 657–666. https://doi.org/10.1101/gr.088112.108

Hwang, B. J., Müller, H. M., & Sternberg, P. W. (2004). Genome annotation by high-throughput 5′ RNA end determination. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(6), 1650–1655. https://doi.org/10.1073/pnas.0308384100

Ibn-Salem, J., Muro, E. M., & Andrade-Navarro, M. A. (2017). Co-regulation of paralog genes in the three-dimensional chromatin architecture. *Nucleic Acids Research*, *45*(1), 81–91. https://doi.org/10.1093/nar/gkw813

Lasda, E. L., & Blumenthal, T. (2011). Trans-splicing. *Wiley Interdisciplinary Reviews: RNA*, *2*(3), 417–434. https://doi.org/10.1002/wrna.71

Lee, J. M., & Sonnhammer, E. L. L. (2003). Genomic gene clustering analysis of pathways in eukaryotes. *Genome Research*, *13*(5), 875–882. https://doi.org/10.1101/gr.737703

Marra, M. A., Hillier, L., & Waterston, R. H. (1998). Expressed sequence tags--ESTablishing bridges between genomes. *Trends in Genetics□: TIG*, *14*(1), 4–7. https://doi.org/10.1016/S0168-9525(97)01355-3

Qian, W., & Zhang, J. (2008). Evolutionary dynamics of nematode operons: Easy come, slow go. *Genome Research*, *18*(3), 412–421. https://doi.org/10.1101/gr.7112608

Ramani, A. K., Calarco, J. A., Pan, Q., Mavandadi, S., Wang, Y., Nelson, A. C., Lee, L. J., Morris, Q., Blencowe, B. J., Zhen, M., & Fraser, A. G. (2011). Genome-wide analysis of alternative splicing in Caenorhabditis elegans. *Genome Research*, *21*(2), 342–348. https://doi.org/10.1101/gr.114645.110

Reinke, V., & Cutter, A. D. (2009). Germline expression influences operon organization in the Caenorhabditis elegans genome. *Genetics*, *181*(4), 1219–1228. https://doi.org/10.1534/genetics.108.099283

Ross, J. A., Koboldt, D. C., Staisch, J. E., Chamberlin, H. M., Gupta, B. P., Miller, R. D., Baird, S. E., & Haag, E. S. (2011). Caenorhabditis briggsae recombinant inbred line genotypes reveal inter-strain incompatibility and the evolution of recombination. *PLoS Genetics*, *7*(7). https://doi.org/10.1371/journal.pgen.1002174

Salehi-Ashtiani, K., Lin, C., Hao, T., Shen, Y., Szeto, D., Yang, X., Ghamsari, L., Lee, H., Fan, C., Murray, R. R., Milstein, S., Svrzikapa, N., Cusick, M. E., Roth, F. P., Hill, D. E., & Vidal, M. (2009). Large-scale RACE approach for proactive experimental definition of C. elegans ORFeome. *Genome Research*, *19*(12), 2334–2342. https://doi.org/10.1101/gr.098640.109

Sinha, A., Langnick, C., Sommer, R. J., & Dieterich, C. (2014). Genome-wide analysis of trans-splicing in the nematode pristionchus pacificus unravels conserved gene functions for germline and dauer development in divergent operons. *Rna*, *20*(9), 1386–1397. https://doi.org/10.1261/rna.041954.113

Spieth, J., & Lawson, D. (2006). Overview of gene structure. *WormBook□: The Online Review of C. Elegans Biology*, 1–10. https://doi.org/10.1895/wormbook.1.65.1

Spieth, J., Lawson, D., Davis, P., Williams, G., & Howe, K. (2014). Overview of gene structure in C. elegans. *WormBook□: The Online Review of C. Elegans Biology*, 1–18. https://doi.org/10.1895/wormbook.1.65.2

Stein, L. D., Bao, Z., Blasiar, D., Blumenthal, T., Brent, M. R., Chen, N., Chinwalla, A., Clarke, L., Clee, C., Coghlan, A., Coulson, A., D'Eustachio, P., Fitch, D. H. A., Fulton, L. A., Fulton, R. E., Griffiths-Jones, S., Harris, T. W., Hillier, L. D. W., Kamath, R., … Waterston, R. H. (2003). The genome sequence of Caenorhabditis briggsae: A platform for comparative genomics. *PLoS Biology*, *1*(2). https://doi.org/10.1371/journal.pbio.0000045

Tourasse, N., Millet, J. R. M., & Dupuy, D. (2017). Quantitative RNA-seq meta analysis of alternative exon usage in C. elegans. *BioRxiv*, 2120–2128. https://doi.org/10.1101/134718

Velculescu, V. E., Zhang, L., Vogelstein, B., & Kinzler, K. W. (1995). Serial analysis of gene expression. *Science*, *270*, 484–487. https://doi.org/10.1038/nprot.2006.269

Wang, F., Huang, S., & Ma, L. (2010). Caenorhabditis elegans operons contain a higher proportion of genes with multiple transcripts and use 3' splice sites differentially. *PLoS ONE*, *5*(8), 8–11. https://doi.org/10.1371/journal.pone.0012456

Wang, X., Zhao, Y., Wong, K., Ehlers, P., Kohara, Y., Jones, S. J., Marra, M. A., Holt, R. A., Moerman, D. G., & Hansen, D. (2009). Identification of genes expressed in the hermaphrodite germ line of C. elegans using SAGE. *BMC Genomics*, *10*. https://doi.org/10.1186/1471-2164-10-213

Williams, C., Xu, L., & Blumenthal, T. (1999). SL1 trans Splicing and 3′-End Formation in a Novel Class of Caenorhabditis elegansOperon. *Molecular and Cellular Biology*, *19*(1), 376–383. https://doi.org/10.1128/mcb.19.1.376

Zahler, A. M. (2012). Pre-mRNA splicing and its regulation in Caenorhabditis elegans. *WormBook□: The Online Review of C. Elegans Biology*, 1–21. https://doi.org/10.1895/wormbook.1.31.2

Zaslaver, A., Baugh, L. R., & Sternberg, P. W. (2011). Metazoan operons accelerate recovery from growth-arrested states. *Cell*, *145*(6), 981–992. https://doi.org/10.1016/j.cell.2011.05.013

List of tables

Table 1: Overview of SL1 and SL2 5’ tag sequence matches in the *C. briggsae* genome.

	Total unique tags	Matches in genome	Unique hits	Multiple hits
All	14,678	10,243	4,753	5,490
SL1	10,400	7,234	3,281	3,953
SL2	□ 4,278	3,009	1,472	1,537

Table 2: Breakdown of tag matches into different categories.

The numbers include both unique and multiple hits. Tag matches termed as 'Others' are those that cannot be placed uniquely into any of the main categories.

Category of tag matches	**SL1**	**SL2**	**Total**
1a	3,537	1,542	5,079 (79.4%)
1b	20	3	23 (0.3%)
Minor misprediction	245	91	336 (5.2%)
Major misprediction	639	291	930 (14.5%)
Others	22	5	27 (0.4%)
TOTAL	**4,463**	**1,932**	**6,395**

Table 3: Breakdown of genes by spliced leader sequences.

	Number of genes
Total	4,252
SL1 type	2,750 (65%)
SL2 type	743 (18%)
SL1/SL2 type	759 (18%)

Table 4. Genes supported by the presence of a single 5’ end (single transcript).

