New gene annotations for three pea aphid genome assemblies allow comparative analyses of genes and gene family evolution

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Abstract: Reliable genome annotation is crucial for analyses of gene function, conservation, and evolution. Factors such as the sequencing technology used to create the assembly and the amount of duplicated sequence within the genome of interest can have a large impact on the quality of gene annotations. In particular, short read-based assemblies tend to mis-assemble duplicated genes as single loci, a problem that requires additional long read sequencing to resolve. Pea aphids exhibit a high level of gene duplication, resulting in mis-assembly and mis-annotation of genes in the short read reference genome. Here, we re-annotate the pea aphid reference genome, along with two long read pea aphid genomes, to facilitate future analyses of gene duplication and function in pea aphids. We use an integrated approach, consolidating both *ab initio* and RNAseq-based annotations into unified gene models. The new annotations contain genes that were missing, mis-annotated, or mis-assembled in the reference, and are generally consistent across assemblies, showing very good agreement between the long read assemblies. Our annotation method is sensitive enough to refine existing gene models, uncovering alternatively used promoters and isoforms, and aids in finding gene duplications. These data provide a useful supplement to the existing reference annotations and a new comparative framework for discovery and analysis of gene function and duplication in this important emerging model insect.

Keywords: gene, annotation, discovery, duplication, paralogs, RNAseq, pea aphids

Introduction

Proper assembly and annotation of genomes is crucial for bioinformatic and molecular biology investigations into gene function and evolution. Arguably one of the most important processes in gene family evolution, gene duplication, presents a unique challenge for the proper assembly and annotation of genes. The method(s) by which a genome assembly is produced have a substantial impact on the accuracy of gene duplicate assembly and annotation. One major deciding factor in accuracy of assembly at the level of individual gene is the choice of read type. Illumina short reads (50-300bp) are readily obtained in high numbers in a cost-effective manner. However, short-read based genome assemblies are the most prone to mis-assemble duplicated genes, especially for recent duplications (Alkan et al., 2011; Mastretta-Yanes et al., 2014; Wang et al., 2021). Long read technologies such as Pacific Bioscience (Eid et al., 2009) or Oxford Nanopore Technologies (Jain et al., 2016), provide the most accurate assembly of overall gene structure, even in the trickiest cases of recent or partial gene duplication (Fadaie et al., 2021; Logsdon et al., 2020; Nguyen, 2024; Showpnil et al., 2024).

Pea aphids (*Acyrthosiphon pisum*) are an important insect model for studying gene duplication (Fernández et al., 2020), the molecular genetic basis of morphological evolution (Li et al., 2020), and aphid-bacterial symbiont interactions (Price et al., 2011), but these types of studies can be hindered by mis-assembly within the short-read pea aphid reference genome (Li et al., 2019). For instance, new long read genome assemblies from winged and wingless males were required to properly assemble and identify the *api* region on the X chromosome controlling male wing dimorphism (Braendle et al., 2005; Li et al., 2020). This region was mis-assembled in the short-read reference genome (Li et al., 2019), but the assembled long read genome revealed the presence of a duplicated gene (*follistatin-3*) as part of an insertion specific to wingless males (Li et al., 2020). More recently, we investigated aphid-specific duplications of wing gene regulatory network components and found that a number of these newly identified paralogs were incorrectly annotated in the reference genome (Saleh Ziabari et al., 2025a). These instances of mis-assembly and mis-annotation highlight the need for higher quality genome assemblies and annotations in this species.

Multiple methods exist for gene annotation, each with their own advantages and drawbacks. Ab initio methods such as AUGUSTUS (Stanke and Morgenstern, 2005) allow annotation of genes from raw genome sequence by predicting groups of exons that code for proteins. While these

methods require no additional data beyond the assembled genome to perform gene annotation, the accuracy and completeness of the resulting annotation may suffer as a result. This accuracy can be increased by providing RNAseq data to better train models of splice sites using BRAKER1 (Hoff et al., 2016). Other RNA-seq based methods of transcript discovery such as genome-guided Trinity (Grabherr et al., 2011) or StringTie (Pertea et al., 2015) utilize read sequence and/or coverage to identify exons and intron-spanning reads to determine how they are spliced into transcripts. These methods can be highly accurate and provide more complete gene models, but they can only identify genes expressed in RNA-seq input (*i.e.* tissue-specific data will provide fewer genes than whole-body data). In addition, Trinity can also assemble transcriptomes *de novo* purely from RNAseq reads (the initial intended use of the software), potentially uncovering genes that are missing or mis-assembled in a given genome (Grabherr et al., 2011).

The software PASA takes a more unified approach, integrating Trinity *de novo*, Trinity genome-guided, BRAKER1, and StringTie transcript predictions into clustered gene models (Haas et al., 2008). These transcripts are first filtered for protein coding potential, quality of alignment to the genome, and intron size before being clustered into genes based on overlap (Haas et al., 2008). The advantage conferred by using PASA is that the complementary strengths of *ab initio* and RNAseq-based methods work synergistically when combined into a unified annotation.

Here we worked to develop a comprehensive reference annotation for the pea aphid genome. To do this, we integrated RNA-seq data from many morphs (asexual females, sexual females, males; winged and wingless when available) and developmental stages (embryos, different nymphal instars, adults) with the reference genome (Li et al., 2019), as well as the high-accuracy long-read assemblies which we previously published (Li et al., 2020). We then used Trinity genome-guided, Stringtie, and BRAKER1 to individually predict genes, followed by PASA to combine those individual annotation sets.

Methods

A summary diagram of the workflow described below is provided (**Supplemental Figure S1**).

Trinity *de novo* transcriptome assembly.

