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DATA NOTE

Improving the annotation of the *Heterorhabditis bacteriophora* genome

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Abstract

Background: Genome assembly and annotation remain exacting tasks. As the tools available for these tasks improve, it is useful to return to data produced with earlier techniques to assess their credibility and correctness. The entomopathogenic nematode *Heterorhabditis bacteriophora* is widely used to control insect pests in horticulture. The genome sequence for this species was reported to encode an unusually high proportion of unique proteins and a paucity of secreted proteins compared to other related nematodes. **Findings:** We revisited the *H. bacteriophora* genome assembly and gene predictions to determine whether these unusual characteristics were biological or methodological in origin. We mapped an independent resequencing dataset to the genome and used the blobtools pipeline to identify potential contaminants. While present (0.2% of the genome span, 0.4% of predicted proteins), assembly contamination was not significant. **Conclusions:** Re-prediction of the gene set using BRAKER1 and published transcriptome data generated a predicted proteome that was very different from the published one. The new gene set had a much reduced complement of unique proteins, better completeness values that were in line with other related species' genomes, and an increased number of proteins predicted to be secreted. It is thus likely that methodological issues drove the apparent uniqueness of the initial *H. bacteriophora* genome annotation and that similar contamination and misannotation issues affect other published genome assemblies.

Keywords: *Heterorhabditis bacteriophora*; genome; annotation

Background

The sequencing and annotation of a species' genome is often the first step in exploiting these data for comprehensive biological understanding. As with all scientific endeavors, genome sequencing technologies and the bioinformatics tool kits available for assembly and annotation are being continually improved. It should come as no surprise therefore that first estimates of genome sequences and descriptions of the genes they contain can be improved. For example, the genome of the nematode *Caenorhabditis elegans* was the first animal genome to be sequenced [1]. The genome sequence and annotations have been updated many times since, as further exploration of this model

organism revealed errors in original predictions such that today, with release WS260 [2, 3], very few of the 19,099 protein-coding genes announced in the original publication [1] retain their original structure and sequence. The richness of the annotation of *C. elegans* is driven by the size of the research community that uses this model species. However, for most species, where the community using the genome data is small or less-well funded, initial genome sequences and gene predictions are not usually updated.

Heterorhabditis bacteriophora is an entomopathogenic nematode that maintains a mutualistic association with the bacterium *Photorhabdus luminescens*. Unlike many other parasitic nematodes, it is amenable to *in vitro* culture [4] and is therefore of

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interest not only to evolutionary and molecular biologists who investigate parasitic and symbiotic systems but also to those concerned with the biological control of insect pests [5, 6]. *Photorhabdus luminescens* colonizes the anterior intestine of the free-living infective juvenile stage (Ij). IJs are attracted to insect prey by chemical signals [7, 8]. On contacting a host, the IJs invade the insect's hemocoel and actively regurgitate *P. luminescens* into the hemolymph. The bacterial infection rapidly kills the insect, and *H. bacteriophora* grow and reproduce within the cadaver. After 2–3 cycles of replication, the nematode progeny develop into IJs, sequester *P. luminescens*, and seek out new insect hosts.

Axenic *H. bacteriophora* IJs are unable to develop past the L1 stage [9], and *H. bacteriophora* may depend on *P. luminescens* for secondary metabolite provision [10, 11]. Mutation of the global post-transcriptional regulator Hfq in *P. luminescens* reduced the bacterium's secondary metabolite production and led to failed nematode development, despite the bacterium maintaining virulence against host (*Galleria mellonella*) larvae [12]. Together, these symbionts are efficient killers of pest (and other) insects, and understanding of the molecular mechanisms of host killing could lead to new insecticides.

Heterorhabditis bacteriophora was selected by the National Human Genome Research Initiative as a sequencing target [13]. Genomic DNA from axenic cultures of the inbred strain *H. bacteriophora* TTO1 was sequenced using Roche 454 technology, and a high-quality 77 Mb draft genome assembly was produced [14]. This assembly was predicted (using JIGSAW [15]) to encode 21,250 proteins. Almost half of these putative proteins had no significant similarity to entries in the GenBank nonredundant protein database, suggesting an explosion of novelty in this nematode. The predicted *H. bacteriophora* proteome had fewer orthologues of Kyoto Encyclopedia of Genes and Genomes loci in the majority of metabolic categories than nine other nematodes. *Heterorhabditis bacteriophora* was also predicted to have a relative paucity of secreted proteins compared to free-living nematodes, postulated to reflect a reliance on *P. luminescens* for secreted effectors [14]. The 5.7 Mb genome of *P. luminescens* has also been sequenced [16]. The *H. bacteriophora* proteome had fewer shared orthologues when clustered and compared to other rhabditine (Clade V) nematodes (including *Caenorhabditis elegans* and the many animal parasites of the Strongyloomorpha) [17].

In preliminary analyses, we noted that while the genome sequence itself had high completeness scores when assessed with the Core Eukaryote Gene Mapping Approach (CEGMA) [18] (99.6% complete) and Benchmarking Universal Single-Copy Orthologs (BUSCO) [19] (80.9% complete and 5.6% fragmented hits for the BUSCO Eukaryota gene set), the predicted proteome scored poorly (47.8% complete and 34.7% fragmented by BUSCO). Another unusual feature of the *H. bacteriophora* gene set was the proportion of noncanonical splice sites (i.e., those with a 5' GC splice donor site, as opposed to the normal 5' GT). Most nematode (and other metazoan) genomes have low proportions of noncanonical introns (less than 1%) [20], but the published gene models had more than 9% noncanonical introns. This is more than double the proportion predicted for *Globodera rostochiensis*, a plant parasitic nematode where the unusually high proportion of noncanonical introns was validated via manual curation [20].

If these unusual characteristics reflect a truly divergent proteome, the novel proteins in *H. bacteriophora* may be crucial in its particular symbiotic and parasitic relationships and be of great interest to development of improved strains for horticulture. However, it is also possible that contamination of the published assembly or annotation artifacts underpin these unusual features. We re-examined the *H. bacteriophora* genome and gene

predictions and used more recent tools to re-predict protein coding genes from the validated assembly. As the BRAKER1 predictions were demonstrably to be better than the original ones, we explored whether some of the unusual characteristics of the published protein set, in particular the level of novelty and the proportion of secreted proteins, were supported by the BRAKER1 protein set.

