

Chromosome Level Assembly of the Comma Butterfly (*Polygonia c-album*)

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Abstract

The comma butterfly (*Polygonia c-album*, Nymphalidae, Lepidoptera) is a model insect species, most notably in the study of phenotypic plasticity and plant-insect coevolutionary interactions. In order to facilitate the integration of genomic tools with a diverse body of ecological and evolutionary research, we assembled the genome of a Swedish comma using 10X sequencing, scaffolding with matepair data, genome polishing, and assignment to linkage groups using a high-density linkage map. The resulting genome is 373 Mb in size, with a scaffold N50 of 11.7 Mb and contig N50 of 11,2Mb. The genome contained 90.1% of single-copy Lepidopteran orthologs in a BUSCO analysis of 5,286 genes. A total of 21,004 gene-models were annotated on the genome using RNA-Seq data from larval and adult tissue in combination with proteins from the Arthropoda database, resulting in a high-quality annotation for which functional annotations were generated. We further documented the quality of the chromosomal assembly via synteny assessment with *Melitaea cinxia*. The resulting annotated, chromosome-level genome will provide an important resource for investigating coevolutionary dynamics and comparative analyses in Lepidoptera.

Key words: linkage map, butterfly genome, quantitative annotation assessment, comparative genomics, *Polygonia c-album*.

Significance

The *Polygonia c-album* butterfly is considered a model species for the study of evolutionary interactions between insects and their host plants. However, it is conspicuously absent in genomic and genetics literature. We provide a chromosome-level genome for this species in order to facilitate the integration of functional and population genomic research with ecology, physiology, and evolutionary findings. Assessment of our annotated genes suggests a high-quality de novo assembly. Chromosome level assembly accuracy was validated via alignment with the genome of another nymphalid species, *Melitaea cinxia*.

Introduction

Butterflies have long served as model species for a wide range of research, from ecology to studies of developmental evolution (Boggs et al. 2003). Within this diverse field of species and questions, research using the comma butterfly, *Polygonia c-album* (Nymphalidae, Lepidoptera), has made extensive

contributions, in particular to the study of plant-insect coevolution. Most butterflies are specialists at the level of individual plant families, making the host plant repertoire of *P. c-album* notable as it includes several families in four different plant orders. Two additional observations make the dramatically higher diversity of the host plant repertoire of *P. c-album*

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even more interesting. First, related species from the same tribe (Nymphalini) have a host repertoire that is mostly a subset of the *P. c-album* hosts (Nylin 1988). Second, the larvae of these other species often can feed on the diverse hosts of *P. c-album*, even when females of these species no longer use those plants for oviposition (Janz et al. 2001). These patterns suggest that the diverse host plant repertoire of *P. c-album* reflects the suite of hostplants used during the evolution of the tribe, an observation that inspired the “oscillation hypothesis” of host range and speciation (Janz and Nylin 2008). The oscillation hypothesis is an important alternative to the classical coevolution hypothesis, for explaining the striking diversification of phytophagous insects as well the ecological and evolutionary patterns seen in other coevolutionary interactions, including pollination and emerging infectious diseases (Sedivy et al 2011; Hardy and Otto 2014; Hamm and Fordyce 2015; Hoberg and Brooks 2015; Braga et al 2018).

Research specifically using *P. c-album* itself as the model has also generated many other insights into insect-plant systems, concerning host repertoires of adults versus larvae (Nylin and Janz 1996), preference-performance correlations (Janz et al. 1994), female host search strategies, and neural constraint and plasticity (Carlsson et al. 2011; Schäpers et al. 2015; van Dijk et al. 2017; Gamberale-Stille et al. 2019) and genetics of host use within and among populations (Nygren et al. 2006). Other research areas that are making considerable use of *P. c-album* and close relatives as model species include effects of temperature and climate change (Braschler and Hill 2007; Hodgson et al. 2011; Audusseau et al. 2013) as well as seasonal plasticity, life history regulation, and seasonal polyphenism (Inoue et al. 2005; Hiroyoshi et al. 2018; Eriksson et al. 2020).

Finally, two studies have investigated transcriptome plasticity in larvae depending on host plants, using RNA-Seq and GeneFishing, respectively (Heidel-Fischer et al. 2009; Celorio-Mancera et al. 2013) but the analysis and interpretation of results were constrained by the lack of a published genome.