Only paired-end reads were used to generate the Trinity *de novo* assembly (Grabherr et al., 2011). Reads were first adapter and quality-trimmed with Trimmomatic v0.39 (Bolger et al., 2014) in paired-end mode using the parameters “ILLUMINACLIP: TruSeq3-PE-2.fa:2:30:10 SLIDINGWINDOW:4:5 LEADING:5 TRAILING:5 MINLEN:25”. Reads were then randomly subsampled to 2% using the view method in Sambamba v0.6.6 (Tarasov et al., 2015) with the parameter “-s 0.02”. Trinity (v2.15.1) *de novo* assembly of transcripts was then performed in singularity-ce v3.11.4 (Kurtzer et al., 2017) using the image file trinityrnaseq.v2.15.1.simg and the parameters “--normalize_by_read_set --no_parallel_norm_stats --min_kmer_cov 2”.

Read mapping and RNAseq-based transcript assembly.

Both single-end and paired-end samples were used for RNA-seq based gene annotation methods. Read accessions (Grantham et al., 2020; Purandare et al., 2014; Saleh Ziabari et al., 2025b) are found in **Supplemental Table S1**). All of the following steps were performed on Winged Long Read, Wingless Long Read (Li et al., 2020), and Modified Reference (Li et al., 2019; Saleh Ziabari et al., 2025b) genome assemblies. Reads were aligned to each assembly with Hisat2 v2.2.1 (Kim et al., 2019) using default mapping parameters. The .sam files for each replicate from each sample were converted to .bam, sorted, merged, and indexed using SAMtools v1.13 (Li et al., 2009). Duplicate reads were removed from the merged replicate .bam files using the markdup function of Sambamba v0.6.6. These files were then each randomly subsampled at a rate of 20% using the view method of Sambamba with the parameter “-s 0.2”. These merged-replicate, duplicate-less, subsampled .bam files were then merged using SAMtools and used for all RNAseq-based annotation methods. Braker was run in ET (Hoff et al., 2016) mode on the respective processed .bam file for each assembly in singularity-ce v3.11.4 (Kurtzer et al., 2017) using the image braker3.sif (Gabriel et al., 2024). Genome-guided transcriptome assembly was performed with these files for each assembly in Trinity v2.15.1 (Grabherr et al., 2011), also run in singularity-ce v3.11.4 with the image trinityrnaseq.v2.15.1.simg, using the flag “--genome_guided_bam” and the parameter “--genome_guided_max_intron 200000”. Transcripts were mapped to the genome using GMAP v2021-08-25 (Wu and Watanabe, 2005). StringTie v2.2.3 (Pertea et al., 2015) was run using default parameters and transcript sequences were extracted from genomic intervals using GffRead v0.12.7-2 (Pertea and Pertea, 2020).

Integrated gene clustering of transcripts using PASA.

Transcript files from Trinity *de novo* (Trinity-DN) and genome-guided (Trinity-GG) assembly, and genomic coordinate files output from StringTie and Braker, were filtered and clustered into genes using PASA v2.5.3 (Haas et al., 2008) for all three assemblies. Following extraction of Trinity-DN accessions, clustering was run in singularity-ce v3.11.4 using the image file pasapipeline.v2.5.3.simg with the following parameters: "--create --run -I 100000 --ALIGNERS gmap --ALT_SPLICE –TRANSDECODER". The final output included nucleotide sequences and genomic intervals for high-confidence protein-coding transcript isoforms with a maximum intron size of 100kb clustered into 'genes'.

Protein prediction, BUSCO analysis, and ortholog clustering.

Protein sequences coded by transcripts from Trinity-DN, and from Trinity-GG, StringTie -> GffRead, Braker, and PASA for each genome assembly, were predicted separately using the "Longest-ORFs.fasta" output of TransDecoder (Haas, n.d.) run on the Galaxy webserver (The Galaxy Community et al., 2024). Gene-transcript maps output from Trinity-DN and Trinity-GG were provided using the "–gene_trans_map parameter". For all transcript files, TransDecoder was run using the Universal Genetic Code with a minimum protein length of 100 amino acids "-m 100" in stranded mode "-S".

Reference protein sequences were obtained from NCBI (O'Leary et al., 2016) from the RefSeq genomes for pea aphids and twenty other invertebrate and vertebrate species [*Aedes aegypti* (GCF_002204515.2), *Aphis gossypii* (GCF_020184175.1), *Apis mellifera* (GCF_003254395.2), *Acyrthosiphon pisum* (GCF_005508785.2), *Bombyx mori* (GCF_030269925.1), *Caenorhabditis elegans* (GCF_000002985.6), *Drosophila melanogaster* (GCF_000001215.4), *Daphnia pulex* (GCF_021134715.1), *Daktulosphaira vitifoliae* (GCF_025091365.1), *Homarus americanus* (GCF_018991925.1), *Homo sapiens* (GCF_000001405.40), *Homalodisca vitripennis* (GCF_021130785.1), *Mus musculus* (GCF_000001635.27), *Macrobrachium nipponense* (GCF_015104395.2), *Myzus persicae* (GCF_001856785.1), *Nilaparvata lugens* (GCF_014356525.2), *Neocloeon triangulifer* (GCF_031216515.1), *Rhopalosiphum maidis* (GCF_003676215.2), *Rhopalosiphum padi* (GCF_020882245.1), *Schistocerca gregaria* (GCF_023897955.1), and *Tribolium castaneum* (GCF_031307605.1)].

Protein files from gene annotation methods of the three assemblies of interest as well as the RefSeq genomes above were used as input proteomes for ortholog clustering using OrthoFinder v2.5.5 (Emms and Kelly, 2019).

BUSCO v5.2.2 (Manni et al., 2021; Tegenfeldt et al., 2025) was run on the TransDecoder Longest ORFs protein output for PASA, Trinity genome-guided, StringTie, and Braker for each

assembly, as well as Trinity *de novo*, and the RefSeq proteins. OrthoDB version 10 protein databases for the Eukaryota, Metazoa, Arthropoda, and Insecta levels were used (Tegenfeldt et al., 2025).

Annotation of genomic interval files.