Numbers refer to genes identified by SL1, SL2 and SL1/SL2 tags. The genes have been divided further into various categories based on distance from the nearest exon (see figure 1). Novel exons and potential paralogs are excluded.

	ALL□	SL1	SL2	SL1/SL2
Matching first exon (1a)	3,288	2,143 (65.2%)	558 (17.0%)□	587 (17.9%)
Matching internal exon (1b)	16	14(87.5%)	1 (6.2%)	1 (6.2%)
Minor misprediction of first or internal exon□	204	146 (71.6%)	34 (16.7%)	24 (11.7%)
Major misprediction of first or internal exon□	517	357 (69%)	127 (24.6%)	33 (6.4%)
TOTAL	4,025	2,660	720	645

Table 5: Genes supported by the presence of multiple 5' ends.

Numbers refer to genes identified by SL1, SL2 and SL1 and SL2 tags. These genes have been divided further into various categories based on distance from the nearest exon (see figure 1). Genes for which exons belong to multiple categories are grouped as 'Others'. Novel exons and potential paralogs are excluded.

	ALL	**SL1**	**SL2**	**SL1/SL2**
Matching first exon (1a) and matching internal exon (1b)	5	2 (25%)	0	3 (75%)
Matching internal exons (1b)	0	0	0	0
Matching first exon (1a) and minor misprediction of one or more internal exons	39	14 (36%)	7 (18%)	18 (46%)
Matching first exon (1a) and major misprediction of one or more internal exons	145	57 (39%)	14 (10%)	74 (51%)
Others	6	2 (33%)	0	4 (67%)
All mispredicted exons (minor and major)	32	16 (50%)	2 (6%)	14 (44%)
TOTAL	227	91	23	113

Table 6: Breakdown of operons based on the number of genes present.

Operons are placed into two broad categories, those consisting entirely of genes with known spliced leader sequences (Tag-supported) and others where the splice leader identity of the first gene is unknown (Predicted operons).

	All	Number of genes in an operon					
		2	3	4	5	6	7
Tag-supported operons	**334**	263	54	14	2	0	1
Predicted operons	**865**	706	125	26	7	1	0

Table 7: Tag-supported operons in *C. briggsae.*

Exact match operons are conserved between *C. briggsae* and *C. elegans*. Partially conserved operons may contain some but not all orthologs that are part of corresponding *C. elegans* operons. Novel operons may contain *C. elegans* orthologs and divergent, *C. briggsae*-specific, genes.

Operon type	**Number** **(% of total)**
Fully conserved operons (Exact match)	134 (40.1%)
Partially conserved operons (Partial match)	127 (38%)
Novel operons	73 (21.9%)
- consisting of both divergent genes as well as orthologs that are not part of *C. elegans* operons	61 (18.3%)
- consisting entirely of divergent genes	12 (3.6%)
TOTAL	334

Table 8: Novel *C. briggsae* operons identified in this study with ICRs of less than 1 kb. None of the genes in these operons have orthologs in *C. elegans*. The numbers in brackets refer to ICR.

***C. briggsae* operon**	**# of genes**	**Gene names (ICR)**
CBROPX0130	3	CBG30062 (172) CBG25686 (105) CBG25687
CBROPX0131	3	CBG27303 (533) CBG27302 (116) CBG27301
CBROPX0135	2	CBG19287 (781) CBG19288
CBROPX0140	2	CBG11551 (162) CBG31489
CBROPX0129	3	CBG21606 (235) CBG30457 (493) CBG21605
CBROPX0139	2	CBG30329 (76) CBG30328
CBROPX0134	2	CBG30811 (611) CBG07748

List of figures

Figure 1: Representative model of locations of tag sequences within the genome. Three broad categories of matches are: valid prediction (termed 1a and 1b), minor misprediction, and major misprediction.

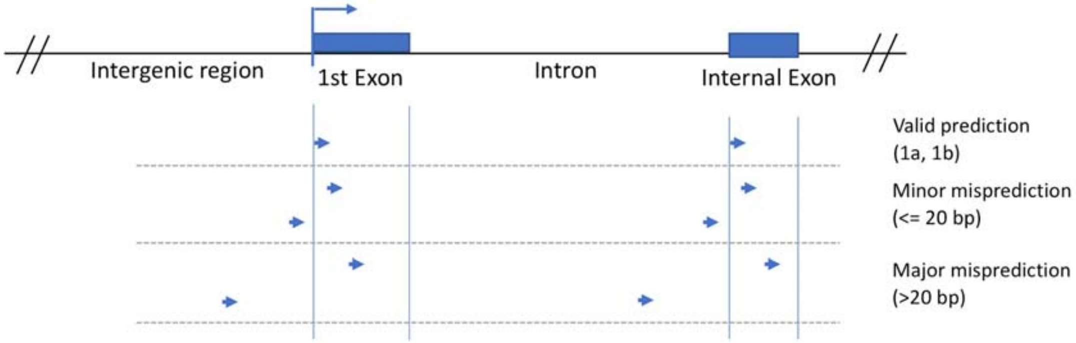


Figure 2. Proportion of tags belonging to different categories. The majority of SL1 (A) and SL2 tags (B) have unique hits in the genome and belong to category 1a, i.e., predicted 5' ends match with Wormbase gene models.

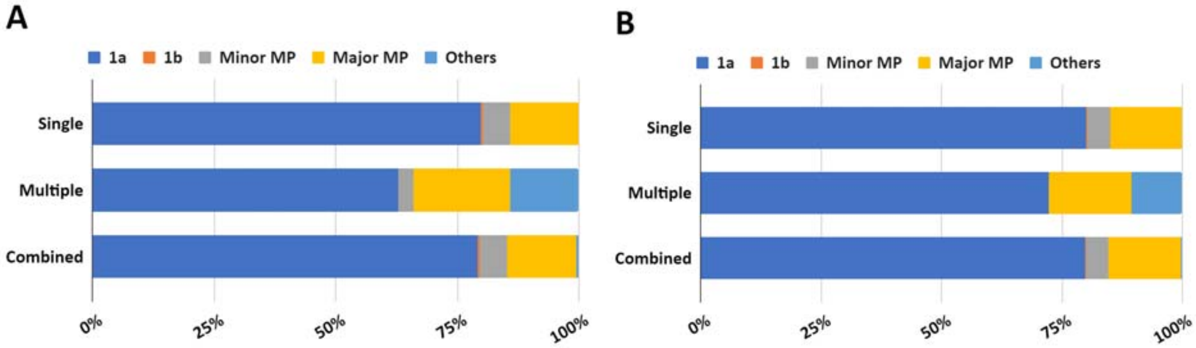


Figure 3: Analysis of the reclassification of genes from various categories in WS176 to category 1a in WS276. Only single transcript genes were compared. (A-C) Venn diagrams, with WS276 genes of category 1a of in black circles and WS176 genes of various categories in coloured circles. Numbers in overlapping circles represent genes of a given category in WS176 that are annotated as 1a type in WS276. Numbers in the middle of black circles (non-overlapping) represent genes that are unique to WS276 analysis (A, 286 or 13.2% of SL1-spliced; B, 107 or 19.0% of SL2-spliced; C, 53 or 9% of SL1/SL2 hybrid-spliced) whereas those in brackets next to colored circles are total genes identified by tag searches in WS176. (D) Histogram showing the proportion of genes with matching 5' ends in WS276 (category 1a) that overlap with various categories in the WS176 analysis.