Findings

No evidence for substantial contamination of the *H. bacteriophora* genome assembly

We used BlobTools [21] to assess the published genome sequence [14] for potential contamination. The raw read data from the published assembly were not available on the trace archive or short-read archive. We thus used new Illumina short-read resequencing data generated from strain G2a1223, an inbred derivative of *H. bacteriophora* strain "Gebre," isolated by Adler Dillman in Moldova. G2a1223 has about 1 single-nucleotide change per ~2,000 nucleotides compared to the originally sequenced TT01 strain. G2a1223 was grown in culture on the non-colonizing bacterium *Photorhabdus temperata*. The majority of these data (96.3% of the reads) mapped as pairs to the assembly, suggesting completeness of the published assembly with respect to the new raw read data. In addition, 99.96% of the published assembly had at least 10-fold coverage from the new raw reads.

The assembly was explored using a taxon-annotated GC-coverage plot, with coverage taken from the new Illumina data and sequence similarity from the National Center for Biotechnology Information (NCBI) nucleotide (nt) database (Fig. 1). *Heterorhabditis bacteriophora* was excluded from the database search used to annotate the scaffolds in order to exclude self hits from the published assembly. All large scaffolds clustered congruently with respect to read coverage and CG content. A few (57) scaffolds had best Basic Local Alignment Search Tool nucleotide (BLASTn) matches to phyla other than Nematoda (Table 1). A small amount (5 kb) of likely remaining *P. luminescens* contamination was noted. We identified 100 kb of the genome of a strain of the common culture contaminant bacterium *Stenotrophomonas maltophilia* [22]. Contamination of the assembly with *S. maltophilia* was acknowledged [14], but removal of scaffolds before annotation was not discussed. Two high-coverage scaffolds that derived from the *H. bacteriophora* mitochondrial genome were annotated as "undefined Eukaryota" because of taxonomic misclassification in the NCBI nt database. Many scaffolds with coverages close to that of the expected nuclear genome had best matches to two unexpected sources: the platyhelminths *Echinostoma caproni* and *Dicrocoelium dendriticum* and several hymenopteran arthropods. Inspection of these matches showed that they were due to high sequence similarity to a family of *H. bacteriophora* mariner-like transposons [23]; thus these were classified as *bona fide* nematode nuclear sequences. A group of scaffolds contained what appears to be a *H. bacteriophora* nuclear repeat with highest similarity to histone H3.3 sequences from Diptera and Hymenoptera. The remaining scaffolds had low-scoring nucleotide matches to a variety of chordate, chytrid, and arthropod sequences from deeply conserved genes (tubulin, kinases) but had coverages similar to other nuclear sequences.

Scaffolds with average coverage of less than 10-fold were removed from the assembly (35 scaffolds spanning 132,949 bases, 0.2% of the total span; see Supporting Data [24],

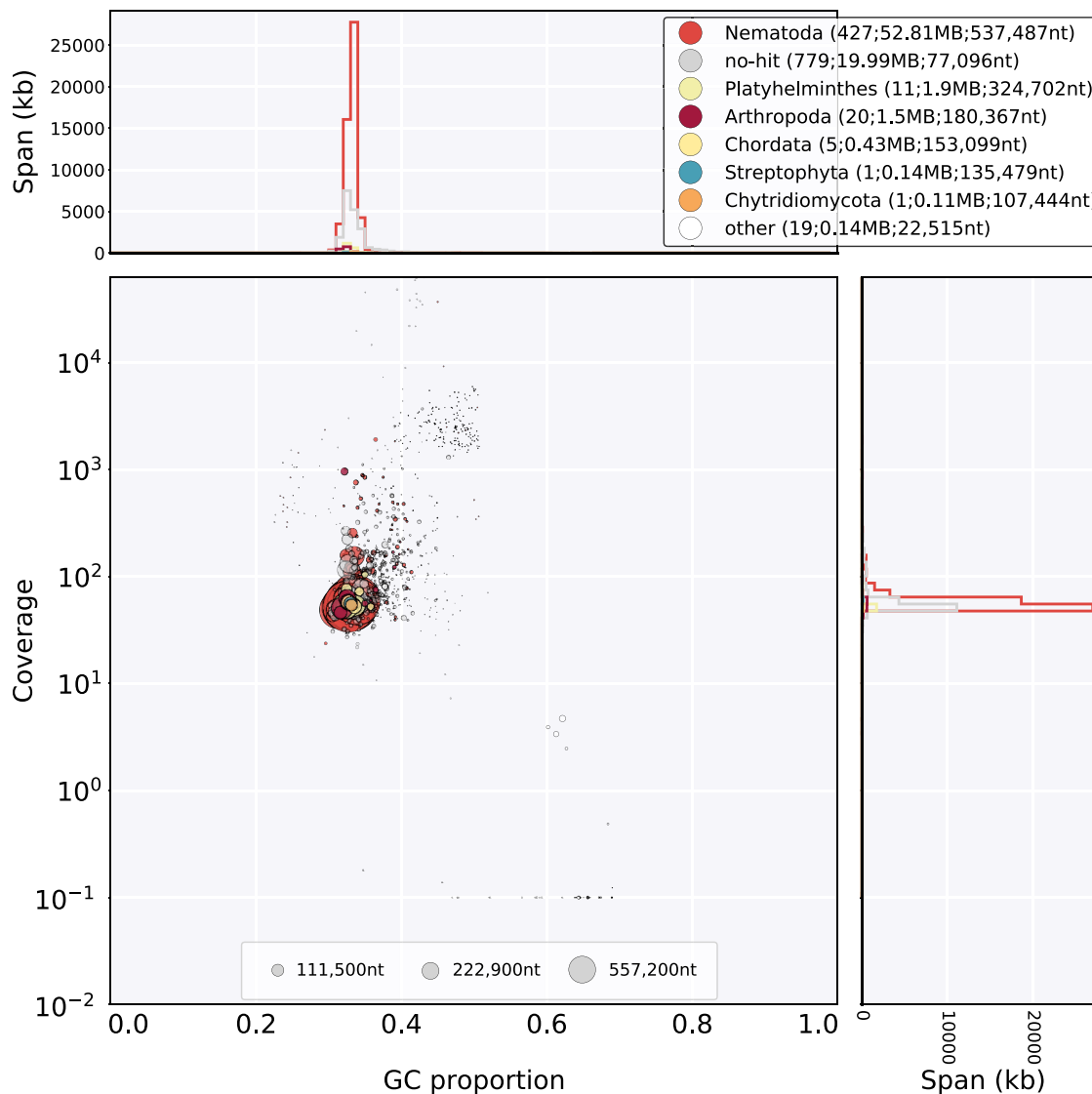


Figure 1: Taxon-annotated GC-coverage plot of the *H. bacteriophora* assembly. Bottom left panel: Each scaffold or contig is represented by a single filled circle. Each scaffold is placed in the main panel based on its GC proportion (X axis) and coverage by reads from the Illumina resequencing project (Y axis). The fill color of the circle indicates the taxon of the top BLASTn hit in the NCBI nt database for that scaffold. The colors are annotated in the top right hand key, which indicates taxon assignment and (in brackets) the number of contigs and scaffolds so assigned, their total span, and their N50 length. The circles are scaled to scaffold length, as indicated in the key at the base of the main panel. Right panel: Nucleotide span in kb at each coverage level. Top panel: Nucleotide span in kb at each GC proportion.