The genomic intervals for mapped transcripts in PASA clusters for each genome assembly were annotated using the OrthoGroups.tsv file output from OrthoFinder, along with header information from the RefSeq protein fasta files for the twenty additional species obtained from NCBI, using custom python scripts. For each protein in a transcript in an orthogroup, the first protein listed for a species within the same orthogroup had its header information appended to the corresponding transcript entry in the genomic intervals file as “Species_ortholog:header information”. In cases where a species had no proteins in that orthogroup, the ortholog is listed as ‘none’ in the genomic intervals file. Annotated genomic interval files can be found in **Supplemental data files**.

Estimating gene number.

The following was performed using custom python scripts. A new orthogroup table was generated replacing the protein(s) (often multiple per locus) from PASA annotations of the three pea aphid assemblies with a single entry for each PASA gene cluster present for each assembly in the orthogroup, along with the proteins present for all other species (and the RefSeq pea aphid proteins). Orthogroups were then analyzed for the number of PASA clusters present for each pea aphid assembly, as well as the number of orthogroup members from the following groups: other pea aphid assemblies (including RefSeq), all non-pea aphid members, as well as all non-aphid members. The total number of PASA clusters for each assembly was then summed based on presence in an Orthogroup containing: >1 pea aphid member, ≥1 non-pea aphid member, ≥1 non-aphid member, ≥10 non-pea aphid members, or ≥10 non-aphid members.

Extracting novel annotations.

Orthogroups containing PASA clusters for all three newly annotated assemblies, as well as ≥10 non-aphid members including *Drosophila melanogaster*, but no RefSeq pea aphid member, were extracted from the orthogroup table generated above for further examination using custom python scripts.

Visualization of results.

Plots were generated from comma-separated value files output from custom python scripts visualized in Microsoft Office 365 Excel v2502 and edited in Adobe Photoshop v24.5.0. Gene diagrams were first visualized in Integrative Genomics Viewer v2.16.1 using annotated genomic interval files for PASA clusters in each assembly using a fixed window size across assemblies. Gene annotations were then isolated and compiled in Adobe Photoshop v24.5.0.

Results

The numbers of transcripts, predicted proteins, proteins in orthogroups, and unassigned proteins, for PASA, Trinity genome-guided, StringTie and Braker for each assembly, as well as for Trinity *de novo* and pea aphid RefSeq annotations, are shown in **Figure 1**. The raw data for this figure can be found in **Supplemental Table S2**. Integration of transcripts from the other four methods into PASA clusters consistently yielded the highest number of transcripts coding for proteins in orthogroups by clustering orthologs of 20 other species with OrthoFinder (**Figure 1A-C**). Braker consistently produced the highest percentage of unassigned proteins of the four genome + RNAseq-based methods (1.8%-3.2%) although Trinity *de novo* produced the highest percentage of unassigned proteins overall (5.6%, **Figure 1A-D, Supplemental Table S2**). Trinity *de novo* also produced the lowest percentage of coding transcripts overall (34.8%), with Trinity genome-guided producing only slightly higher percentages of coding transcripts for each assembly (WD Male Long Read: 35.8%, WL Male Long Read: 35.9%, Modified Reference: 36.1%, **Figure 1A-C, Supplemental Table S2**). Still, both Trinity methods produced a large number of total assembled transcripts relative to other methods, and thus still identified a high number of protein coding transcripts in orthogroups despite the low percentages (**Figure 1A-D**, **Supplemental Table S2**). StringTie proved more conservative, consistently predicting much lower numbers of transcripts across each assembly (~55.9k-58.6k) compared to Trinity genome guided, but with a much higher percentage of coding transcripts (81.6%-87.9%) and the lowest number and percentage of unassigned proteins of any of the methods (144-196, 0.3%-0.4%, **Figure 1 A-C**, **Supplemental Table S2**).

To assess completeness of the proteomes produced by the various methods outlined above, we employed Benchmarking Universal Single-Copy Orthologs (BUSCO, Manni et al., 2021;

240 Tegenfeldt et al., 2025). We evaluated proteome completeness for all methods, including the
241 RefSeq annotations, at the Eukaryote, Metazoan, Arthropod, and Insect levels using version 10
242 databases from OrthoDB (Tegenfeldt et al., 2025). The results of this analysis are shown in
243 **Figure 2** (see **Supplemental Table S4** for exact percentages). Integration of transcripts from
244 the four other methods into PASA clusters yielded highly complete predicted proteomes
245 comparable to RefSeq (**Figure 2 A-C, E**). Focusing on the Insecta level, the methods that
246 produced the most complete predicted proteomes were Braker (94.0%-95.6%) and PASA
247 (93.7%-94.9%). The proteomes produced by those two methods were only slightly less
248 complete than the RefSeq annotation (96.7%). This is despite Braker producing the highest
249 percentages of unassigned proteins, likely through detection Universal Single Copy Orthologs
250 that are not expressed in our RNAseq dataset, which were then incorporated into PASA
251 annotations. StringTie proteomes were less complete than those produced by Braker and PASA
252 (89.6%-91.1%), but still considerably more complete than those produced by Trinity genome-
253 guided (54.0%-55.2%) or Trinity *de novo* (49.7%).
254
255 Given widespread gene duplication known to occur in pea aphids (Li et al., 2019; The
256 International Aphid Genomics Consortium, 2010), we next asked how many genes are predicted
257 within each of these genomes by our novel annotations. A recent gene number estimate, based
258 on ESTs, cDNA libraries, and WQ-maker predictions, made during assembly of the
259 chromosome-level reference genome, came to 40,090 (Li et al., 2019). We find that our
260 annotation methods produced comparatively high numbers of PASA clusters (141,397 [WD
261 Male LR], 145,815 [WL Male LR], 115,952 [Modified Reference]), likely containing
262 pseudogenes, transposons, and potentially endosymbiont contamination.
263
264 We assessed more stringent gene estimates based on the clustering of proteins from PASA
265 clusters in orthogroups with members from other pea aphid assemblies or twenty other species
266 (**Figure 3**). Requiring that a PASA cluster from a given assembly codes for a protein in an
267 orthogroup with at least one other protein from another pea aphid assembly produced gene
268 count estimates more similar to the 40,090 estimate of Li et al., 2019 (48,932 [WD Male LR],
269 47,898 [WL Male LR], 31,888 [Modified Reference]) (**Figure 3, Supplemental Table S5**). The
270 number of genes in orthogroups drops sharply as requirements on the number of orthogroup
271 members from more distantly related species increases (**Figure 3**).
272