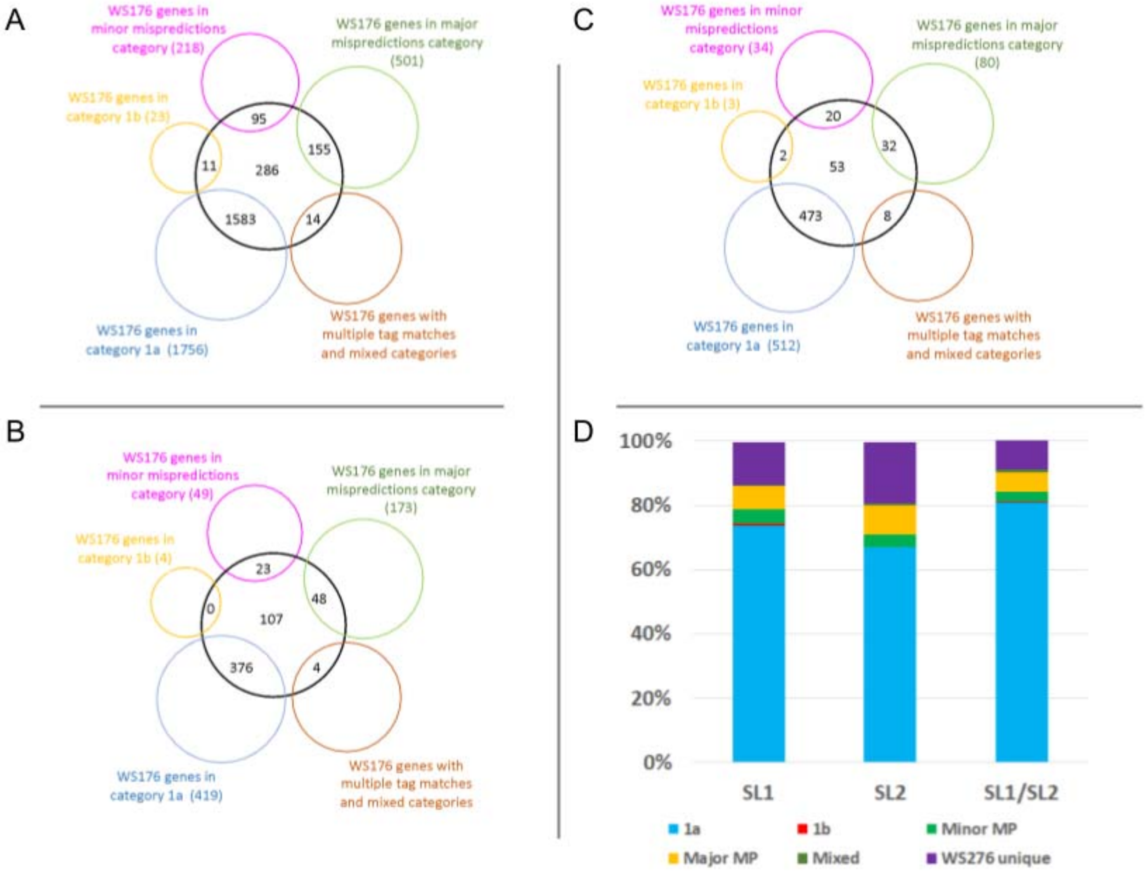


Figure 4: Frequencies of ICR lengths between SL2 and hybrid-spliced genes in operons. ICRs are sorted in bins of 100 nucleotides. For pairs of genes where the second gene is within the first gene, ICR is calculated as a negative value. For bin sizes, round brackets indicate exclusive bound, square brackets indicate inclusive bounds. Genes with larger than 2kb ICRs are shown as a single peak.

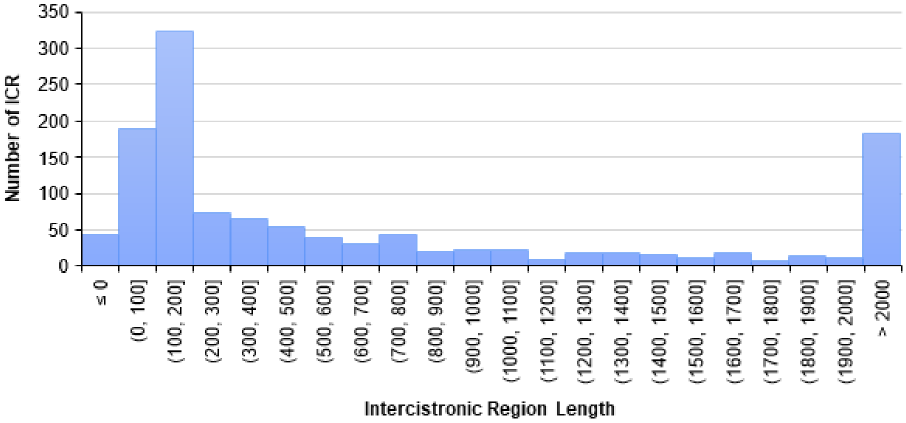


Figure 5: A: Intergenic regions (IGRs) of genes identified by tag matches. Box plots show IGRs for SL1-spliced, SL2-spliced, and SL1/SL2-spliced genes. The inside line marks the median, lower and upper lines represent the borders of the 25th and 75th quartile of the data sample, respectively. Whiskers enclose the 10-90% range of the data. B: 100% stacked columns of intergenic region (IGR) length. IGR lengths are sorted in bins of 500 nucleotides. For pairs of genes where the second gene is overlapping or inside the first gene, IGR length was calculated as a negative value. For bin sizes, round brackets indicate exclusive bound, square brackets indicate inclusive bounds.

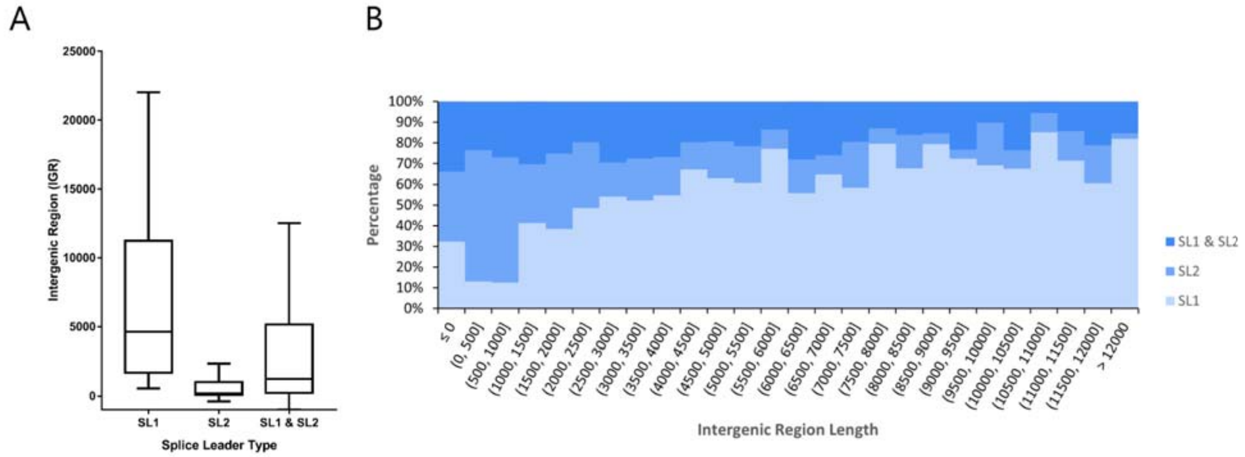


Figure 6. Genomic regions of *C. briggsae* CBROPX0001 and *C. elegans* CEOP2496. A: CBROPX0001 is proposed to contain at least 7, and possibly 8, genes depending on the inclusion of CBG03063. B: Homologous *C. elegans* operon CEOP2496 contains 7 genes. This and other similar images are modified versions of Wormbase Jbrowse.