Low_coverage_scaffolds.txt). This removed all scaffolds aligning to *S. maltophilia* and to *Photorhabdus* spp. (104 kb). The origins of the additional 28 kb were not investigated. In the published annotation [14], 76 genes were predicted from these scaffolds.

Improved gene predictions are biologically credible and have unexceptional novelty

New gene predictions were generated from a soft-masked version of the filtered assembly using the RNA-sequencing (RNA-seq)-based annotation pipeline BRAKER1 v1.9 [25], generating 16,070 protein predictions from 15,747 protein-coding genes (see Supporting Data [24], *BRAKER1.soft.masked.output.files.zip*). We compared the soft-masked predictions to those from the published analysis [14] (Fig. 2, Table 2). The predicted proteins from the new BRAKER1/soft-masked gene set were, on average, longer (Fig. 2A). While the average number of introns per gene was

the same in the BRAKER1/soft-masked and published predictions, the BRAKER1/soft-masked gene set had more single-exon genes (Fig. 2B). Hard masking of the genome and re-prediction resulted in fewer single exon genes, suggesting that many of these putative genes could be derived from a repetitive sequence (Supporting Data [24], *BRAKER1.hard.masked.output.files.zip* and *BRAKER1.annotation.comparisons.txt*), but only 316 of the single exon genes from the BRAKER1/soft-masked assembly had similarity to transposases or transposons. The BRAKER1/soft-masked annotations were taken forward for further analysis.

Four-fifths (83.3%) of the published protein-coding gene predictions [14] overlapped to some extent with the BRAKER1/soft-masked predictions at the genome level, with a mean of 67% of the nucleotides of each BRAKER1/soft-masked gene covered by a published gene (Fig. 2C). Half (8,061) of the 15,747 BRAKER1/soft-masked gene predictions had an overlap proportion of ≥ 0.9 with the published predictions. At the level of protein sequence, only

Table 1: Contamination screening of the *H. bacteriophora* assembly

Number of scaffolds	Sum of scaffold spans (bp)	Mean coverage ^a	Best matches in NCBI nt database	Assignment
12	99,556	2.8	<i>Stenotrophomonas maltophilia</i> genome	Bacterial culture contaminant ^b
4	4,709	0.1	<i>Photorhabdus</i> sp. genomes	Symbiont culture contaminant ^b
2	2,144	756.0	Poorly annotated mitochondrial matches	<i>H. bacteriophora</i> mitochondrial fragments
22	3,051,844	69.6	Mariner transposons in Metazoa, especially Hymenoptera and Platyhelminthes	<i>H. bacteriophora</i> nuclear genome mariner transposon family (highest coverage 960-fold)
10	334,100	76.6	Low score match to several histone H3.3 across Metazoa	<i>H. bacteriophora</i> nuclear sequence
7	713,932	56.5	Chance nucleotide matches to conserved genes in other taxa	<i>H. bacteriophora</i> nuclear sequences

^aThe average read coverage of the whole assembly was 85.3.

^bThese scaffolds were removed by the low-coverage filter.

Table 2: Comparison of the published and BRAKER1/soft-masked protein coding gene predictions

Prediction set	Published [14]	BRAKER1/soft-masked
Number of protein coding genes predicted	20,964	15,747
Mean protein length (amino acids)	218.8	344.5
Number of single exon genes	1,728	2,326
Mean number of exons per gene ^a	5.9	7.8
Proportion of noncanonical (GC-AG) introns	8.87%	0.79%
Percentage mapping to publicly available transcriptome reads		
Sanger ESTs	80.45%	84.26%
Roche 454 reads	37.18%	58.03%
BUSCO score for proteome		
Complete	47.8%	94%
Fragmented	34.7%	4.3%
Number of proteins with no hits in Uniref90	8,962	2,889
Protein singletons in clustering	5,442	1,112
Conserved, single-copy orthologues ^b		
Total	2,089	2,330
Missing	377	141
Expanded	184	84

^aNumber of exons: number of coding DNA sequence (CDS) entries per gene for BRAKER1 predictions. CDS features, not exons, are outputted by AUGUSTUS in GFF files.

^bThe list of strict one-to-one orthologues was augmented with protein clusters where 75% of species had single-copy representatives ("fuzzy-1-to-1" orthologues identified by KinFin).

836 proteins were identical between the two predictions, and only 2,099 genes had identical genome start and stop positions.

The BRAKER1/soft-masked and published gene sets were checked for completeness using BUSCO [19], based on the Eukaryota lineage gene set, and *Caenorhabditis* as the species parameter for orthologue finding. The BRAKER1/soft-masked gene set contained a substantially higher percentage of complete and a lower percentage of fragmented BUSCO genes than the published set (Table 2). Two *H. bacteriophora* transcriptome datasets, publicly available Roche 454 data and Sanger expressed sequence tags, were mapped to the published and BRAKER1/soft-masked transcriptomes to assess gene set completeness. This suggested that the BRAKER1/soft-masked transcriptome predictions were more complete than the original (Table 2).

Nearly half (9,893/20,964; 47.2%) of the published proteins were reported to have no significant matches in the NCBI nonredundant (nr) protein database [14]. This surprising result could be due to a paucity of data from species closely related to *H. bacteriophora* in the NCBI nr database at the time of the search,

or to inclusion of poor protein predictions in the published set, or both. Targeted investigation of these 9,893 orphan proteins here was not possible due to inconsistencies in gene naming in the publicly available files. The published and BRAKER1/soft-masked proteomes were compared to the Uniref90 database [26] using DIAMOND v0.9.5 [27] with an expectation value cutoff of $1e^{-5}$. In the published proteome, 8,962 proteins (42.7%) had no significant matches in Uniref90. Thus, a relatively poorly populated database was not the main driver for the high number of orphan proteins reported in the published proteome. In the BRAKER1/soft-masked proteome, only 2,889 proteins (18.3%) had no hits in the Uniref90 database (Table 2).