273 While a large number of newly annotated genes appear to be lineage-specific, there are also
274 ~1,000-1,500 more genes with ten or more non-aphid orthologs in the long read genomes
275 versus the short read reference (**Figure 3, Supplemental Table S5**). We searched for genes in
276 orthogroups with no RefSeq member but ten or more non-aphid members, including *Drosophila*
277 *melanogaster*, to identify newly annotated pea aphid orthologs of conserved fly genes. Using
278 this method, we found genes that are missing from the RefSeq annotations (**Figure 4A**), as well
279 as genes whose mis-annotation or mis-assembly disrupted protein clustering (**Figure 4B**).
280 These include missing annotations for *Centrocortin* (*Cen*), *Nad-kinase 2* (*Nadk2*), *Rad17*
281 *checkpoint clamp loader component* (*Rad17*), *Cytochrome-c oxidase assembly factor 5* (*COA5*)
282 (**Figure 4A**), and mis-annotation of the genes *trithorax* (*trx*), *Valette* (*Vlet*), and *snail* (*sna*)
283 (**Figure 4B**).
284
285 Based on our ability to detect and correct previously unannotated and mis-annotated genes, we
286 checked if these data support the previous identification of four paralogs of the BMP-branch
287 TGF-β ligand *dpp* (Brisson et al., 2010; Saleh Ziabari et al., 2025a). We were able to confirm the
288 presence and gene structure of all four paralogs across all three assemblies (**Figure 5A**).
289 Predicted gene structures, however, did differ between RefSeq features (red) and PASA clusters
290 (black). Our PASA clusters contain novel isoforms using alternative promoters for *dpp-1* and
291 *dpp-3* and predict that the promoter for *dpp-4* is upstream of the RefSeq annotations (**Figure**
292 **5A**). These refined promoter annotations for the *dpp* paralogs are consistent across all three
293 genome assemblies.
294
295 Recent findings have shown that paralogs of the gene *follistatin* are important for pea aphid
296 wing dimorphisms (Li et al., 2020; Saleh Ziabari et al., 2025b). While we have speculated that
297 morph-specific transcripts from *dpp* duplicates may play a role in these dimorphisms (Saleh
298 Ziabari et al., 2025b), potentially interacting with *follistatin* duplicates, Dpp is not known to
299 interact with Follistatin *in vivo* (Bickel et al., 2008; Pentek et al., 2009). For this reason, we set
300 out to identify potential gene duplication for the Activin branch TGF-β ligand and primary
301 Follistatin target, *myoglianin* (Bickel et al., 2008; Pentek et al., 2009). We identified three
302 paralogs of *myoglianin* present in all three assemblies (**Figure 5B**). Two fragmented RefSeq
303 annotations for *myo-1* are actually a single gene. PASA clusters in the Long Read assemblies
304 agree on additional upstream promoters for *myo-1* that are absent from the RefSeq features
305 and PASA clusters in the Modified Reference, indicating potential mis-assembly of this region in
306 the refence (**Figure 5B**). The RefSeq annotation of *myo-2* indicates an alternative downstream

307 exon not supported by RNAseq-based annotation in any of the three genomes (**Figure 5B**).
308 *myo-3* is a pseudogene, with different intact ORFs present in each of the three genome
309 assemblies, responsible for differences in ortholog clustering between them (**Figure 5B**). That
310 is, PASA clusters from the Modified Reference and WD male Long Read assemblies code for
311 proteins that cluster with other *myoglianin* paralogs and orthologs, but the RefSeq annotation
312 and the PASA cluster in the WL Male Long Read assembly do not.
313
314
315 **Discussion**
316
317 Here we present new gene annotations leveraging RNAseq samples spanning life stages,
318 morphs, and sex, for three pea aphid genome assemblies. We integrated annotations from
319 multiple methods (Trinity *de novo*, Trinity genome-guided, StringTie, Braker-ET) into PASA gene
320 clusters, maximizing the number of proteins in orthogroups (**Figure 1**) and overall completeness
321 as determined by BUSCO (**Figure 2**).
322
323 Gene count estimates from our annotations of the long read (48,932 / 47,898) and short read
324 (31,888) genomes lie above and below, respectively, the previous estimate of 40,090 for the
325 reference genome (**Figure 3, Supplemental Table S5**) (Li et al., 2019). For the long read
326 genomes, >20k of these genes are predicted to be lineage-specific, while only half as many
327 lineage-specific genes are annotated in the short read reference (**Figure 3**). This large disparity
328 in gene count is not observed for more broadly conserved genes. New annotations on all three
329 assemblies and RefSeq agree on 9,283-10,854 genes which cluster with orthologs in at least
330 ten non-aphid species (**Figure 3, Supplemental Table S5**). There are likely a number of factors
331 contributing to this large disparity in count estimates for new genes.
332
333 The difference in pea aphid gene counts for the reference genome (31,888 in this study, 40,090
334 in Li et al., 2019) may stem from the numerous differences in estimation methods, as well as our
335 requirement that proteins cluster with those from another pea aphid assembly or the RefSeq
336 proteins. One likely major contributor is that most of the 40,090 predicted genes do not have
337 associated RefSeq annotations or reference proteins. Additionally, for rapidly evolving gene
338 duplicates that are not part of the reference protein set, mis-assembly in the short read
339 reference genome may lead to predicted protein sequences that do not cluster with proteins
340 from the properly assembled loci in the long read genomes. Thus, these lineage specific genes

