A Haplotype-resolved Chromosome-scale Genome Assembly and Annotation for the Leafcutter Ant, *Acromyrmex octospinosus*

Gaurav Agavekar [1,*], Yoshihiko Suzuki [2], Miyuki Suenaga [1], Miguel Pacheco-Leiva[3], Michael Hiller [4,5,6], Adrián A. Pinto-Tomas [7], Eugene Myers [2], Evan P. Economo [1,8]

[1]Biodiversity and Biocomplexity Unit, Okinawa Institute of Science and Technology Graduate University, Onna-son, Japan

[2]Algorithms for Ecological and Evolutionary Genomics Unit, Okinawa Institute of Science and Technology Graduate University, Onna-son, Japan

[3]Center for Research in Cellular and Molecular Biology, University of Costa Rica, San José, Costa Rica

[4]LOEWE Centre for Translational Biodiversity Genomics, Frankfurt, Germany

[5]Senckenberg Research Institute, Frankfurt, Germany

[6]Faculty of Bioscience, Goethe-University, Frankfurt, Germany

[7]Center for Research in Microscopic Structures and Department of Biochemistry, School of Medicine, University of Costa Rica, San José, Costa Rica

[8]Department of Entomology, University of Maryland, College Park, USA

*Corresponding author: E-mail: gaurav.agavekar@oist.jp.

Accepted: March 03, 2025

Abstract

Leafcutter ants are ecologically important insects that cultivate fungal gardens for sustenance, playing crucial roles in Neotropical ecosystems. Due to their ecological and evolutionary significance, high-quality genomic assemblies for the species in this fascinating group can provide a foundation for understanding their evolution. Here, we present a chromosome-scale, haplotype-resolved genome assembly for *Acromyrmex octospinosus*, a common leafcutter ant species broadly distributed in the Neotropics. Using PacBio HiFi sequencing (99x coverage) and Hi-C scaffolding (51x coverage), we generated both haplotype-resolved assemblies (312–314 Mb) and a haplotype-collapsed assembly (320 Mb), each containing 19 chromosomes. One hundred percent of the assembly is anchored to chromosome-level scaffolds, and the assemblies exhibit high contiguity (contig N50: 6.13–8.28 Mb), base accuracy (QV 61.5–61.8), and gene completeness (BUSCO scores: 98.3% to 98.4%). Synteny analysis between haplotypes revealed high concordance (96.0% to 96.8%) with minor structural variations, consistent with expectations for a diploid individual. Combining transcriptomic and homology-based protein evidence with ab initio predictions, we annotated 12,123 genes, achieving a near-complete BUSCO gene completeness of 99.6%. The high-quality assemblies significantly enhance the current genomic resources available for leafcutter ants, providing a foundation for future comparative genomic studies within *Acromyrmex* and across fungus-farming ants.

Key words: fungus-farming ants, insect genomics, hymenoptera, diploid assembly.

Significance

This study presents the first fully phased, haplotype-resolved genome assembly for an ant species derived from PacBio HiFi and Hi-C data from a single individual. The resulting assembly has exceptional contiguity, accuracy, and completeness, providing a high-quality reference genome for *A. octospinosus*. This work opens new avenues for research into the genomic basis of unique adaptations in leafcutter ants. By serving as a reference genome, this resource will facilitate comparative genomic analyses across the broader clade of fungus-farming ants, advancing our understanding of these ecologically important insects and their complex symbioses.

Introduction

Fungus-farming ants in the Neotropics are one of the few animal clades that have evolved agriculture as a primary means of sustenance. Among these, leafcutter ants of the genera *Atta*, *Acromyrmex*, and *Amoimyrmex* exhibit the most sophisticated form of agriculture, using vegetation material as a substrate to cultivate extensive fungal gardens (Mehdiabadi and Schultz 2010). As ecologically significant herbivores, leafcutter ants play a crucial role in nutrient cycling and ecosystem dynamics in the Neotropics (Swanson et al. 2019). Their intricate symbiotic relationships with fungal cultivars have been the subject of a wide range of studies, from coevolutionary dynamics and population genetics among the mutualism partners (Mikheyev et al. 2007; Mueller et al. 2011; Schultz et al. 2024), the evolution of symbiotic adaptations (De Fine Licht et al. 2014), to ecosystem engineering (Soper et al. 2019) and major evolutionary transitions (Schultz and Brady 2008).