OrthoFinder v1.1.4 [28] was used to define orthologous groups in the proteomes of 23 rhabditine (Clade V) nematodes (Supporting Data [24], *Orthofinder.analysis*) and just the published *H. bacteriophora* protein-coding gene predictions, or just the BRAKER/soft-masked proteome, or both. All proteins <30 amino acids long were excluded from clustering (see Supporting Data: *Orthofinder.analysis*). We identified 5,442 singletons (26.8% of the

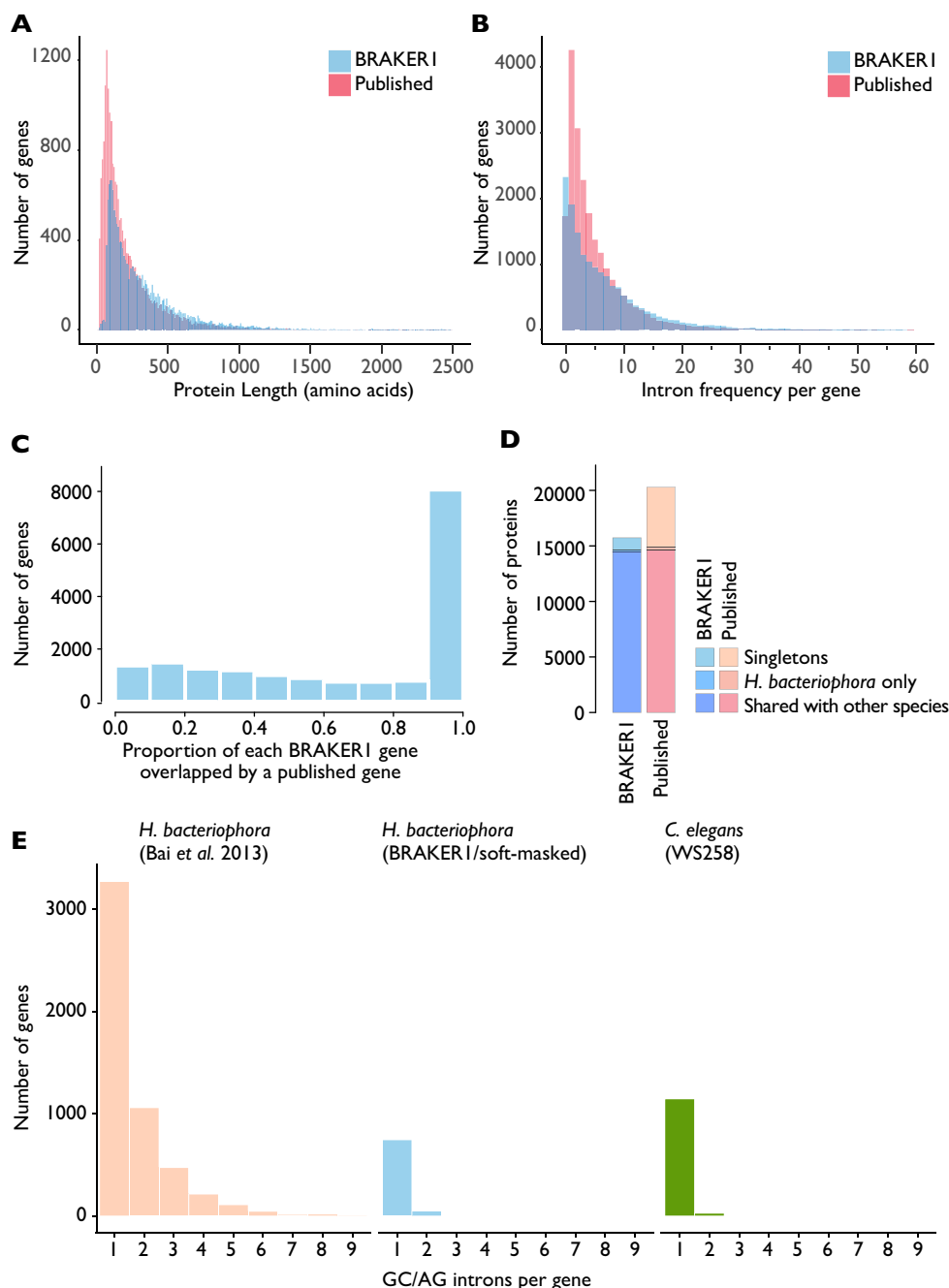


Figure 2: Comparisons of BRAKER1/soft-masked and original gene predictions from *H. bacteriophora*. A, B) Frequency histograms of intron count (A) and protein length (B) in BRAKER1/soft-masked (blue) and published (yellow) protein coding gene predictions. Outlying proteins longer than >2,500 amino acids ($n = 40$) or genes containing >60 introns ($n = 20$) are not shown. C) Frequency histogram of the proportion of each BRAKER1 gene prediction overlapped by a published gene prediction at the nucleotide level. D) Comparison of singleton, proteome-specific, and shared proteins in the published and BRAKER1/soft-masked protein sets. E) Counts of noncanonical GC/AG introns in gene predictions from the published and BRAKER1 *H. bacteriophora* gene sets and the model nematode *Caenorhabditis elegans* (WS258). Counts are of genes containing at least one noncanonical GC/AG intron with the specified number of noncanonical introns.

proteome) when the analysis included only the published *H. bacteriophora* protein set. An additional 248 proteins formed *H. bacteriophora*-specific orthogroups. Orthology analysis including only the BRAKER/soft-masked protein set predicted 1,112 *H. bacteriophora* singletons (7.1% of the proteome) with 167 proteins in *H. bacteriophora*-specific orthogroups (Fig. 2D). In comparison, when the orthology analysis included the BRAKER1/soft-masked predictions, there were 1,858 *C. elegans* singletons (9.2%

of the *C. elegans* proteome). Very few universal, single-copy orthologues were defined in either analysis. Exploring “fuzzy-1-to-1” orthogroups (where true 1-to-1 orthology was found for more than 75% of the 24 species, i.e., 18 or more species), the published protein predictions had more missing fuzzy-1-to-1 orthologues than did the BRAKER1/soft-masked predictions (Table 2). In the clustering that included both proteomes, 2,019 clusters contained more proteins from the BRAKER1/soft-masked than

the published proteome, whereas 2,714 contained a larger number contributed from the published than from the BRAKER1/soft-masked proteome (Supporting Data [24], kinfin.zip).

The published *H. bacteriophora* gene set had additional peculiarities. The published set of gene models included 102,274 introns, 9,069 of which (8.9%) had noncanonical splice sites (i.e., 5' GC—AG 3'). Some of the genes in the published gene set had up to nine noncanonical introns (Fig. 2E). In the BRAKER1/soft-masked gene set, there were 109,767 introns, 868 (0.8%) of which had noncanonical splice sites. This proportion is in keeping with that found in most other rhabditine nematodes. For example, the extensively manually annotated *C. elegans* has 2,429 (0.6%) noncanonical (5' GC—AG 3') introns. In *C. elegans* noncanonical introns are frequently found only in alternately spliced, and shorter, isoforms, and more than 93–99% were in genes that had homologues in other species, depending on the species used in the protein orthology clustering. However, in the published *H. bacteriophora* gene set, 34–49% of the genes with GC—AG introns were in *H. bacteriophora*-unique proteins.