341 would not show up in our gene count estimate. Further, even when mis-assembly of duplicates
342 into a single locus in the reference does not disrupt clustering with properly assembled paralogs
343 in the long read genomes, the gene count estimate for the reference only increases by one,
344 while it increases by more than one for the long read assemblies.
345
346 The two long read genomes (WD and WL Male) were derived from closely related, inbred lines.
347 Thus, when paralogs are properly assembled in one long read genome, it is more likely that
348 they will be similarly assembled, annotated and clustered, keeping gene estimates similar.
349 However, there is also the possibility that the gene counts in the long read genomes (48,932 /
350 47,898) are artificially inflated by redundant contig fragments. Given these factors, we find it
351 reasonable that we obtained gene counts above and below the 40,090 predicted by Li et al.,
352 2019 for our long read and reference genomes, respectively. Still, further analyses are required
353 to determine where the true gene count lies within the range shown in **Figure 3**.
354
355 The new annotations provided by integrating multiple methods into PASA gene clusters have
356 identified previously missing or mis-annotated genes in the reference assembly (**Figure 4**).
357 These annotations have also helped in confirming previous reports on *dpp* gene duplication
358 (**Figure 5A**) as well as identified a previously unknown *myo* duplicate pseudogene (**Figure 5B**).
359 The new annotations also identify novel promoters for many of these gene duplicates, and
360 consistency of the gene models across the three assemblies strongly suggests that they are
361 accurate (**Figure 5**).
362
363 Differences were observed between the assemblies in the annotated gene structures and
364 protein clustering for the *myo3* pseudogene (**Figure 5B**). This seems to be due to differential
365 fragmentation of the ancestral ORF by different premature stop codons in each assembly,
366 disrupting transcript discovery, gene structure annotation, and protein clustering. This highlights
367 an additional challenge in initial pseudogene annotation and detection with a genome assembly
368 generated from a single genetic line. However, such disagreement in structure and coding
369 potential across assemblies may be a hallmark of pseudogenes. Detection of such
370 discrepancies requires a comparative approach, which the data presented here help facilitate
371 for pea aphids.
372
373 These data provide a useful supplement to existing RefSeq annotations by identifying and
374 correcting missing and mis-annotated genes. The annotations of the two long read genomes

375 provide a platform for investigating mis-assembly of multiple loci into a single locus in the short-
376 read reference genome. These annotations will aid in future investigations into the structure,
377 function, and evolution of genes and gene families.

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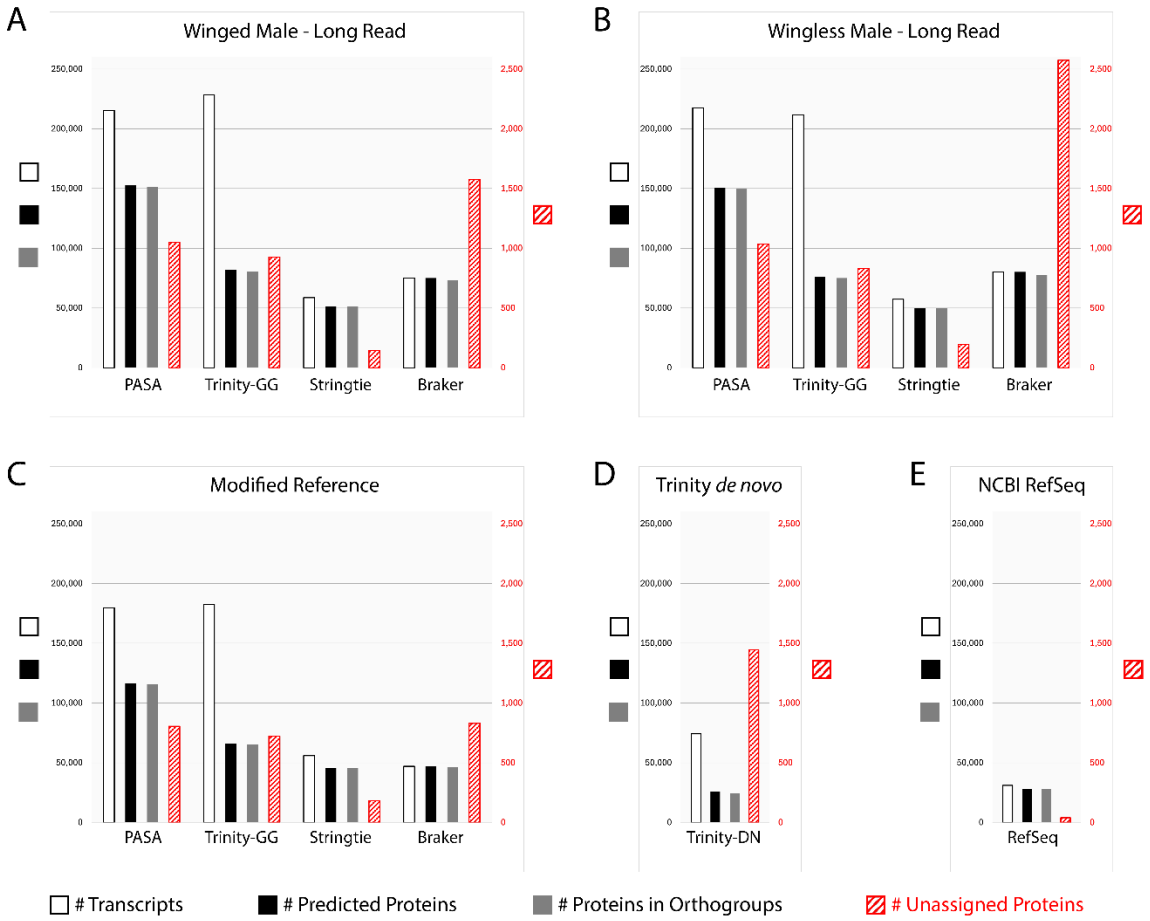
540 **Figures:**



541

542 **Figure 1. Relative amounts of coding and non-coding transcripts annotated by various**
543 **methods.**

544 Number of predicted transcripts (white), protein-coding transcripts (black), and protein-coding
545 transcripts in orthogroups (gray) are shown on the left axis. Number of predicted transcripts
546 coding for proteins that were not assigned to any orthogroup (red hatched) are indicated on the
547 right axis. All plots share the same scales for both axes. (A-C) Numbers are shown for genome
548 + RNAseq-based methods for each assembly. (D) Numbers for Trinity *de novo* assembly of
549 transcripts directly from RNAseq reads, integrated with predicted transcripts from the other three
550 methods to produce PASA annotations for each assembly. (E) Numbers for RefSeq annotations
551 of transcripts and proteins in the reference genome.