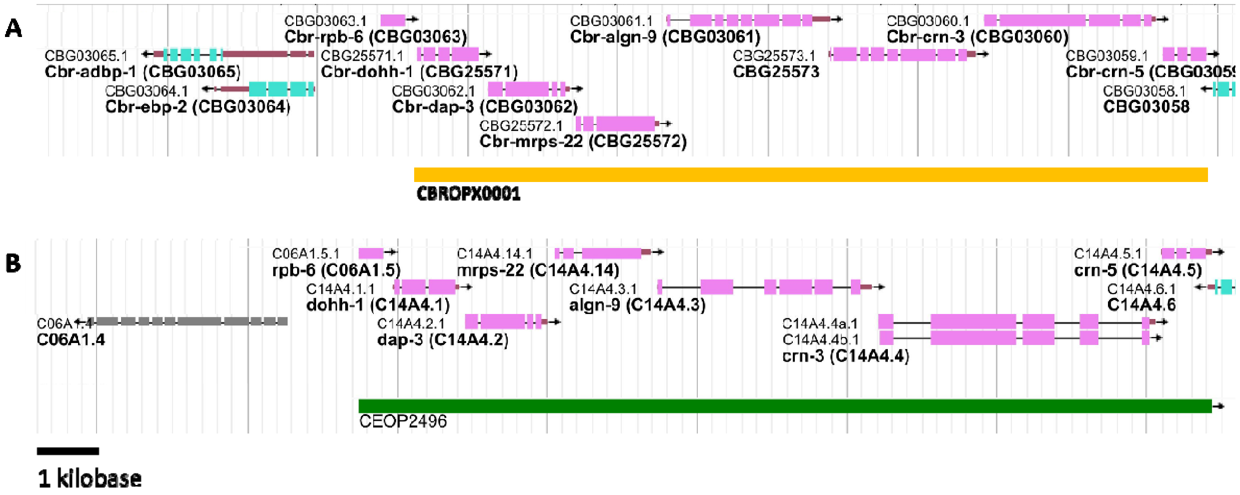


Figure 7: *C. briggsae* operon CBROPX0007. A: A cluster of four genes that define CBROPX0007. B: The orthologs of the four genes are split between two *C. elegans* operons - CEOP2396 and CEOP2749.

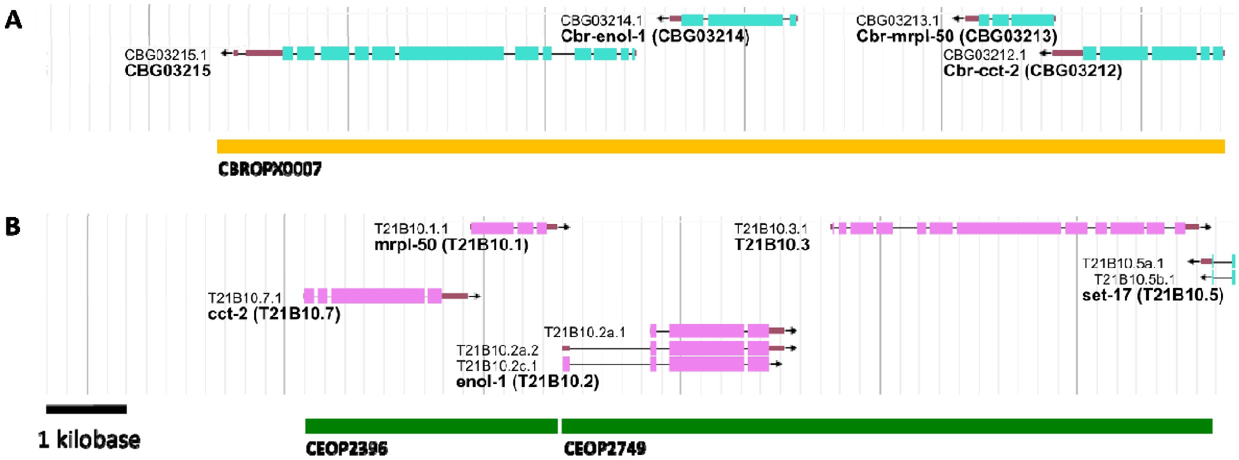


Figure 8. Partially conserved operon and its *C. elegans* ortholog. A: CBROPX0003 is an example of a partially conserved operon identified in this study. B: CEOP1484, *C. elegans operon* orthologous to *C. briggsae* operon CBROPX0003.

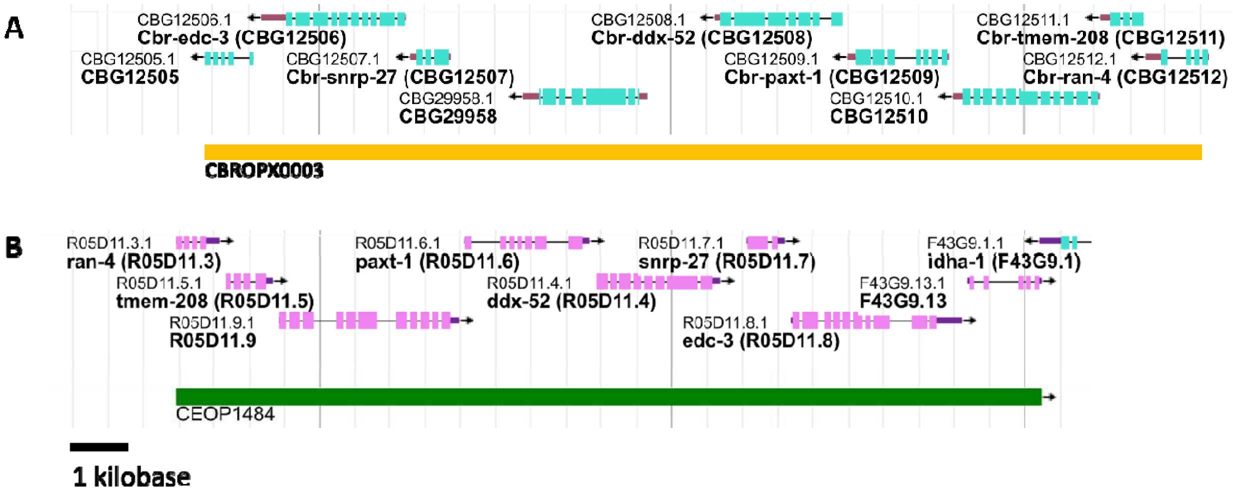
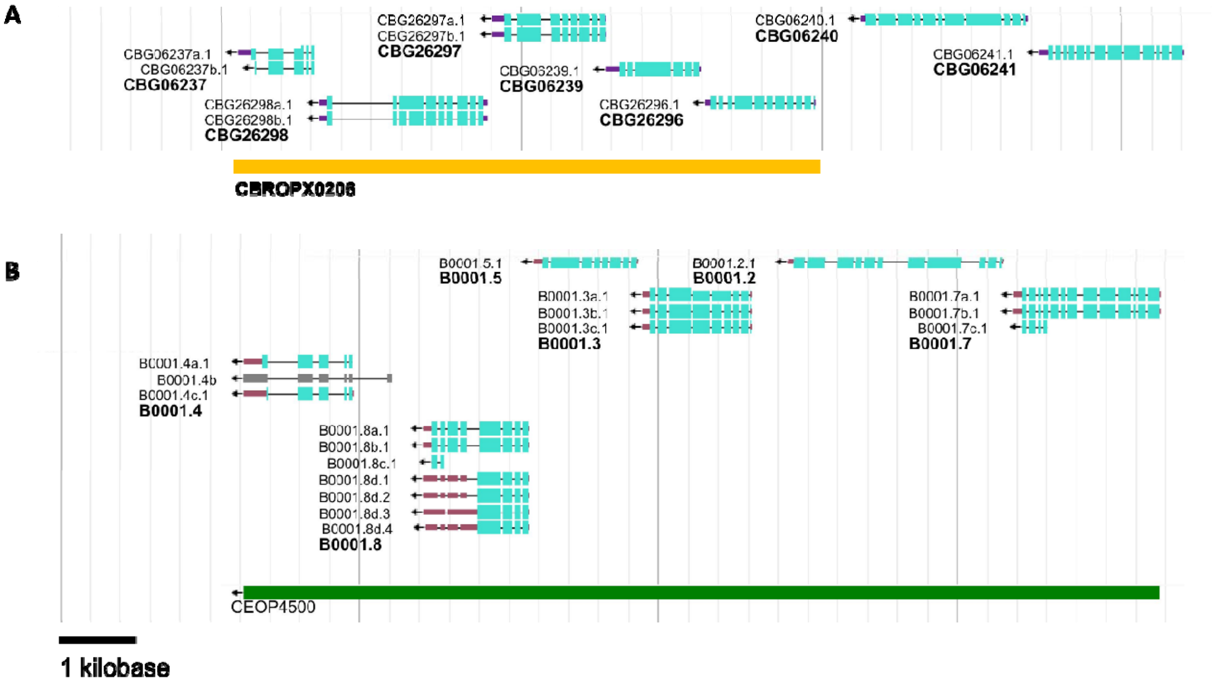