A supermatrix maximum likelihood phylogeny was generated from the fuzzy-1-1 orthologues in the clustering that included both *H. bacteriophora* proteomes (Fig. 3; see Supporting Data [24], *Phylogenetic analyses*). The phylogeny, rooted with *Pristionchus* spp., shows the *H. bacteriophora* proteomes as sisters. However, the BRAKER1/soft-masked proteome has a shorter branch length to *Heterorhabditis*' most recent common ancestor with other Clade V nematodes, suggesting that the published proteome includes uniquely divergent sequences.

The secretome of *H. bacteriophora* has been of particular interest as it may contain proteins involved in symbiotic interactions with *P. luminescens* and proteins crucial to invasion and survival within the insect hemocoel. In the original publication, only 603 proteins (2.8% of the proteome) were predicted to be secreted [14]. This proportion is much lower than in free-living nematodes, such as *C. elegans*, and it was postulated that *H. bacteriophora* relies on *P. luminescens* for secreted effectors [14]. The signal peptide detection method used in the original analyses was not described [14]. We used SignalP version 4.1 within Interproscan to annotate proteins in both the BRAKER1 and published *H. bacteriophora* proteomes. Proteins having a predicted signal peptide but no transmembrane domain were classified as secreted. We identified 1,023 (6.5%) putative secreted proteins in the BRAKER1/soft-masked proteome and 1,067 (5.1%) in the published proteome. By the same method, other rhabditine (Clade V) nematodes that do not have known symbiotic associations with bacteria, such as *Teladorsagia circumcincta*, had secretome sizes comparable to that of *H. bacteriophora* (Supporting Data [24], *Secretome analysis.txt*). This suggests that *H. bacteriophora* does not have a reduced secretome compared to other, related nematodes that do not have symbiont partners.

Interproscan was also used to annotate the BRAKER1 and published proteomes by identifying matches against the databases TIGRFAM v15.0, ProDom v2006.1, SMART-7.1, PrositePatterns v20.119, PRINTS v42.0, SuperFamily v1.75, Pfam v29.0, and PrositeProfiles v20.119. The BRAKER1 proteome had a greater number of proteins annotated, with at least one domain compared to the published proteome, and a greater number of total domains identified (Supporting Data [24], *IPR.domain.analysis.txt*).

Discussion

Assembly of and gene finding in new genomes are challenging tasks, especially in larger genomes and those phylogenetically distant from any previously analyzed exemplar. When applied *de novo* to datasets from extremely well-assembled and well-annotated model species, even the best methods fail to recover fully contiguous assemblies and yield predicted gene sets that have poor correspondence with the known truth [29]. A major issue with primary assemblies and gene sets arises when exceptional findings are taken at face value and used to assert exceptional biology in a target species [30]. Where these exceptions are, in fact, the result of methodological failings, the scientific record, including public databases, becomes contaminated. At best, erroneous assertions can be quickly checked and corrected, but at worst, they can mislead and inhibit subsequent work.

A second concern arises from the recognition that while no method can currently produce perfect assemblies and perfect gene sets from raw data, analyses using the same tool sets will resemble each other and reflect the successes and failings of the particulars of the algorithms used. However, when comparing genome assemblies and gene sets produced by different pipelines, it may be that the disparity in output generated by different pipelines dominates any signal from biology. Genomes assembled and annotated with the same tools will look more similar, and in a pool of assemblies and protein sets, the one species that used a variant process will be flagged as exceptional. Again, the model organisms show the way; as new data and new scrutiny are added to the genome, better and better analyses are available. With additional analysis and additional independent data, genome and gene predictions can be improved markedly for any species [31].

Here, we examined the “outlier” whole-genome protein predictions from the entomopathogenic nematode *H. bacteriophora* [14]. The original publication noted that the number of novel proteins (those restricted to *H. bacteriophora*) was particularly large while the number of secreted proteins was rather small and suggested that these genome features might be a result of evolution to the species' novel lifestyle (which includes an essential symbiosis with the bacterium *P. luminescens*). Overall, we found that while the published genome sequence had a small amount of bacterial contamination and a small number of “nematode” genes were predicted from these contaminants, the assembly itself was of high quality. Our re-prediction of the gene set of *H. bacteriophora*, however, suggested that the excess of unique genes, the lack of secreted proteins, and several other surprising features of the original gene set were likely to be artifacts of the gene prediction pipeline chosen. While our gene set was by no means perfect (e.g., we identified an excess of single exon genes that derive from likely repetitive sequence), it had better biological completeness and credibility.

We used the RNA-seq-based annotation pipeline BRAKER1 [25], not available to the authors of the original genome publication, who used JIGSAW [15] (see Supplementary File 1). While JIGSAW achieved high sensitivity and specificity at the level of nucleotide, exon, and gene predictions in the nematode genome annotation assessment project, nGASP [29], direct comparison of the sensitivity and specificity of JIGSAW and BRAKER1 has not been published to the best of our knowledge. BRAKER1 has been shown to give superior prediction results over *ab initio* GeneMark-ES and *ab initio* AUGUSTUS alone [25]. In particular, BRAKER1 is able to better use transcriptome data for gene finding. While we supplied only a partial Roche 454 transcriptome to BRAKER1, the resulting gene set has much improved numerical