In order to facilitate insights at the genomic level into these extensively studied coevolutionary dynamics and plastic phenotypes, here we present a chromosomal assembly of the *P. c-album* genome, the result of combining Illumina sequencing data from 10X and matepair data, with a high-density linkage map. Together with our validated functional annotation, this genomic resource will greatly facilitate future studies using the species as a model, as well as provide an important genome for comparative evolutionary analyses of the Lepidoptera.

Results and Discussion

Genome Assembly

Using 197 million 10X reads, 11 genomes were assembled using Supernova with a range of data input (15–100%;

supplementary material F1, [Supplementary Material](#) online), an optimal assembly using 70% data were identified, based on contiguity and lowest percentage of missing BUSCOs (scaffold N50 of 76.5 kb and 4.2% missing BUSCOs; fig. 1). Scaffolding with a 3-kb mate-pair library increased the N50 to 519.1 kb, with subsequent haplotype merging further increasing N50 to 572.5 kb. Genome polishing with Pilon using three different mapping programs found that bam files generated by NGM outperformed the rest by displaying the longest N50, high genome completeness, and lowest recovery of duplicates which may indicate erroneous assembly of haplotypes (supplementary material F2, [Supplementary Material](#) online) and was thus used for downstream steps.

Linkage Map

To generate a chromosome-level assembly, we used a linkage mapping data set, which also provides information on recombination rate, providing insights into the relationship between physical and genetic distance. Using RAD-seq data from 287 sexed individuals, composed of two families with full-sibs and corresponding parents, we identified 84,422 candidate SNPs, which allowed us to identify 12,541 markers in 31 linkage groups. This is consistent with the reported *P. c-album* karyotype of 30 autosomes and one sex chromosome (Robinson 1971). Comparisons between physical and genetic distance revealed variable recombination landscapes across chromosomes (fig. 1), with an overall high level of recombination across chromosomes typical of butterfly species (Martin et al. 2016). Using this we were able to anchor 1,366 scaffolds, of which we could orient 550, totaling 86% and 69% of the assembly length, respectively. The resulting chromosome-level assembly consisted of 31 scaffolds with an N50 of 11.7 Mb, with 13,625 unplaced scaffolds (ranging from 502 to 390,522 bp in length, an N50 = 4,741 bp, and total length of 51.7 Mb). We then validated the chromosome structure of our assembly via alignment to the chromosome-level assembly of *M. cinxia*, which last shared a common ancestor with *P. c-album* approximately 42 Ma (Chazot et al. 2019), finding a high concordance across chromosomes (fig. 2).

Genome Annotation and Validation

The chromosomal genome completeness was assessed using BUSCO, which identified 90.1% of the Lepidoptera ortholog data set ($N = 5,286$) as complete and single copy, 0.3% duplicated, 4.7% fragmented and 4.9% missing (supplementary material F2, [Supplementary Material](#) online). We next compared genome annotations generated either using a protein sequence data set for Arthropoda, RNA-Seq data from our focal species, or both, using Braker2 v.2.1.5, expecting similar ability to predict genes when training the algorithm with the different data sets (Büna et al. 2021). Quantitatively, the RNA-Seq data set allowed the software