Despite the ecological and evolutionary significance of leafcutter ants, only a few genome assemblies are currently available for the ∼50 extant leafcutter ant species (Bolton 2025). The first genomes for these ants, those of *Atta cephalotes* and *Acromyrmex echinatior*, were published in 2011 (Nygaard et al. 2011; Suen et al. 2011). These initial assemblies, generated with short-read sequencing technologies, provided valuable insights into the genomic correlates of obligate fungus-farming in ants but were highly fragmented. The Global Ant Genomics Alliance (GAGA), a recent community-wide large-scale genome sequencing effort, has substantially expanded genomic resources for ants, including new long-read-based genome assemblies for 2 *Atta* and 5 *Acromyrmex* species (Boomsma et al. 2017; Vizueta et al. 2024). These assemblies represent an important advancement over earlier short-read-based resources and have improved contiguity and completeness. However, they do not utilize PacBio HiFi sequencing or phased assemblies, which provide improved accuracy and haplotype-level resolution.

Here, we present a haplotype-resolved, chromosome-scale genome assembly of *Acromyrmex octospinosus*. Historically, this taxon has encompassed multiple valid species, including *A. volcanus*, which was the identification we had initially assigned to our samples at the time of collection. A recent taxonomic revision synonymized *A. volcanus* with *A. octospinosus*, and we therefore refer to our samples as *A. octospinosus* in accordance with the latest revision (Mera-Rodríguez et al. 2024). We note, however, that the *A. octospinosus* species complex is taxonomically challenging, and future studies may further refine species boundaries.

Leveraging the large body size of female alates, we were able to prepare both PacBio HiFi and Dovetail (Omni) Hi-C libraries from a single individual pupa. This approach allowed us to obtain high-coverage HiFi and Hi-C data from the same individual, overcoming a common challenge in sequencing phased assemblies of small insects like ants. The resulting assembly contains complete sequences of the 2 haplotypes with chromosome-level scaffolding. Additionally, we generated short-read RNA-seq data from multiple developmental stages that we used to produce a highly complete gene set for this newly sequenced assembly. This genomic resource represents a significant enhancement to the current resources available for leafcutter ants and complements the recent large-scale effort for ant genomic resources led by GAGA. The high-quality, chromosome-scale assembly of *A. octospinosus* will facilitate comparative genomic studies within the *Acromyrmex* genus and across the broader clade of fungus-farming ants.

Results and Discussion

Genome Assembly and Quality Assessment

We generated a diploid haplotype-phased assembly for the leafcutter ant *A. octospinosus* using a combination of PacBio HiFi reads (99x coverage) for contig assembly and Dovetail (Omni) Hi-C reads (51x coverage) for phasing and scaffolding. The final dataset obtained with hifiasm (Cheng et al. 2021) using HiFi and Hi-C reads yielded 2 haplotypes (hereafter hap1 and hap2) as well as a haplotype-collapsed assembly. Each of our final curated assemblies was scaffolded to 19 chromosomes (Fig. 1a), consistent with the karyotype ($2n = 38$) reported for related *Acromyrmex* species (Barros et al. 2021). In these assemblies, 100% of the contigs assembled with hifiasm were assigned to chromosome-level scaffolds.