552

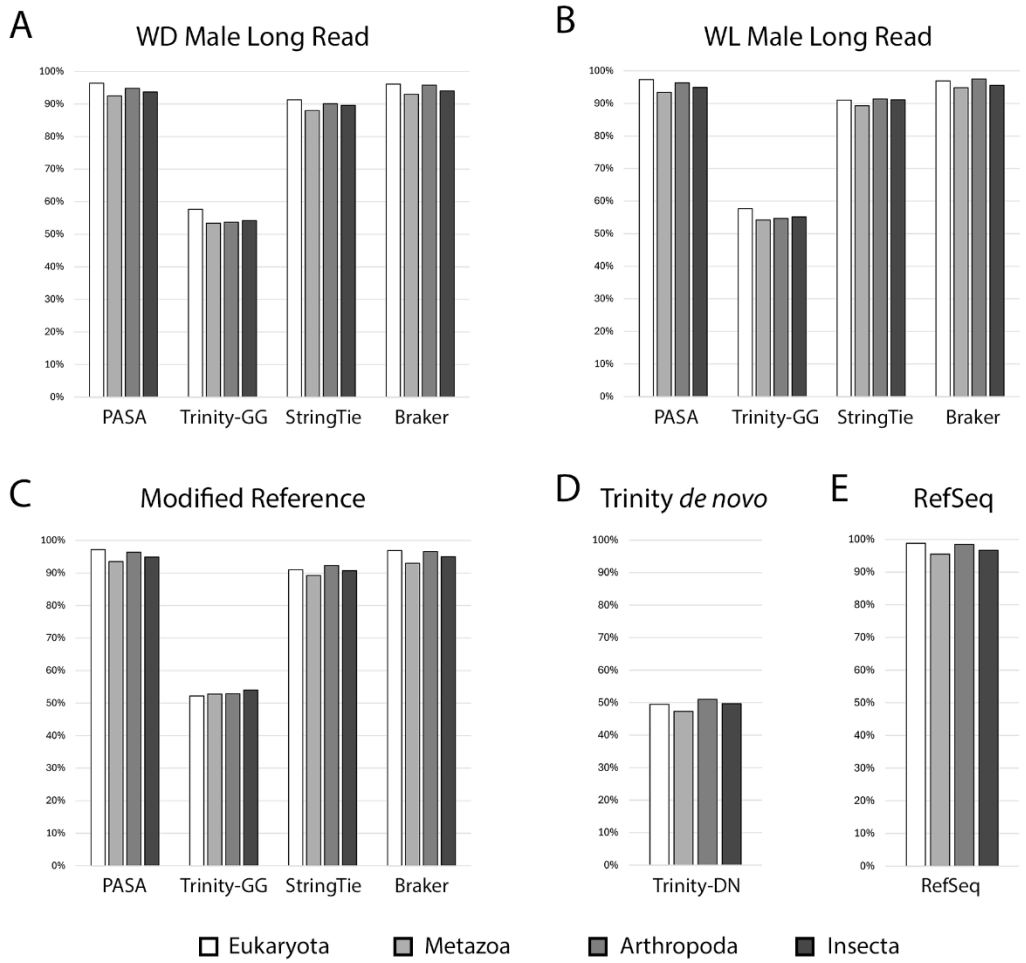


Figure 2. BUSCO analysis of proteomes

The percent of complete BUSCO's contained in PASA, Trinity genome-guided (Trinity-GG), StringTie, and Braker proteomes for the WD Male Long Read (A), WL Male Long Read (B), and Modified Reference (C) assemblies, as well as Trinity *de novo* (Trinity-DN, D) and RefSeq proteomes (E). Bar shade corresponds to the OrthoDB v10 dataset used: Eukaryota (white), Metazoa (light gray), Arthropoda (gray), or Insecta (dark gray).