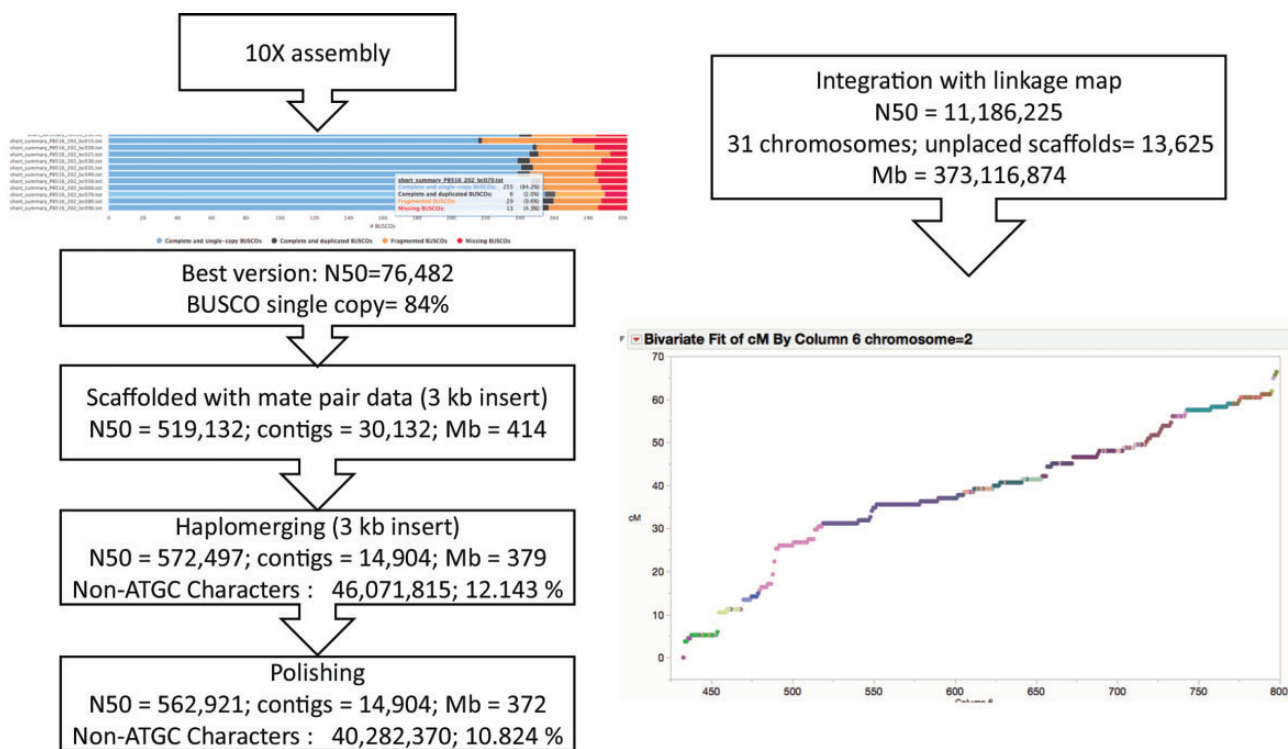


FIG. 1.—Pipeline for genome assembly and linkage map construction of the *P. c-album* genome. Details of the results from each step are indicated within each box.

to predict 358 more unique genes than the protein data set regardless of isoform number per gene. When the number of genes was limited to only those consisting of one isoform, the protein data set predicted 1,011 more. For a qualitative assessment, the longest ortholog hit ratio (OHR) (O’Neil et al. 2010; Hornett and Wheat 2012) between the predicted gene sequences by either data set (protein- or transcript-based) and the *B. mori* gene set (fig. 2) was calculated, finding no differences in gene prediction success between the two algorithm-training strategies. When using both the Arthropod protein database and the *P. c-album* RNA-Seq data, and considering only one isoform per gene, the joined training set predicted 199 more unique genes than when using the RNA-Seq set and 812 less unique genes when using the protein set only. However, there was an improvement in training capacity of the algorithm when using both data sets together, as more complete homologs in the *P. c-album* genome were identified (fig. 2); using both data sets generated more accurate annotations.

Here, we report a chromosome-level genome assembly for the Nymphalid butterfly *P. c-album*. Using a linkage map, we were able to place 86% our assembly into a chromosomal context, with the number of chromosomes and their genic content highly syntenic in comparison to a related butterfly approximately 42 Myr divergent. Quantitative assessment of alternative genome polishing methods, as well as genome annotation methods, supports our chosen pipeline for a

high-quality assembly. Together with our validated functional annotation, this genomic resource will greatly facilitate future studies using the species as a model, as well as provide an important genome for comparative evolutionary analyses of the Lepidoptera.

Materials and Methods

Biological Samples

Material for the genome was generated from *P. c-album* butterflies, collected in Stockholm area (years: 2013–2015). The laboratory population was inbred for five generations, with a last generation, female pupa used for DNA extraction. The offspring (F1 pupae) of two additional mating pairs (wild female x inbred male) was used for the mapping analysis (Family B = 140 F1, and Family H = 141 F1).

DNA and RNA Sampling and Sequencing

Two DNA extraction protocols were used. A phenol-chloroform procedure combining salting out extraction (Woronik et al. 2019) was used for inbred female pupae, and a robot-based protocol for the samples used for linkage mapping, following manufacturer’s instructions (KingFisher Cell and Tissue DNA Kit and the KingFisher Duo Prime System, Thermo Scientific, MA). RNA was extracted from adult antenna, tarsi, and larval gut tissue (34 samples total,