The haplotype-collapsed assembly spans a total of 320,115,327 bases, whereas hap1 and hap2 span

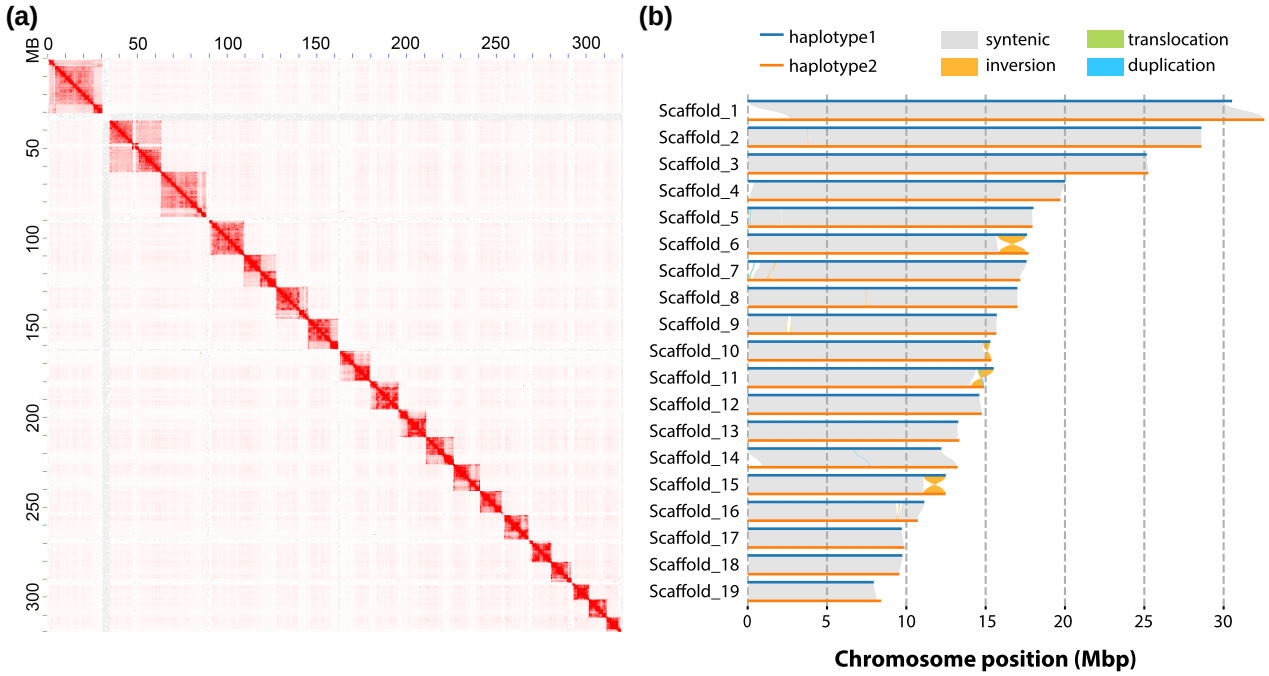

Fig. 1. a) Hi-C density map for the haplotype-collapsed assembly of A. octospinosus. b) Synteny and structural rearrangements between 2 haplotypes of the A. octospinosus genome. The scaffolds presented on the y-axis are those of hap1.

312,135,816 and 314,278,106 bases, respectively. These genome sizes are slightly larger than those reported for other *Acromyrmex* species on NCBI (e.g. *A. echinatior* at 295.9 Mb), likely reflecting methodological differences in sequencing and assembly.

We characterized the genome quality for accuracy, contiguity, and completeness using a variety of metrics (Table 1). The contig N50 values demonstrate high contiguity: 8.2 Mb for the haplotype-collapsed assembly, 6.6 Mb for hap1, and 6.1 Mb for hap2. Of particular note is the high accuracy of QV 61.4–61.8 for the assemblies as estimated with Merqury (Rhie et al. 2020). QV of 61.5 corresponds to ~99.99993% base-level accuracy or an error rate of ~1 in 1.4 million bases. Additionally, high BUSCO scores of 98.2% to 98.4% of the assemblies indicate high completeness.