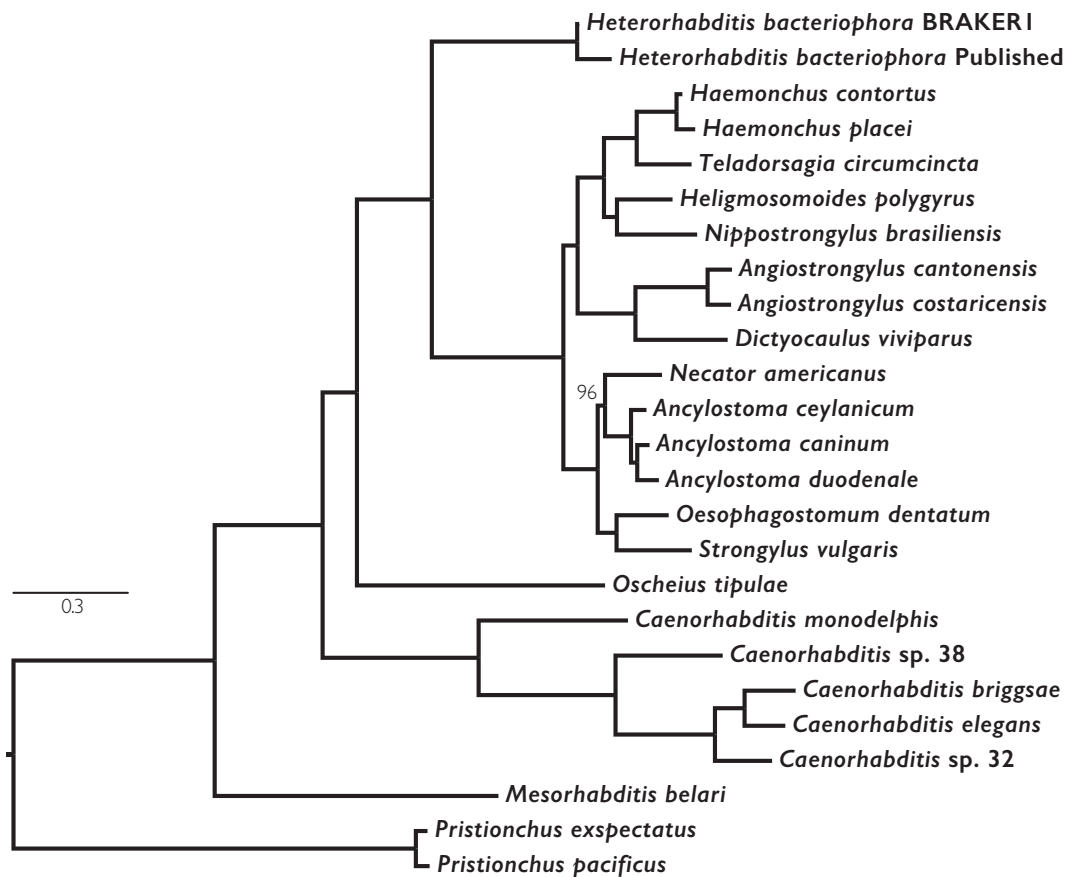


Figure 3: Maximum likelihood phylogeny of selected rhabditine (Clade V) nematodes. A supermatrix of aligned amino acid sequences from orthologous loci from both *H. bacteriophora* predictions and a set of 23 rhabditine (Clade V) nematodes (see Supporting Data, *Orthofinder.analysis*) were aligned and analyzed with RaxML using a PROTGAMMAGTR amino acid substitution model. *Pristionchus* spp. were designated as the outgroup. Bootstrap support values (100 bootstraps performed) were 100 for all branches except one.

and biological scores. In particular, we note that the biological completeness of the predicted gene set now matches that of the genome sequence from which it was derived (Table 2).

The published gene set had an unusually high proportion (8.9%) of noncanonical (5' GC—AG 3') introns. While most genomes have a low proportion of noncanonical introns (usually approximately 0.5% of all introns), some species have markedly higher proportions [20]. The high proportion found initially in *H. bacteriophora* could perhaps have been taken as a warning that the prediction set was of concern. We note that gene predictors can be set to disallow any predictions that require noncanonical splicing, and many published genomes have zero noncanonical introns. These gene prediction sets are likely to categorically miss true noncanonically spliced genes.

The new BRAKER1 gene prediction set had many fewer species-unique genes (7.1%) than did the original (42.7%) when compared to 23 other related nematodes. We regard this reduction in novelty as indicative of a better prediction. For example, *C. elegans*, the best-annotated nematode genome, had only 9.2% of species-unique genes in our analysis. Having a large proportion of orphan proteins is not unique to the published *H. bacteriophora* predictions. Nearly half (47%) of the gene predictions in *Pristionchus pacificus* were reported to have no homologues in 15 other nematode species [32]. Evaluation of proteomic and transcriptomic evidence, as well as patterns of synonymous and nonsynonymous substitution, suggested that as many as 42–

81% of these genes were, in fact, expressed [33]. Therefore, the high proportion of orphan genes in *H. bacteriophora* is not *prima facie* evidence of poor gene predictions. Expanded transcriptomic and comparative data are needed to build on the work we have presented in affirming the true *H. bacteriophora* gene set.

Biological pest control agents may become increasingly important for ensuring crop protection in the future [34]. A number of factors currently limit the commercial applicability of *H. bacteriophora*, including their short shelf life, susceptibility to environmental stress, and limited insect tropism [13, 35]. Accurate genome annotation will assist in the analysis of *H. bacteriophora*, facilitating the exploration of genes involved in its parasitic and symbiotic interactions and supporting genetic manipulation to enhance its utility as a biological control agent.

Methods

Methods supplementary note

A detailed description of the command lines used in the generation of the BRAKER1 gene predictions and the associated analysis can be found in Supplementary File 2.

Contaminant screening and removal of low-coverage scaffolds

The assembly scaffolds were aligned to the NCBI nt database, release 204, using Nucleotide-Nucleotide BLAST v2.6.0+ (available at [36]) in megablast mode, with an e-value cutoff of $1e^{-25}$ and a culling limit of 2 [37]. *Heterorhabditis bacteriophora* hits were excluded from the search using a list of all *H. bacteriophora*-associated gene identifiers downloaded from NCBI GenBank nucleotide database, release 219. Raw, paired-end Illumina reads from the resequencing project were mapped against the assembly, as paired, using Burrows-Wheeler Aligner (BWA) v0.7.15 (available at [38]) in mem mode with default options [39]. The output was converted to a BAM file using Samtools v1.3.1 (SAMTOOLS, [RRID:SCR.002105](#)) [40]; overall mapping statistics were generated in flagstat mode.

Blobtools v0.9.19 [21] was used to create taxon annotated GC-coverage plots for the published assembly, using the Nucleotide-Nucleotide BLAST and raw read mapping results. Scaffolds that did not have Nematoda as a top BLAST hit at the phylum level were identified, and the species-level top BLAST hit, length of scaffold, and scaffold mean base coverage were extracted from the Blobology output. Scaffolds with a mean base coverage of <10x were identified from the output of the Blobology pipeline and removed from the assembly. A list of excluded scaffolds is available in Supporting Data [24]; *Low.coverage.scaffolds.txt*.

Generation of BRAKER1 gene predictions

Before annotation, the published assembly was soft-masked for known Nematoda repeats from the RepeatMasker Library v4.0.6 using RepeatMasker v4.0.6 (RepeatMasker, [RRID:SCR.012954](#)) [41] with default options. The two publicly available Roche 454 RNA-seq data files were adaptor and quality-trimmed using BBDuk v36.92 (unpublished tool kit from Joint Genome Institute, n.d.). Reads below an average quality of 10 or shorter than 25 nucleotides were discarded. Regions with average quality below 20 were trimmed. The cleaned reads were mapped to the soft-masked assembly using STAR v2.5 (STAR, [RRID:SCR.015899](#)) with default options [42, 43]. The soft-masked assembly was annotated with BRAKER1 v1.9 [25] with guidance from the mapping output from STAR. An identical annotation method was applied to a hard-masked version of the assembly. The assembly was hard-masked for known Nematoda repeats from the RepeatMasker Library v4.0.6 using RepeatMasker v4.0.6 with default options. The published and BRAKER1 proteomes were compared using DIAMOND v0.9.5 [27] in BLASTP mode to the Uniref90 database (release 03/2017) [26] with an expectation value cutoff of $1e^{-5}$ and no limit on the number of target sequences. Hits to *H. bacteriophora* proteins were removed using its TaxonID.