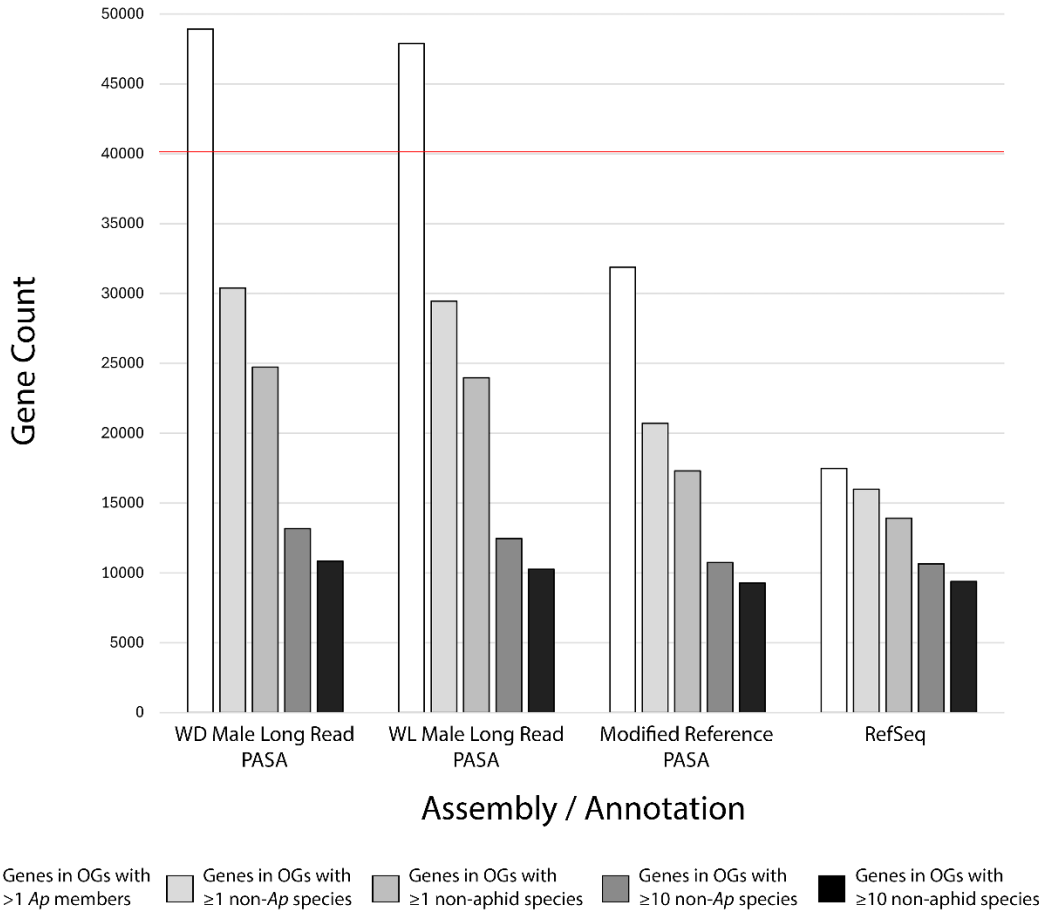


Figure 3. Gene count estimates in pea aphid assemblies with orthologs of varying evolutionary distance.

Numbers of PASA gene clusters for each assembly, or RefSeq genes, producing protein-coding transcripts assigned to orthogroups containing members of varying evolutionary distance. The categories are as follows: at least one other member from another pea aphid assembly (white), at least 1 non-pea aphid species (light grey), at least one non-aphid species (medium grey), at least 10 non-pea aphid species (dark gray), or at least 10 non-aphid species (black). The red line indicates the 40,090 gene estimate from the most recent genome assembly (Li et al., 2019).