To test for contamination with possible nontarget sequences, we used the NCBI Foreign Contamination Screen on the final assemblies. No significant contaminant scaffolds or regions were detected, indicating that our genome is free of notable contamination. We note a visible gap in the Hi-C map near the end of Scaffold_1 (Fig. 1a). Further examination of this region revealed a block of predominantly repetitive DNA (~4 Mb), explaining the low Hi-C contact density rather than indicating an absent segment. Such reduced coverage is commonly observed in highly repetitive genomic regions. Finally, we examined the assembled chromosomes for telomeric repeats using the tool tidk (Brown et al. 2025). We focused on the motif AACCT, which is frequently reported as a telomeric or subtelomeric element in arthropods and was the top hit during telomeric repeat sequence exploration in our assembly with tidk. By quantifying its distribution in 10 kb windows across the genome, we found that in 8 scaffolds the repeat was enriched on only 1 terminus, whereas in 5 scaffolds it occurred on both termini (supplementary fig. S1, Supplementary Material online). Although we did not detect this motif at both ends of every chromosome, its presence near several scaffold termini supports the chromosome-scale organization of the assembly.

Structural Variations in the 2 Haplotypes of *A. octospinosus* Genome

To characterize structural variation between haplotypes, we aligned hap1 and hap2 using minimap2 and analyzed synteny and rearrangements with SyRI (Goel et al. 2019). The 2 haplotypes showed extensive synteny consistent with expectations for a single diploid individual, representing 96.8% syntenic regions in hap1 and 96.0% in hap2 (Fig. 1b).

For hap1, the remaining regions constituted 1.57% inversions, 0.25% translocations, 0.12% duplications, and 1.38% unaligned regions. These proportions are nearly identical for hap2 (supplementary table S1, Supplementary Material online). While the biological implications of these structural variants remain to be investigated, their presence

Table 1 Genome assembly statistics for the newly sequenced *A. octospinosus* genome

Statistic	Haplotype1 (hap1)	Haplotype 2 (hap2)	Haplotype-collapsed assembly
Assembly length (bp)	312,135,816	314,278,106	320,115,327
No. of contigs	82	81	50
No. of scaffolds	19	19	19
Contig N50	6,606,488	6,132,152	8,286,559
Scaffold N50	17,580,990	17,186,683	17,604,077
Merqury QV	61.5	61.8	61.4
Reliable block N50	4,669,802		9,838,991
Assembly BUSCO (Hymenoptera, 5991 genes)	C:98.2% [S:98.0%, D:0.2%], F:0.7%, M:1.1%.	C:98.3% [S:98.1%, D:0.2%], F:0.6%, M:1.1%.	C:98.4% [S:98.2%, D:0.2%], F:0.6%, M:1.0%.
Annotation BUSCO (Hymenoptera, 5991 genes)	C:99.6% [S:52.8%, D:46.8%], F:0.1%, M:0.3%.		

For BUSCO scores, C, complete; S, complete and single-copy; D, complete and duplicated; F, fragmented; M, missing BUSCOs.

underscores the value of haplotype-resolved assemblies for revealing intraspecific chromosomal variation.

Genome Annotation

We annotated the final haplotype-collapsed assembly with the publicly accessible NCBI Eukaryotic Genome Annotation Pipeline (EGAPx) at https://github.com/ncbi/egapx. This pipeline combines the use of RNA-seq data with homology-based evidence from NCBI for gene prediction. The resulting annotation contains 12,123 genes, of which 10,487 are protein-coding. The overall number of genes is comparable to the 12,253 genes reported for the *A. echinatior* reference assembly on NCBI. However, it is worth noting that gene counts vary considerably among *Acromyrmex* species in the NCBI database (e.g. *A. insinuator*: 14,546 genes; *A. charruanus*: 8,986 genes; *A. heyeri*: 9,118 genes), which may reflect differences in annotation methods rather than true biological variation. To assess the completeness of our annotation, we ran BUSCO in transcriptome mode with the Hymenoptera dataset (5991 BUSCOs). We found that our annotation is 99.6% complete, containing nearly all genes from the benchmark dataset. This high level of gene recovery places the *A. octospinosus* assembly among the most complete ant genomes currently available, supporting its utility for downstream functional and comparative analyses.