Figure 9: Two predicted operons in *C. briggsae* along with their *C. elegans* counterparts.

A, B: CBROPX0206 with five genes and its orthologous operon CEOP4500 in *C. elegans*. Three genes are conserved between these two operons. C: Rows containing *C. elegans* CEOP4500 genes (row 1), CBROPX0206 genes (row 2), and *C. elegans* orthologs of CBROPX0206 genes (row 3). The genes are presented in the order they are located in operons. D, E: CBROPX0207 with five genes and its orthologous operon CEOP5428 with 7 genes. All five genes of the *C. briggsae* operon are conserved in CEOP5428. Two additional genes are present in CEOP5428.



C

C. elegans CEOP4500	B0001.7	B0001.2	B0001.3	B0001.5	x	B0001.8	B0001.4
C. briggsae CBROPX0206			CBG26296	CBG06239	CBG26297	CBG26298	CBG06237
C. elegans ortholog			B0001.3	B0001.8	x	B0001.5	B0001.2/B0001.4

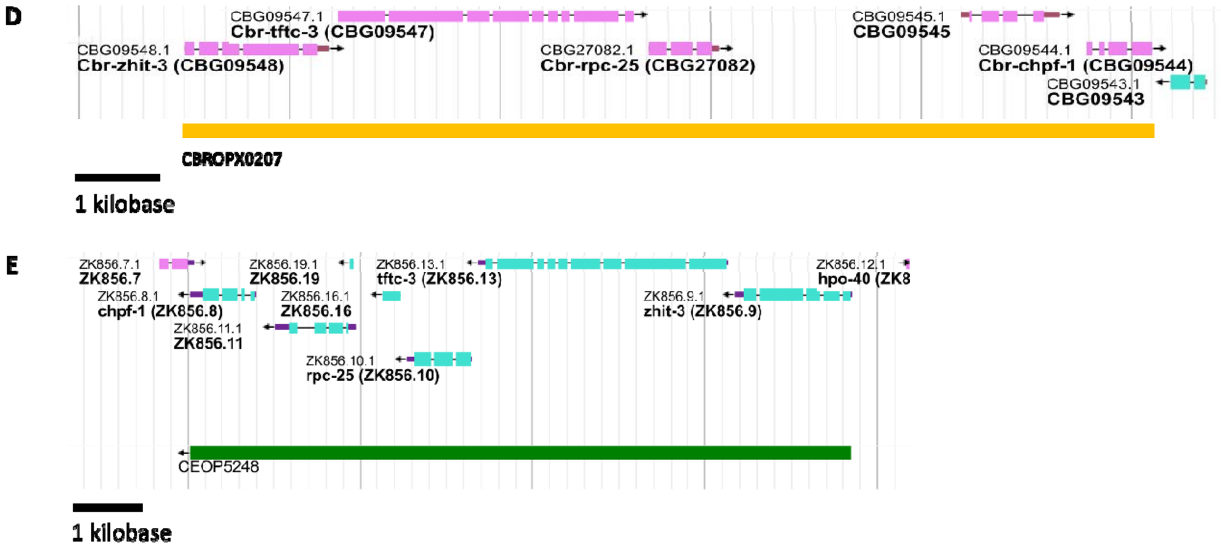
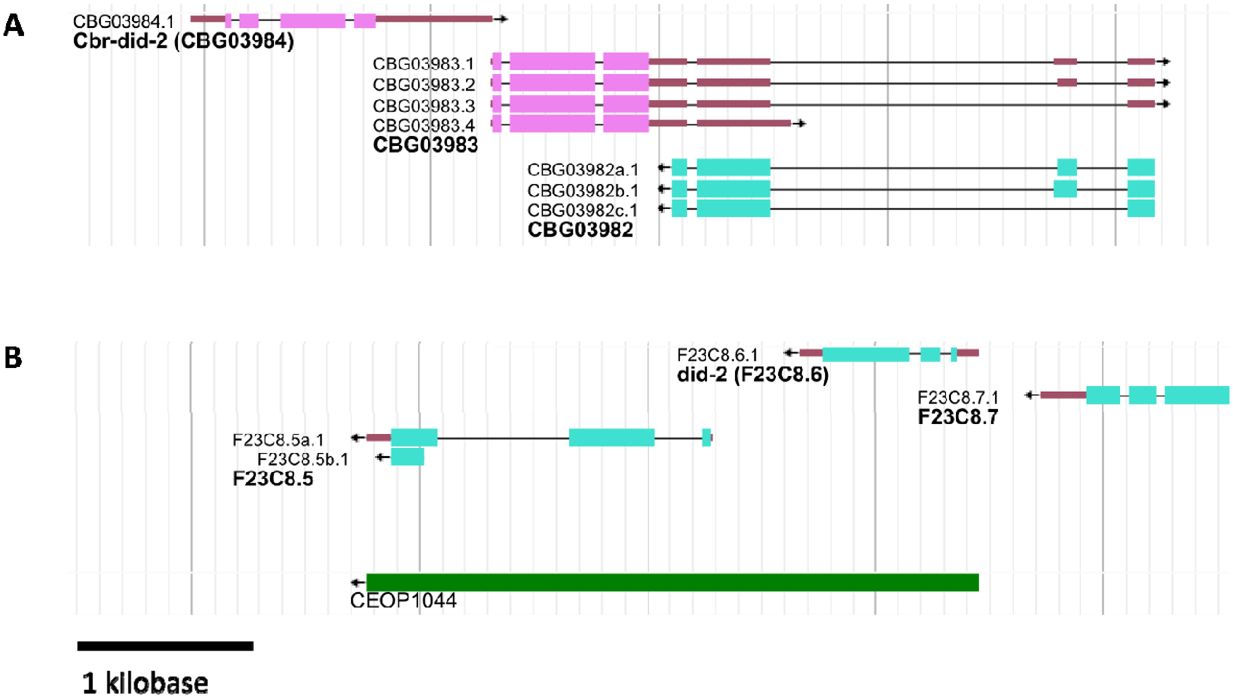


Figure 10. A predicted SL1-type operon in *C. briggsae.* A: *C. briggsae* genes CBG03984 and CBG03983 have a 1 bp ICR. Both CBG03984 and CBG03983 are spliced with a SL1 leader sequence. B: *C. elegans* orthologs did-2 and F23C8.5, respectively, depicted sharing operon CEOP1044.



