



DATA NOTE

The genome sequence of the Four-spotted Footman moth,

Lithosia quadra (Linnaeus, 1758)

[version 1; peer review: 2 approved, 1 approved with reservations]

Finley Hutchinson¹, Liam M. Crowley ²,
 University of Oxford and Wytham Woods Genome Acquisition Lab,
 Darwin Tree of Life Barcoding collective,
 Wellcome Sanger Institute Tree of Life Management, Samples and Laboratory
 team,
 Wellcome Sanger Institute Scientific Operations: Sequencing Operations,
 Wellcome Sanger Institute Tree of Life Core Informatics team,
 Tree of Life Core Informatics collective, Darwin Tree of Life Consortium

¹University of Exeter, Penryn, England, UK²University of Oxford, Oxford, England, UK

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Abstract

We present a genome assembly from a male *Lithosia quadra* (Four-spotted Footman; Arthropoda; Insecta; Lepidoptera; Erebidae). The genome sequence has a total length of 456.27 megabases. Most of the assembly (99.91%) is scaffolded into 31 chromosomal pseudomolecules, including the Z sex chromosome. The mitochondrial genome has also been assembled and is 15.38 kilobases in length.

Keywords

Lithosia quadra, Four-spotted Footman, genome sequence, chromosomal, Lepidoptera



This article is included in the [Tree of Life gateway](#).

Open Peer Review

Approval Status   

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1. **Axel Künstner** , University of Lübeck, Lübeck, Germany
2. **Yu-Feng Huang** , National Chung Hsing University, Taichung, Taiwan
3. **Sivasankaran Kuppusamy** , Loyola College, Chennai, India

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Darwin Tree of Life Consortium (mark.blaxter@sanger.ac.uk)

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Species taxonomy

Eukaryota; Opisthokonta; Metazoa; Eumetazoa; Bilateria; Protostomia; Ecdysozoa; Panarthropoda; Arthropoda; Mandibulata; Pancrustacea; Hexapoda; Insecta; Dicondylia; Pterygota; Neoptera; Endopterygota; Amphiesmenoptera; Lepidoptera; Glossata; Neolepidoptera; Heteroneura; Ditrysia; Obtectomera; Noctuoidea; Erebidae; Arctiinae; Lithosiini; *Lithosia*; *Lithosia quadra* (Linnaeus, 1758) (NCBI:txid987974)

Background

Lithosia quadra, or the Four-spotted Footman, is a moth of the family Erebidae. Its range includes southern and central Europe, extending eastward across the Palaearctic to the Amur River and Japan. In the north, it occurs in southern Great Britain, Scandinavia, and Ireland (GBIF Secretariat, 2023).

This species exhibits marked sexual dimorphism. Males have smaller wingspans (18–22 mm) with grey forewings with black and yellow blotches at the base and a blue-black stripe along the edge. Females are larger (20–26 mm) with yellow forewings marked by two distinct dark spots (Waring *et al.*, 2017). With a wingspan of 35–55 mm, *L. quadra* is a large Footman moth.

The Four-spotted Footman inhabits mature broadleaved woodlands and shows annual fluctuations in population, often reinforced by immigration. Its larvae feed on lichens and algae, overwintering in the larval stage before pupating in late spring (Waring *et al.*, 2017).

Here we present a chromosomally complete genome sequence for *Lithosia quadra*, based on a male specimen from Wytham Woods, Oxfordshire, United Kingdom (Figure 1).

Genome sequence report

Sequencing data

The genome of a specimen of *Lithosia quadra* (Figure 1) was sequenced using Pacific Biosciences single-molecule HiFi long



Figure 1. Photograph of the *Lithosia quadra* (iLitQuad1) specimen used for genome sequencing.

reads, generating 27.69 Gb from 2.31 million reads. GenomeScope analysis of the PacBio HiFi data estimated the haploid genome size at 450.36 Mb, with a heterozygosity of 2.02% and repeat content of 23.25%. These values provide an initial assessment of genome complexity and the challenges anticipated during assembly. Based on this estimated genome size, the sequencing data provided approximately 58.0x coverage of the genome. Chromosome conformation Hi-C sequencing produced 84.60 Gb from 560.29 million reads. Table 1 summarises the specimen and sequencing information, including the BioProject, study name, BioSample numbers, and sequencing data for each technology.

Assembly statistics

The primary haplotype was assembled, and contigs corresponding to an alternate haplotype were also deposited in INSDC databases. The assembly was improved by manual curation, which corrected 17 misjoins or missing joins and removed 2 haplotypic duplications. The final assembly has a total length of 456.27 Mb in 43 scaffolds, with 67 gaps, and a scaffold N50 of 16.41 Mb (Table 2).

The snail plot in Figure 2 provides a summary of the assembly statistics, indicating the distribution of scaffold lengths and other assembly metrics. Figure 3 shows the distribution of scaffolds by GC proportion and coverage. Figure 4 presents a cumulative assembly plot, with separate curves representing different scaffold subsets assigned to various phyla, illustrating the completeness of the assembly.