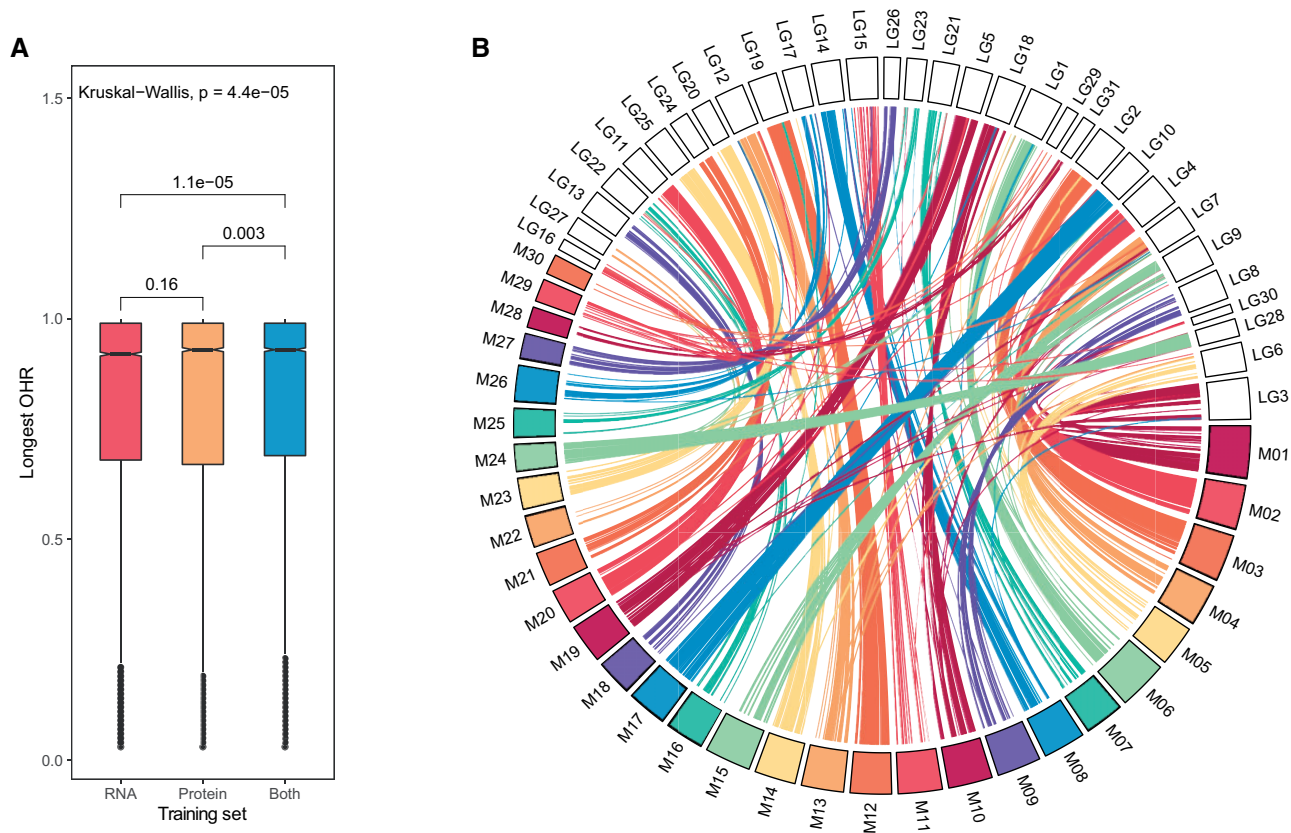


FIG. 2.—Genome validation and genetic diversity. (A) Ortholog homology ratio improved with the combination of RNA-Seq and protein data. There was greater homology between *B. mori* proteins and proteins predicted using both the RNA-Seq and protein trained annotation (blue) than using either RNA-Seq trained annotation (red) or protein-trained annotation (yellow) only. (B) Synteny between the *M. cinxia* genome (colored chromosomes) and the *P. c-album* genome (noncolored linkage groups).

~30 M reads) using a phenol-guanidinium thiocyanate protocol (TRIzol, Thermo Fisher Scientific, MA) and followed by either BCP (1-bromo, 3-chloropropane; Merck KGaA, Darmstadt, Germany) or column-based chemistry (Direct-zol RNA MiniPrep, Zymo, CA). Samples were quantified using fluorometry (Qubit, Thermo Scientific), and the corresponding library preparation, sequencing, and Falcon assemblies were performed at the National Genomics Infrastructure Sweden (NGI, Stockholm, Sweden). For genome assembly, three Illumina DNA libraries were produced: short insert (180 bp), long insert (3 kb) mate-pair libraries (Illumina TruSeq PCR-free and Nextera libraries, respectively), and 10X Chromium Genome library, all sequenced on Illumina HiSeqX. Read trimming and quality filtering used bbmap, following previous work (Woronik et al. 2019).