Supplementary data

Jhaveri and van den Berg et al.

Gene identification and genome annotation in *Caenorhabditis briggsae* by high throughput 5' RNA end determination

Supplementary data files (Microsoft Excel spreadsheets)

File name	**Description**
Supplementary data file 1	Exons identified in our analysis
Supplementary data file 2	Unique genes identified
Supplementary data file 3	Genes uniquely spliced in *C. briggsae*
Supplementary data file 4	Validation based on overlap with WS176
Supplementary data file 5	New exons identified by our study
Supplementary data file 6	Manual curation of 1b and major mispredictions based on *C. elegans* orthologs
Supplementary data file 7	List of operons
Supplementary data file 8	Intergenic region values
Supplementary data file 9	Germline genes present in operons and GO analysis
Supplementary data file 10	Proposed paralog sets

Supplementary tables

Supplementary table 1: Primers used to generate Biotin-RT-PCR products

Primers	**Sequence (5’ to 3’)**
RT primer	GTGATGTCTCGAGTAGTTCGAAATGGCC (T)22
5’ SL1-Bpm I RT-PCR primer	Biotin/ AGACGCAAGGTTTAATTACCCAAGCTGGAG
5’ SL2-Bpm I RT-PCR primer	Biotin/ AGACGCAAGGTTTTAACCCAGTTACTGGAG
3’ RT-PCR primer	GAGGTGATGTCTCGAGTAGTTCGAAATGGC

Supplementary table 2: PCR primers used to generate mono-TAGs from the 5’ biotin-adaptor DNA fragments

Primers	**Sequence (5’ to 3’)**
5’ SL1-Xho I primer	AGACGCAAGGTTTAATTACCCAAGCTCGAG
5’ SL2-Xho I primer	AGACGCAAGGTTTTAACCCAGTTACTCGAG
3’ for adaptor 1 (KpnI)	CTATAGGGCTCAAAGATGACGAGAGGA
3’ for adaptor 2 (HindIII)	CAAGATTCTCACGACGATGTTCGGAGT
3’ for adaptor 3 (EagI)	TGAAGATTGCACAGAGGAGAGACCGCT
3’ for adaptor 4 (SacI)	CAGTTGGAATGAATGAAGCTATACCAT
3’ for adaptor 5 (MluI)	CTAGTATACGTTCTAGTATCAGAGGAA
3’ for adaptor 6 (NheI)	TCTTGCAGTGATTAGCGTCAGTGCCTG

Supplementary table 3: Adaptors used for ligation onto Bpm I-digested, 5’ biotin-DNA fragments

Adapter	**Sequence (5’ to 3’)**	**Sequence (3’ to 5’)**
Adapter 1 (KpnI)	CTATAGGGCTCAAAGATGACGAGAGGAGGTACC	TGCTCTCCTCCATGG
Adapter 2 (HindIII)	CAAGATTCTCACGACGATGTTCGGAGTAAGCTT	CAAGCCTCATTCGAA
Adapter 3 (EagI)	TGAAGATTGCACAGAGGAGAGACCGCTCGGCCG	CTCTGGCGAGCCGGC
Adapter 4 (SacI)	CAGTTGGAATGAATGAAGCTATACCATGAGCTC	GATATGGTACTCGAG
Adapter 5 (MluI)	CTAGTATACGTTCTAGTATCAGAGGAAACGCGT	AGTCTCCTTTGCGCA
Adapter 6 (NheI)	TCTTGCAGTGATTAGCGTCAGTGCCTGGCTAGC	GTCACGGACCGATCG

Supplementary Table 4: Chromosomal locations of 4,252 unique genes identified by TEC-RED. Chr: Chromosome, Un: unmapped genomic region.*Ross et al. (2011).

Chr	**Total gene count**	**SL1 genes**	**Fraction**	**Density**	**SL2 genes**	**Fraction**	**Density**	**SL1/SL2 genes**	**Fraction**	**Density**	**Chr length (Mb)***
I	763	447	16.26	28.93	158	21.27	10.23	158	20.79	10.23	15.45
II	752	473	17.21	28.46	147	19.78	8.84	132	17.37	7.94	16.62
III	751	444	16.15	30.47	145	19.52	9.95	162	21.32	11.12	14.57
IV	717	447	16.26	25.57	133	17.90	7.61	137	18.03	7.84	17.48
V	749	534	19.43	27.40	105	14.13	5.39	110	14.47	5.64	19.49
X	515	400	14.55	18.57	54	7.27	2.51	61	8.03	2.83	21.54
Un	5	4			1			0			
	4252	**2749**		26.14	**743**		7.07	**760**		7.23	**105.15**

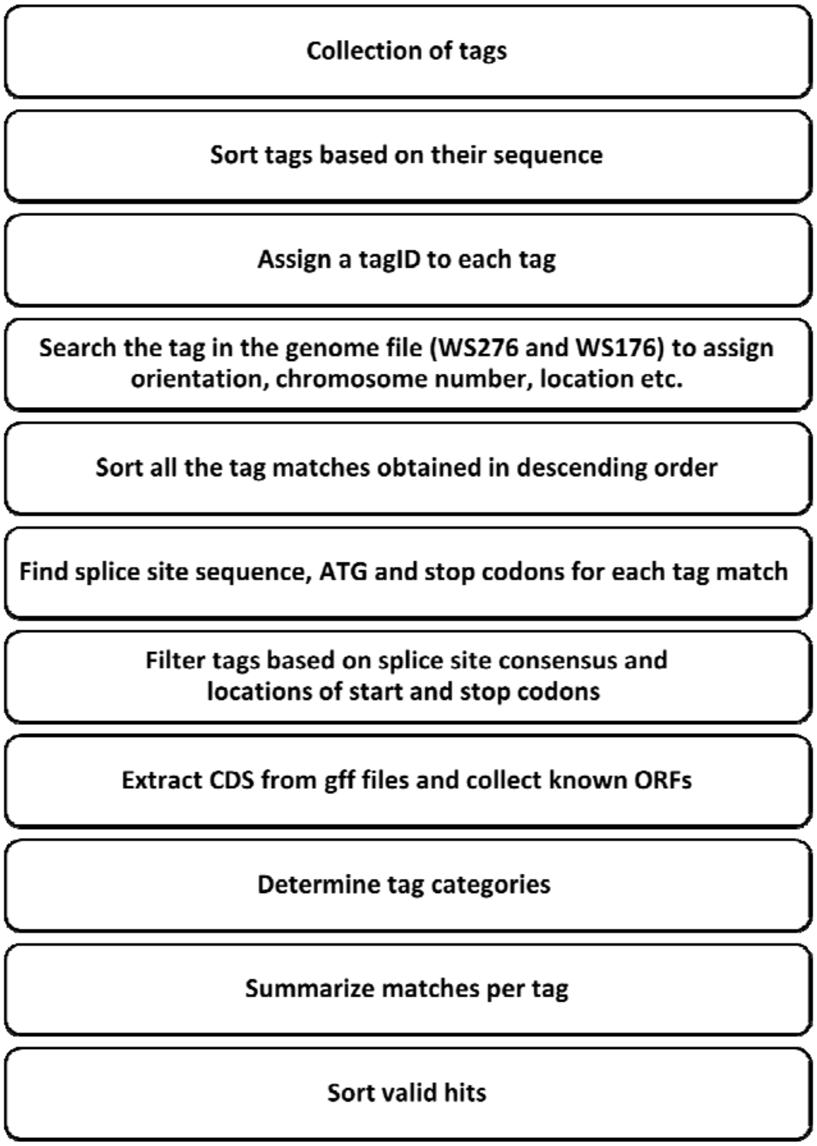
Ross, J. A., Koboldt, D. C., Staisch, J. E., Chamberlin, H. M., Gupta, B. P., Miller, R. D., Baird, S. E., & Haag, E. S. (2011). Caenorhabditis briggsae recombinant inbred line genotypes reveal inter-strain incompatibility and the evolution of recombination. *PLoS Genetics*, *7*(7). https://doi.org/10.1371/journal.pgen.1002174