Gene prediction statistics

Gene-level statistical summaries were calculated, including only the longest isoforms of the BRAKER1 gene predictions. The longest isoform for each gene in the BRAKER1 *H. bacteriophora* annotation was identified from the general feature format (GFF) file and then selected from the protein FASTA files. The GFF file for the published gene predictions did not contain any isoforms and was analyzed in its entirety. *f* Introns were inferred for the published GFF file using GenomeTools v1.5.9 in -addintrons mode [44]. Intron frequencies were then calculated for the published and BRAKER1 annotations from their respective GFF files. Exon frequencies were calculated for the published annotations

directly from the GFF file. For the BRAKER1 annotations, exon frequency per gene was assumed to be equivalent to coding DNA sequence (CDS) frequency and inferred from the GFF file, as exon features were not included in the GFF file. Intron frequency histograms and bar plots were generated in Rstudio v1.0.136 (RStudio, [RRID:SCR.000432](#)) with R v3.3.2 (R Project for Statistical Computing, [RRID:SCR.001905](#)) and, in some instances, the package ggplot2 v2.2.1. As intron frequency lists did not contain single exon genes (those with no introns), these were added manually to the intron frequency lists in Microsoft Excel before importing the data into Rstudio.

The proportion of introns with GC—AG splice junctions was assessed for the gene models of *C. elegans* (WS258) and the published and BRAKER1/soft-masked gene models of *H. bacteriophora*. Intronic features were added to general feature format version 3 (GFF3) files using GenomeTools v1.5.9 [44] (“gt gff3 -sort -tidy -retainids -fixregionboundaries -addintrons”), and splice sites were extracted using the script extractRegionFromCoordinates.py [20]. Results were visualized using the script plot_GCAG_counts.R (available at [45]).

Gene features, extracted from the GFF files, were assessed for overlap using bedtools v2.26 (BEDTools, [RRID:SCR.006646](#)) in intersect mode [46]. Only genes on the same strand were considered to be overlapping. To calculate the number of identical proteins shared between the published and BRAKER1 proteomes, nonredundant protein fasta files were generated using cd-hit v4.6.1 (CD-HIT, [RRID:SCR.007105](#)) [47] for the BRAKER1 and published predictions. The files were concatenated, sorted, and unique sequences counted using unix command line tools.

BUSCO v2.0.1 (BUSCO, [RRID:SCR.015008](#)) [19], with Eukaryota as the lineage dataset and *Caenorhabditis* as the species parameter for orthologue finding, was applied to both proteomes and the published assembly to calculate BUSCO scores. CEGMA (CEGMA, [RRID:SCR.015055](#)) [18] was run on the published genome sequence. BWA was used with default settings to map the RNA-seq datasets (the Sanger expressed sequence tags (ESTs) in assembled form) to the CDS transcripts from the published and BRAKER1 annotations and the summary statistics obtained with Samtools v1.3.1 in flagstat mode.

Protein orthology analyses

OrthoFinder v1.1.4 [28] with default settings was used to identify orthologous groups in the proteomes of 23 Clade V nematodes with the addition of either the BRAKER1/soft-masked and published *H. bacteriophora* proteomes separately or simultaneously. The proteomes for the 23 Clade V nematodes were downloaded from WBPS8 (available at [48]) or GenomeHubs.org (available at [49]); detailed source information is available in the Supporting Data [24], *Secretome.analysis.txt*. All proteomes were filtered to contain only the longest isoform of each gene, and for all proteomes (except the BRAKER1/soft-masked *H. bacteriophora* protein set), proteins less than 30 amino acids in length were excluded before clustering. For the *H. bacteriophora* BRAKER1/soft-masked protein set, proteins less than 30 amino acids in length (SF5.2) were removed manually from the orthofinder clustering statistics after clustering. None of these proteins seeded new clusters and therefore will not have influenced the clustering results. Kinfn v0.9 [50] was used with default settings to identify true and fuzzy 1-to-1 orthologues and their associated species-specific statistics. Fuzzy 1-to-1 orthologues are true 1-to-1 orthologues for greater than 75% of the species clustered. For the clustering analysis presented in Supporting Data [24], *Orthofinder.analysis*, the BRAKER1/soft-masked and published pro-

teomes were clustered simultaneously to the 23 other Clade V nematode proteomes; singletons and species-specific clusters were excluded.

Interproscan and search for transposons

Interproscan v5.19–58.0 (Interproscan, [RRID:SCR.005829](#)) [51] was used in protein mode to identify matches in the BRAKER1 and published proteomes in the following databases: TIGRFAM v15.0, ProDom v2006.1, SMART-7.1, SignalP-EUK v4.1, PrositePatterns v20.119, PRINTS v42.0, SuperFamily v1.75, Pfam v29.0, and PrositeProfiles v20.119. For secretome analysis of the 23 Clade V nematodes, Interproscan v5.19–58.0 was run against the SignalP-EUK v4.1 database alone. InterProScan was run with the option for all match calculations to be run locally and with gene ontology annotation activated. The number of single exon genes with similarity to transposons or transposases in the BRAKER1/soft-masked predictions was calculated by searching the full InterProScan results for the strings “Transposon,” “transposon,” “Transposase,” or “transposase” and counting the number of single exon gene InterProScan results containing these terms. InterProScan results from searching the SignalP-EUK-4.1 database were queried to identify putative secreted proteins. Those with a predicted signal peptide but no transmembrane region were considered to be secreted.

Phylogenetic analyses

Both *H. bacteriophora* proteomes were clustered simultaneously with the 23 Clade V nematode proteomes into orthologous groups using Orthofinder v1.0 [28]. The fuzzy 1-to-1 orthologues were extracted and processed using GNU parallel [52]. They were aligned using MAFFT v7.267 (MAFFT, [RRID:SCR.011811](#)) [53], and the alignments trimmed with NOISY v1.5.12 [54]. A maximum likelihood gene tree was generated for each orthologue using RAxML v8.1.20 (RAxML, [RRID:SCR.006086](#)) with a PROTGAM-MAGTR amino acid substitution model [55]. Rapid Bootstrap analysis and search for the bestscoring maximum-likelihood (ML) tree within one program run with 100 rapid bootstrap replicates was used. The trees were pruned using PhyloTreePruner v1.0 [56] to remove paralogues, with 0.5 as the bootstrap cut-off and a minimum of 20 species in the orthogroup after pruning for inclusion in the supermatrix. Where species had more than one putative orthologue in an orthogroup, the longest was selected. The remaining 897 orthogroups were re-aligned using MAFFT v7.267, trimmed with NOISY v1.5.12, and concatenated into a supermatrix using FASconCAT v1.0 [57]. A supermatrix ML tree was generated using RAxML with the rapid hill climbing algorithm (default), with a PROTGAMMAGTR amino acid substitution model and 100 bootstrap replicates. *Pristionchus* spp. were designated as the outgroup. The tree was visualized in Dendroscope v3.5.9 [58].