Genome Assembly

De novo genome assemblies were made using Supernova v. 1.21 (Weisenfeld et al. 2017) by following the authors' recommendation to barcode subsample genomes smaller than 1.6 Gb, that is, using the parameters “-bcfrac” and “-maxreads.” Assemblies were generated for barcode fractions

0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.5, 0.6, 0.7, 0.9 and 1.0 and subsequently evaluated using QUAST version 4.5.4 (Gurevich et al. 2013), MultiQC v. 1.3 (Ewels et al. 2016) and BUSCO v. 2.0.1 (Simao et al. 2015) with its “eukaryota_odb9” data set. Scaffolding of this genome using the mate-pair data started with read data filtering using NEXTCLIP (Leggett et al. 2014) followed by scaffolding using BESST v2.0 (Sahlin et al. 2016). Alternative haplotypes were then merged, after soft masking repeats using RED v. 05/22/2015 (Girgis 2015), using Haplomerger2 v. 20180603 (Huang et al. 2017), with detected tandem duplicates removed settings. Polishing of the merged genome using the short insert library was performed using Pilon v. 1.23 (Walker et al. 2014), with reads mapped using NextGenMap-0.5.0 (Sedlazeck et al. 2013), bwa v. 0.7.17 (Li and Durbin 2009) and SNAP (Zaharia et al. 2011).

Linkage Map Construction and Scaffold Anchoring

We obtained RAD-seq data from 287 sexed individuals composed of two families with full-sibs and corresponding parents. The data were generated with nextRAD methodology implemented by SNPsaurus (OR). Reads were mapped to the

genome using bwa mem and together with samtools (Li et al. 2009) sorted individual bam files were created. The samtools mpileup and Lep-MAP3 (Rastas 2017) pipeline was used to get genotype likelihoods for the map construction. The map construction pipeline used default parameters, except 1) ZLimit=2 in ParentCall2 to call Z/W markers, 2) dataTolerance=0.0001 in Filtering2, and 3) informativeMask=2 in SeparateChromosomes2 in order to find linkage groups robustly using only nonrecombining female information and lodLimit=30 and lodDifference=5 in JoinSingles2All to add male informative markers to the map, recombination2=0 and informativeMask=13 and calculate Intervals in OrderMarkers2 to ignore the nonrecombining female information in the final maps and to output information on the map uncertainty. The scaffold anchoring was obtained using a preliminary version of Lep-Anchor (Rastas 2020) using the linkage map. This linkage map was re-evaluated in the found scaffold order (parameter evaluateOrder in OrderMarkers2), and based on these maps some minor manual fixes on 5 chromosomes were performed.

Genome Annotation, Validation, and Functional Annotation

After soft-masking the final genome version, annotations were performed using Braker2 v. 2.1.5 (Brůna et al. 2021 and references herein), in the genome mode, training Augustus using either the RNA-Seq, the protein mode or both, with reference proteins from the Arthropoda section of OrthoDB v. 10 (Kriventseva et al. 2019) and our RNA-Seq data mapped against the genome using HiSat2 2.1.0 (Kim et al. 2019).

The qualities of the RNA, protein, and RNA + protein annotations were assessed using the longest OHR (O'Neil et al. 2010; Hornett and Wheat 2012). Protein sequences in each annotation were collapsed (CD-Hit; 90% identity) and converted to protein databases (NCBI BLAST v. 2.5.0). Protein sequences from a published *Bombyx mori* annotation (accessed from NCBI; GCF_000151625.1_ASM15162v1) were then blasted against the databases. The longest hit for each *B. mori* protein was identified and OHR was calculated as the ratio of the hit length to that of the *B. mori* protein.

Comparative Analysis of Chromosomal Structure

We used nucmer (MUMmer4 v. 4.0.0beta2; Marçais et al. 2018) to align the polished genome to that of the best chromosome level genome assembly from the same subfamily (Nymphalinae) as *P. c-album*, *Melitaea cinxia* (Blande et al. 2020). The alignment file was filtered to retain only those aligned sequences that were longer than 200 bp and had less than 90% identity between the two genomes in contigs that were at least 1 Mb long. Alignments were visualized using the R-package circlize (Gu et al. 2014).

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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Data Availability

The chromosome level assembly fasta sequence file of *Polygonia c-album* is available on ENA (accession number ERZ1744298). The scripts in the bioinformatic pipeline are available at https://github.com/bioinfowheat/Polygonia_calbum_genomics.

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