Supplementary table 5: Intergenic distances of selected Category 3 genes that are less than 10 kb apart. ***** BLAST match showed some similarity in a very small 5' region.

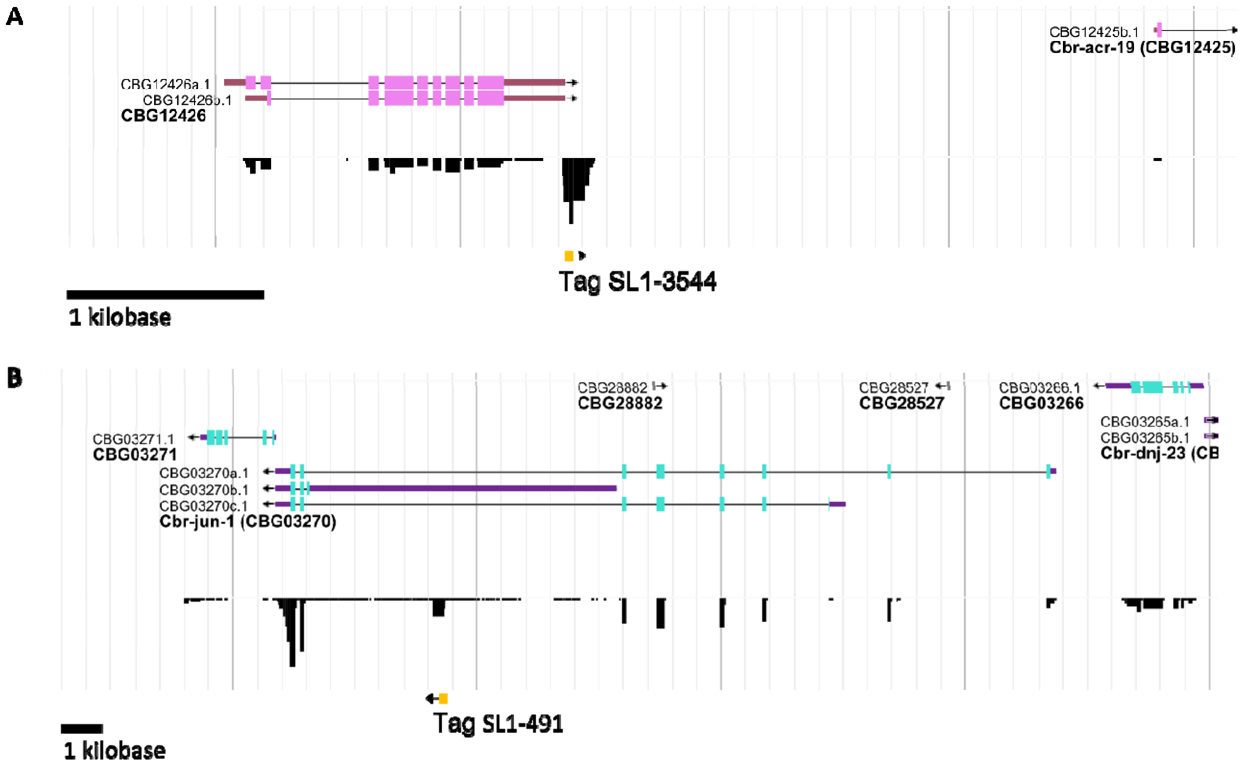
Adjacent genes identified by tags	**IGR**	**BLAST alignment**	***C. elegans* orthologs**	***C. briggsae* gene orientation**
CBG25816, CBG00473	2,903 bp	Yes	none	Opposite
CBG08766, CBG08768a	578 bp	No*	F25E5.8 and *nhr-117*	Opposite
CBG25203, CBG29819	2,251 bp	No	F59A3.2 and *ubl-5*	Opposite
CBG26374, CBG05421	3,564 bp	No*	None, *fan-1*	Same
CBG26845, CBG26846	8,559 bp	Yes	None	Same

Supplementary Figures

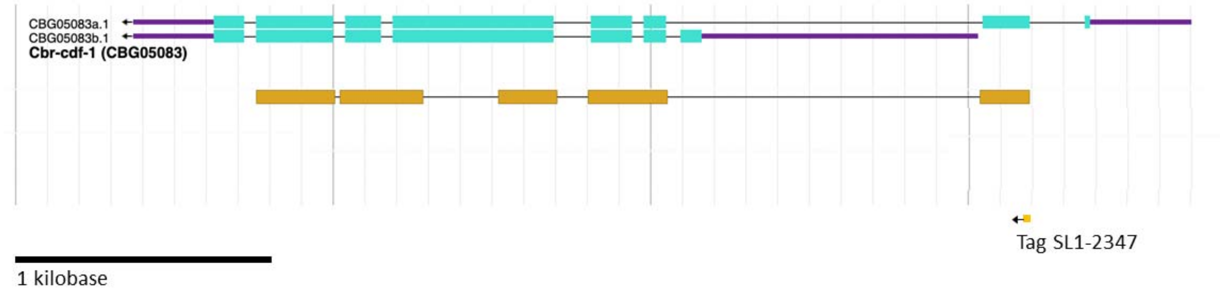
Supplementary Figure 1: Flowchart of steps used to analyze 5' tag sequences and genes.