Input data and data availability

The *H. bacteriophora* genome and annotations [14] were downloaded from Wormbase Parasite (WBPS8) (see Supporting Data [24], *Publicly-available-assembly-details.txt*). The ESTs [59, 60] were obtained from NCBI dbEST [61] (accessions listed in Supporting Data [24], *EST.acc.txt*); the assembled versions used in the analysis are available in the Supporting Data [24], *EST.assembled.fas*. Roche 454 transcriptome data [14] were obtained from the Short Read Archive (accession numbers SRX001441 and SRX001440). *Heterorhabditis bacteriophora* strain Gebre, a gift from Adler Dill-

man, was inbred by selfing single hermaphrodites for five generations to generate the strain G2a1223. New Illumina HiSeq2000, paired-end, 75 base data were generated from *H. bacteriophora* G2a1223 genomic DNA by the Millard and Muriel Jacobs Genetics and Genomics Laboratory at Caltech (Short Read Archive accession number SRP135845).

The revised gene annotations for *H. bacteriophora* have been submitted to Zenodo [62]. The supporting data for this manuscript are available via the GigaScience repository, GigaDB [24].

Availability of supporting data

The Supporting Data [24, 62] for this work are described below: augustus.aa: BRAKER1/soft-masked annotations of *Heterorhabditis bacteriophora*. The amino acid sequences of the protein predictions in FASTA format.

augustus.gff: BRAKER1/soft-masked annotations of *Heterorhabditis bacteriophora*. The GFF format file.

augustus.gtf: BRAKER1/soft-masked annotations of *Heterorhabditis bacteriophora*. The GTF format file.

augustus.hm.aa: BRAKER1/hard-masked annotations of *Heterorhabditis bacteriophora*. The amino acid sequences of the protein predictions in FASTA format.

augustus.hm.gff: BRAKER1/hard-masked annotations of *Heterorhabditis bacteriophora*. The GFF format file.

augustus.hm.gtf: BRAKER1/hard-masked annotations of *Heterorhabditis bacteriophora*. The GTF format file.

Blobtools.coverage-analysis.txt: COV file (raw output from the blobology pipeline) detailing the base/read coverage of the published assembly with reads from the re-sequencing project. Text file.

BRAKER1.annotation-comparisons.txt: Comparison of the BRAKER1/soft-masked and BRAKER1/hard-masked gene predictions from *Heterorhabditis bacteriophora*. Tab-delimited text file.

Contaminant.scaffolds.txt: A list of the scaffolds/contigs identified by contamination screening and presented in Table 1. Text file.

EST.acc.txt: Accession numbers for the publically available ESTs used for the EST assembly. Text file.

EST.assembled.fas: Assembled ESTs derived from the publically available ESTs detailed in EST.acc.txt. FASTA .fas format file.

HBACT.BRAKER1.signalPNoTM.txt: Secretome predictions from the BRAKER1/soft-masked predictions. Text file.

HBACT.published.signalPNoTM.txt: Secretome predictions from the published Bai et al. (2013) protein predictions. Text file.

Individual.gene.alignments: Alignments of orthogroups used to build the supermatrix. Directory of aligned sequences in fasta format.

IPR.domain.analysis.txt: Comparative Interproscan statistics. Text file.

kinfin.zip: KinFin analyses from the OrthoFinder analyses of *Heterorhabditis bacteriophora* predicted proteomes. Zipped archive (42.6 Mb).

Low.coverage.scaffolds.txt: Scaffolds and contigs removed from the *Heterorhabditis bacteriophora* assembly because of low coverage in the new whole genome sequencing dataset. Text file.

Newick.tree.txt: Phylogenetic analysis output files. NEWICK format text file.

Orthofinder.zip: The OrthoFinder output files. A zipped archive of the three OrthoFinder clustering result files (published *H. bacteriophora* + 23 species; BRAKER1/soft-masked + 23

species: published + soft-masked + 23 species). Zipped archive (20.9 Mb)

Orthogroup_count_ratios.txt: Table with count of orthogroups at each contribution ratio from the BRAKER1/soft-masked and published proteomes after clustering with 23 other Clade V nematodes. Empty cells denote contribution combinations with no orthogroups. Text file.

Proteomes_in_clustering.txt: A list of the proteomes included in the OrthoFinder analyses. Text file.

Publicly_available_assembly_details.txt: Details of the published, publicly available *Heterorhabditis bacteriophora* genome assembly re-analysed in this study using BRAKER1. Text file.

Scaffolds_included.txt: Scaffolds and contigs in the *Heterorhabditis bacteriophora* assembly included in re-annotation and further analysis. Text file.

Secretome_analysis.txt: Secretome statistics for 23 Clade V nematodes. Text file.

Short BRAKER1_genes_list.txt: List of *Heterorhabditis bacteriophora* proteins of length <30 amino acids excluded from the OrthoFinder analyses. Text file

Supermatrix.fas: Supermatrix of aligned sequences. FASTA .fas format file.

Additional files

Supplementary file 1: BRAKER1 and JIGSAW annotation pipelines. Figure illustrating the differences between the BRAKER1 and the Bai et al 2013 JIGSAW prediction methods used for *Heterorhabditis bacteriophora*. PDF file.

Supplementary file 2: Methods Supplementary Note. A note detailing the command lines used in the generation of the BRAKER1 gene predictions, and the associated analysis. PDF file.

Abbreviations

BUSCO: Benchmarking Universal Single-Copy Orthologs; CDS: coding DNA sequence; CEGMA: Core Eukaryote Gene Mapping Approach; GFF: general feature format; IJ: infective juvenile; ML: maximum likelihood; NCBI: National Center for Biotechnology Information; nr: nonredundant; nt: nucleotide; RNA-seq: RNA sequencing.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Conceptualization, M.B.; methodology, F.M., D.B., and M.B.; formal analysis, F.M., D.R.L., and M.B.; supervision, M.B.; writing the original draft, F.M. and D.R.L.; writing, review, and editing, F.M., M.B., D.R.L., D.B., and H.T.S.; resources, H.T.S.

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