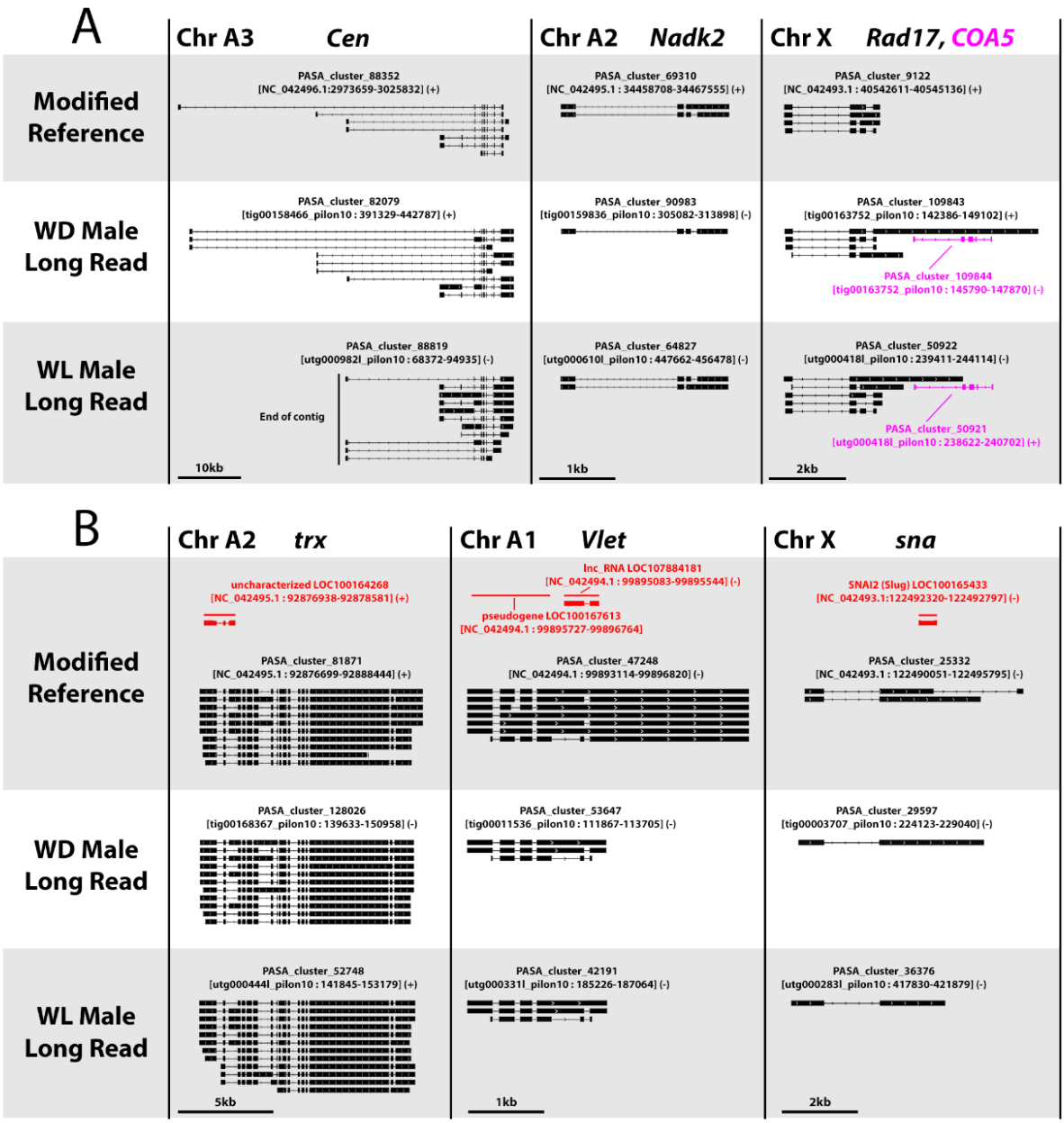


Figure 4. PASA assemblies of missing and mis-annotated genes

Gene structure diagrams for PASA-assembled genes that are either missing or incorrectly annotated in the RefSeq features. RefSeq annotations are shown in red, while newly annotated PASA clusters in the Modified Reference, WD Male Long Read, and WL Male Long Read assemblies are shown in black or magenta. The chromosomal location within the Modified Reference genome is indicated next to the gene name(s). Genes are shown at different scales, indicated by scale bars at the bottom. For clarity, overlapping annotations of other genes are not shown. (A) PASA-assembled gene structures for the genes *Centrocortin* (*Cen*), *Nad-kinase 2*

579 (*Nadk2*), *Rad17 checkpoint clamp loader component* (*Rad17*) (all black), and *Cytochrome-c*
580 *oxidase assembly factor 5* (*COA5*, magenta) that are absent from the RefSeq annotation. The
581 edge of the contig adjacent to the WL Male Long Read annotation of *Cen* is marked by a black
582 line. (B) PASA assemblies for the genes *trithorax* (*trx*), *Valette* (*Vlet*) and *snail* (*sna*) that are
583 incorrectly annotated in the RefSeq features.
584

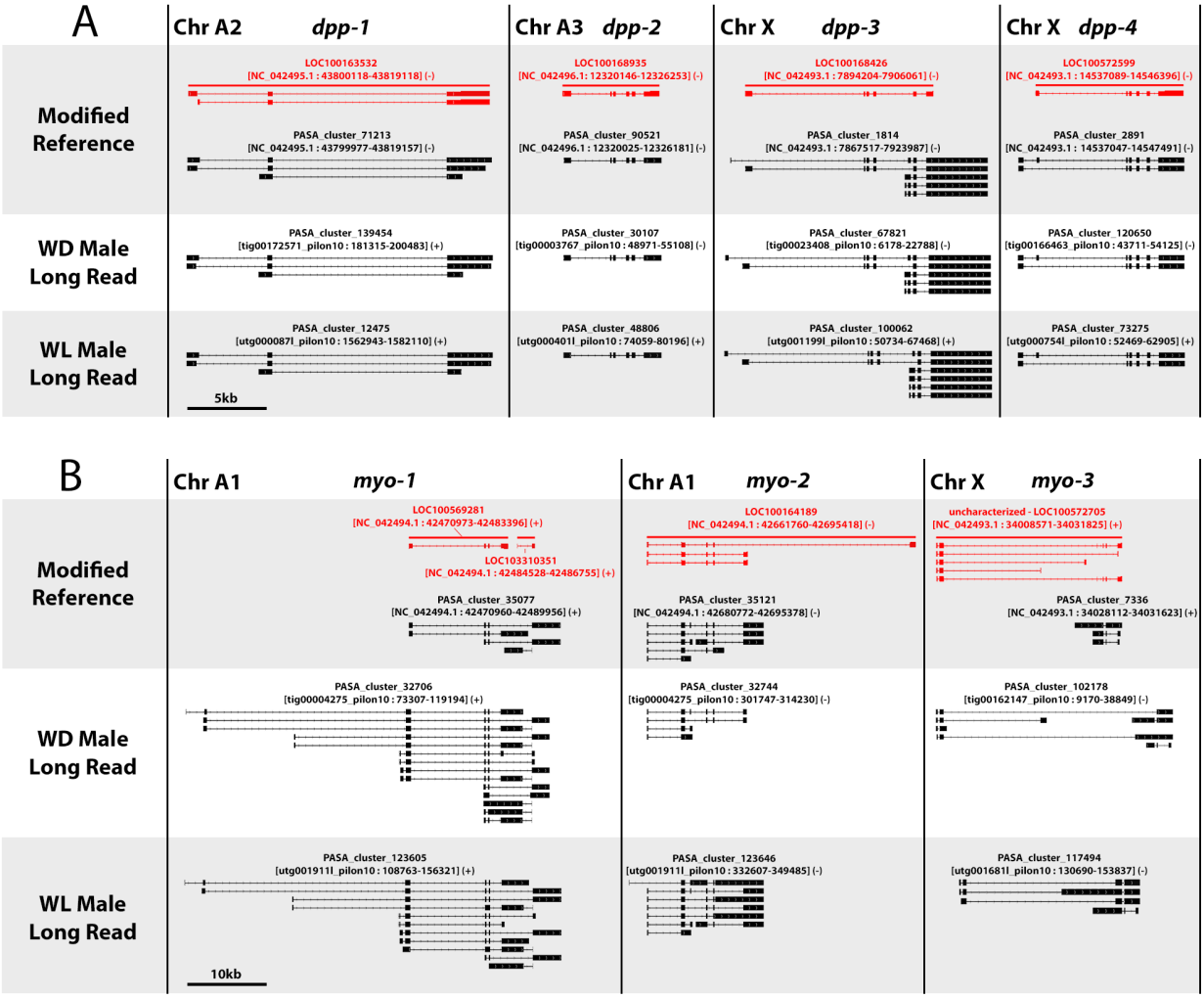


Figure 5. Annotations of duplicated TGF-β signaling ligands

Gene structure diagrams for paralogs of the TGF-β signaling ligands *decapentaplegic* (*dpp*) (A) and *myoglianin* (*myo*) (B) across pea aphid genome assemblies. RefSeq annotations are shown in red, PASA annotations are shown in black. For clarity, overlapping annotations of other genes are not shown. A) Annotations for all four *dpp* paralogs, shown at the same scale. Scale bar: 5kb B) Annotations for the three paralogs of the gene *myo*, shown at the same scale. Scale bar: 10kb