Collection of tags
Sort tags based on their sequence
Assign a tagID to each tag
Search the tag in the genome file (WS276 and WS176) to assign orientation, chromosome number, location etc.
Sort all the tag matches obtained in descending order
Find splice site sequence, ATG and stop codons for each tag match
Filter tags based on splice site consensus and locations of start and stop codons
Extract CDS from gff files and collect known ORFs
Determine tag categories
Summarize matches per tag
Sort valid hits

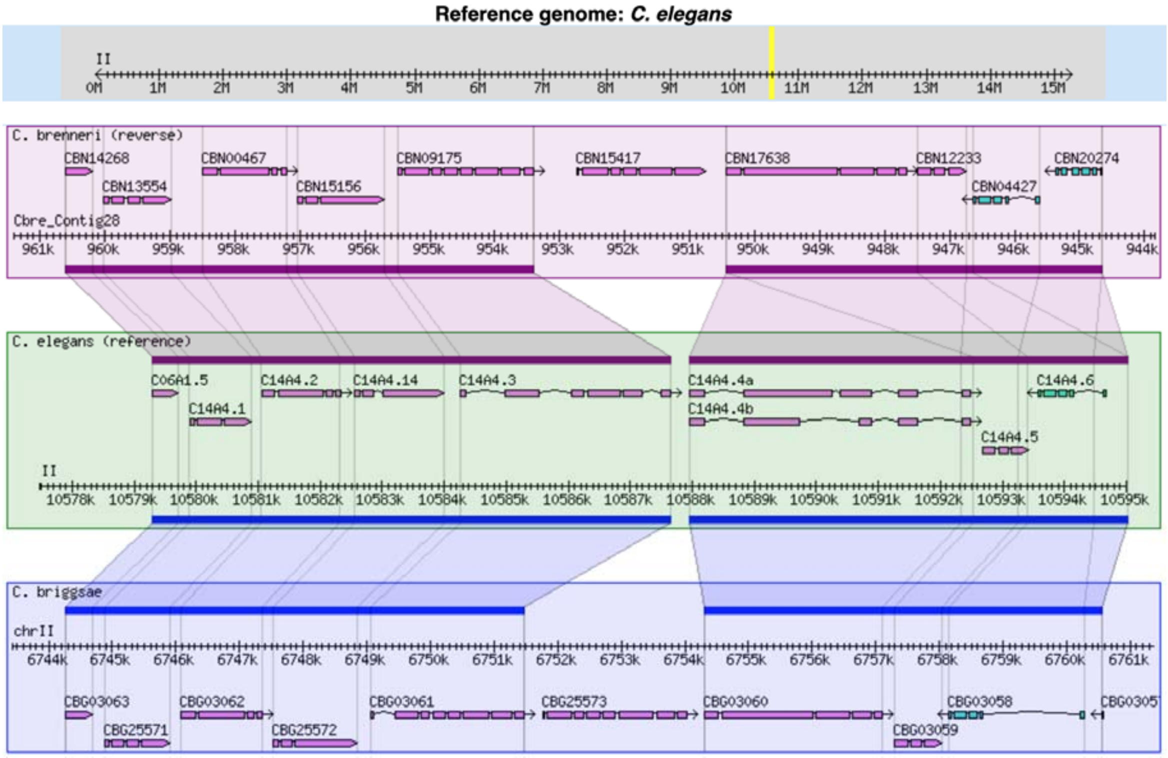
Supplementary Figure 2: Selected examples of novel exons supported by Wormbase RNASeq data. The top track shows currently curated genes. Second track shows alignments of short read sequences from all available RNASeq projects on Wormbase. The number of reads has been normalized by averaging over the number of libraries. The height of reads boxes indicates the relative score of the feature. The bottom track shows a TEC-RED tag binding at a genome location predicted to contain the 5’ start site of a new exon. A: New exon between CBG12426b.1 & CBG12425b.1. B: New exon inside CBG03270a.1.



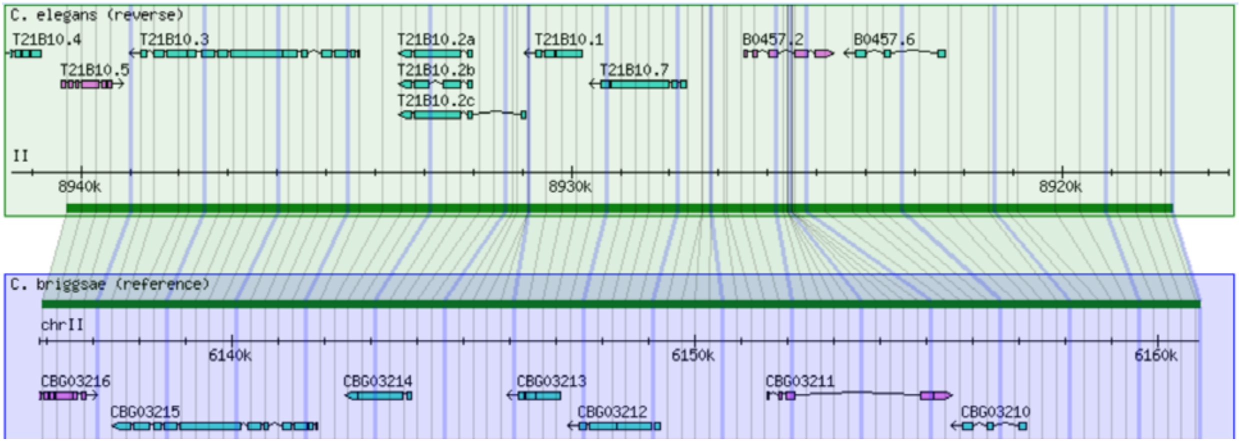
Supplementary Figure 3: An example of the 1b category in *Cbr-cdf-1*. The top track shows curated gene *Cbr-cdf-1*. The middle track shows the *C. elegans* C15B12.7a.1 (*cdf-1*) gene model, which is indicated in orange. The bottom track shows a category 1b TEC-RED tag binding at the 5’ start site of exon 2 of *Cbr-cdf-1*. The *C. elegans* gene model supports the 5’ start site of an unknown transcript variant for *Cbr-cdf-1*.



Supplementary Figure 4. *C. briggsae* operon CBROPX0001 genes, displayed in *C. brenneri*, *C. elegans* and *C. briggsae* using the Wormbase synteny browser.


Reference genome: C. elegans
II
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M 13M 14M 15M
C. brenneri (reverse)
CBN14268
CBN13554
CBN00467
CBN15156
CBN09175
CBN15417
CBN17638
CBN12233
CBN04427
CBN20274
Cbre_Contig28
961k 960k 959k 958k 957k 956k 955k 954k 953k 952k 951k 950k 949k 948k 947k 946k 945k 944k
C. elegans (reference)
C06A1.5
C14A4.1
C14A4.2
C14A4.14
C14A4.3
C14A4.4a
C14A4.4b
C14A4.5
C14A4.6
II
10578k 10579k 10580k 10581k 10582k 10583k 10584k 10585k 10586k 10587k 10588k 10589k 10590k 10591k 10592k 10593k 10594k 10595k
C. briggsae
chrII
6744k 6745k 6746k 6747k 6748k 6749k 6750k 6751k 6752k 6753k 6754k 6755k 6756k 6757k 6758k 6759k 6760k 6761k
CBG03063
CBG25571
CBG03062
CBG25572
CBG03061
CBG25573
CBG03060
CBG03059
CBG03058
CBG0305

Supplementary figure 5: A cluster of the four genes that define the CBROPX0007 operon, displayed in *C. elegans* and *C. briggsae* using the Wormbase synteny browser


C. elegans (reverse)
T21B10.4
T21B10.3
T21B10.5
T21B10.2a
T21B10.2b
T21B10.2c
T21B10.1
T21B10.7
B0457.2
B0457.6
II
8940k
8930k
8920k
C. briggsae (reference)
chrII
6140k
6150k
6160k
CBG03216
CBG03215
CBG03214
CBG03213
CBG03212
CBG03211
CBG03210