In conclusion, our high-quality, chromosome-scale assemblies of *A. octospinosus* significantly enhance the genomic resources available for leafcutter ants. While the current work focuses primarily on reporting the assembly and its quality, this new resource adds to the growing collection of genomic tools for investigating ant evolution more broadly. It also lays the groundwork for future in-depth studies of gene families, genomic regions associated with species-specific adaptations, and comparative analyses within the *Acromyrmex* genus and across the broader clade of fungus-farming ants.

Materials and Methods

Sample Collection and Library Preparation for Genome Assembly

Biodiversity access permits for the University of Costa Rica (UCR) project C1-248 were granted by the Institutional Biodiversity Commission (Resolution 308), and ant collection was authorized by the La Selva Biological Station in Sarapiquí, Heredia. An *A. octospinosus* colony was collected there in September 2021 (code AP210924-01) and maintained in the laboratory at UCR until June 2022, when we sampled different developmental stages and preserved them in a −80°C freezer. These samples were then shipped to the Okinawa Institute of Science and Technology (OIST) for sequencing following a Materials Transfer Agreement in accordance with the Nagoya Protocol, with export permits granted by the Costa Rican Environmental Ministry (CUSBSE-832-2022). A female alate pupa was picked and split dorsoventrally on dry ice, separating the head and part of the thorax from the remainder of the thorax and abdomen. These sections were then processed separately: the head-containing section was used for HiFi library construction, while the thorax-abdomen section was used for Hi-C library construction.

We performed high molecular weight (HMW) DNA extraction for HiFi library preparation using the Qiagen MagAttract HMW DNA Extraction Kit (Qiagen N.V.). The HMW DNA was then sheared to 10–15 kb fragments using Megaruptor 3 (Diagenode SA). We followed the low DNA input protocol from PacBio to prepare HiFi libraries with the SMRTbell Express Template Prep Kit 2.0 (PacBio, Menlo Park, CA, USA). At each stage, DNA concentration was assessed using a Qubit dsDNA HS Assay kit on the Qubit Fluorometer (Thermo Fisher Scientific), and quality control of extracted DNA and library was performed with the Agilent Femto Pulse system (Agilent Technologies). The prepared library was submitted to the OIST Sequencing Section (SQC), where it was sequenced on

the PacBio Sequel II instrument. CCS (HiFi) reads were returned by the SQC following the standard PacBio post-sequencing protocols.

The other half of the pupa was used to generate long-range interaction data using Hi-C. We followed the sample preparation protocol for insects provided by the manufacturer in the Dovetail Omni-C Proximity Ligation Assay Kit (Cantata Bio). The fragment size distribution of the library was checked using TapeStation 4200 (Agilent Technologies) while concentration was measured with the Qubit Fluorometer and Qubit dsDNA HS reagents Assay kit (Thermo Fisher Scientific). The final library was sequenced at the OIST SQC on an Illumina NovaSeq 6000 instrument in paired-end mode with a read length of 150 bp.

RNA Sequencing

To assist gene annotation, we performed short-read RNA sequencing. Three samples of total RNA were obtained by extracting RNA using Trizol reagent (Invitrogen) from the following samples: (i) a mix of 4 larvae of different sizes, (ii) 2 pupae, and (iii) 5 adult workers. We prepared RNA libraries for Illumina short-read sequencing after enriching mRNA using NEBNext Poly(A) mRNA Magnetic Isolation Module (#E7490) with the NEBNext Ultra II Directional RNA library Prep Kit for Illumina (#E7760S) according to the manufacturer's instructions. We multiplexed the libraries and sequenced them paired-end at the OIST SQC on an Illumina NovaSeq 6000 instrument with a read length of 150 bp. The quality and concentration of each RNA extraction and library were assessed using TapeStation 4200 (Agilent Technologies) and Qubit Fluorometer with the RNA HS Reagents Assay Kit (Thermo Fisher Scientific, Waltham, MA). All RNA extractions had RIN >8.

Genome Assembly and Quality Assessment

We first obtained a haplotype-collapsed contig assembly by running hifiasm (v0.16.1) with the HiFi data, using default parameters (Cheng et al. 2021). To remove redundant haplotigs and ensure a high-quality assembly, we ran purge_dups with the parameters $L = 10$, $M = 38$, and $U = 250$ (Guan et al. 2020). To obtain haplotype-resolved contig assemblies, we reran hifiasm, this time integrating Hi-C reads to generate phased contigs. The resulting contig assemblies were scaffolded with YaHS (Zhou et al. 2023) with default parameters. The input for YaHS was a BAM file containing Hi-C read mappings to the contigs, created with BWA (v0.7.17) (Li 2013). Hi-C interaction maps were generated with the fasta2assembly module from the 3D-DNA pipeline (Dudchenko et al. 2017). Finally, manual curation of scaffolds was performed using the Juicebox suite of tools, similar to instructions under the "Manual curation with Juicebox" section on https://github.com/c-zhou/yahs. The details of the methods we used for genome quality assessments are provided in the Supplementary Material online.

Structural Variation Between Haplotypes

To identify structural variations between the haplotypes of *A. octospinosus*, we used SyRI (Goel et al. 2019). First, we aligned the 2 haplotypes using minimap2 (Li 2018) (v2.24) with the –eqx and asm5 options, and sorted the resulting alignments with samtools (v.1.9). We applied fixchr (https://github.com/schneebergerlab/fixchr) to select homologous chromosomes between the 2 haplotypes and to ensure that the homologous scaffolds were correctly matched, scaffold names were standardized using a custom Python script. SyRI was then run with default parameters and the alignment BAM file as input. Finally, the resulting structural variation patterns were visualized using plotsr (Goel and Schneeberger 2022).

Genome and Repeat Annotation

We performed genome annotation for the haplotype-collapsed version of the *A. octospinosus* assembly. For this purpose, we used the publicly accessible version (v. 0.3.2) of the NCBI EGAPx from https://github.com/ncbi/egapx. The pipeline was executed using Nextflow on Deigo, the high-performance computing cluster at OIST. Briefly, this pipeline takes as input a genome assembly in fasta format along with RNA-seq data. It then fetches taxon-appropriate proteins from NCBI and aligns both the RNA-seq reads and proteins to the genome. These alignments are then used for gene prediction with Gnomon, the NCBI eukaryotic gene prediction tool. To test the completeness of the resulting annotation, we ran BUSCO (v.5.1.3) in transcriptome mode with the Hymenoptera_odb10 dataset.

To annotate repeat regions in the genome, we used RepeatModeler 2.0.6 with default parameters and generated a de novo repeat library for the haplotype-collapsed assembly. We then used RepeatMasker 4.1.7 with parameters -engine ncbi with the resulting library from RepeatModeler to softmask the genome.

Supplementary Material

Supplementary material is available at *Genome Biology and Evolution* online.

Acknowledgments

We are grateful for the help and support provided by the Sequencing Section (SQC) of Core Facilities at Okinawa Institute of Science and Technology Graduate University (OIST). We also acknowledge the support from the Scientific Computing and Data Analysis Section (SCDA) at OIST.

Funding

The sequencing data presented here was supported by subsidy funding to Okinawa Institute of Science and Technology Graduate University.

Data Availability

The new sequencing data, assembled genomes, and annotation files have been deposited in NCBI's BioProject under the accession number PRJNA1218502.

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Associate editor: John Wang