Most of the assembly sequence (99.92%) was assigned to 31 chromosomal-level scaffolds, representing 30 autosomes and the Z sex chromosome. These chromosome-level scaffolds, confirmed by Hi-C data, are named according to size (Figure 5; Table 3). During curation, chromosome Z was assigned based on synteny with the genome of *Nudaria mundana* (GCA_963556515.1) (Crowley *et al.*, 2024).

The mitochondrial genome was also assembled. This sequence is included as a contig in the multifasta file of the genome submission and as a standalone record in GenBank.

Assembly quality metrics

The estimated Quality Value (QV) and *k*-mer completeness metrics, along with BUSCO completeness scores, were calculated for each haplotype and the combined assembly. The QV reflects the base-level accuracy of the assembly, while *k*-mer completeness indicates the proportion of expected *k*-mers identified in the assembly. BUSCO scores provide a measure of completeness based on benchmarking universal single-copy orthologues.

The primary haplotype has a QV of 66.7, and the combined primary and alternate assemblies achieve an estimated QV of 66.5. The *k*-mer completeness for the primary haplotype is 68.01%, and for the alternate haplotype it is 68.02%. The combined primary and alternate assemblies achieve a *k*-mer completeness of 99.36%. BUSCO analysis using the

Table 1. Specimen and sequencing data for *Lithosia quadra*.

Project information			
Study title	Lithosia quadra (four-spotting footman)		
Umbrella BioProject	PRJEB66748		
Species	<i>Lithosia quadra</i>		
BioSpecimen	SAMEA112775013		
NCBI taxonomy ID	987974		
Specimen information			
Technology	ToLID	BioSample accession	Organism part
PacBio long read sequencing	iLitQuad1	SAMEA112775073	head and thorax
Hi-C sequencing	iLitQuad1	SAMEA112775073	head and thorax
Sequencing information			
Platform	Run accession	Read count	Base count (Gb)
Hi-C Illumina NovaSeq 6000	ERR12102419	5.60e+08	84.6
PacBio Sequel IIe	ERR12102453	2.31e+06	27.69

lepidoptera_odb10 reference set ($n = 5,286$) indicated a completeness score of 98.8% (single = 98.0%, duplicated = 0.8%).

Table 2 provides assembly metric benchmarks adapted from Rhie *et al.* (2021) and the Earth BioGenome Project (EBP) Report on Assembly Standards September 2024. The assembly achieves the EBP reference standard of **6.C.Q66**.

Methods

Sample acquisition and DNA barcoding

An adult female *Lithosia quadra* (specimen ID Ox003043, ToLID iLitQuad1) was collected from Wytham Woods, Oxfordshire, United Kingdom (latitude 51.77, longitude -1.34) on 2022-07-22, using a light trap. The specimen was collected by Finley Hutchinson and Liam Crowley (University of Oxford), identified by Finley Hutchinson, and preserved on dry ice.

The initial identification was verified by an additional DNA barcoding process according to the framework developed by Twyford *et al.* (2024). A small sample was dissected from the specimen and stored in ethanol, while the remaining parts were shipped on dry ice to the Wellcome Sanger Institute (WSI) (Pereira *et al.*, 2022). The tissue was lysed, the COI marker region was amplified by PCR, and amplicons were sequenced and compared to the BOLD database, confirming the species identification (Crowley *et al.*, 2023). Following whole genome sequence generation, the relevant DNA barcode region was also used alongside the initial barcoding data for sample tracking at the WSI (Twyford *et al.*, 2024). The standard operating

procedures for Darwin Tree of Life barcoding have been deposited on protocols.io (Beasley *et al.*, 2023).

Metadata collection for samples adhered to the Darwin Tree of Life project standards described by Lawniczak *et al.* (2022).

Nucleic acid extraction

The workflow for high molecular weight (HMW) DNA extraction at the Wellcome Sanger Institute (WSI) Tree of Life Core Laboratory includes a sequence of procedures: sample preparation and homogenisation, DNA extraction, fragmentation and purification. Detailed protocols are available on protocols.io (Denton *et al.*, 2023b). The iLitQuad1 sample was prepared for DNA extraction by weighing and dissecting it on dry ice (Jay *et al.*, 2023). Tissue from the head and thorax was homogenised using a PowerMasher II tissue disruptor (Denton *et al.*, 2023a).

HMW DNA was extracted in the WSI Scientific Operations core using the Automated MagAttract v2 protocol (Oatley *et al.*, 2023). The DNA was sheared into an average fragment size of 12–20 kb in a Megaruptor 3 system (Bates *et al.*, 2023). Sheared DNA was purified by solid-phase reversible immobilisation, using AMPure PB beads to eliminate shorter fragments and concentrate the DNA (Strickland *et al.*, 2023). The concentration of the sheared and purified DNA was assessed using a Nanodrop spectrophotometer and Qubit Fluorometer using the Qubit dsDNA High Sensitivity Assay kit. Fragment size distribution was evaluated by running the sample on the FemtoPulse system.

Table 2. Genome assembly data for *Lithosia quadra*.

Genome assembly		
Assembly name	ilLitQuad1.1	
Assembly accession	GCA_963576445.1	
Alternate haplotype accession	GCA_963576435.1	
Assembly level for primary assembly	chromosome	
Span (Mb)	456.27	
Number of contigs	110	
Number of scaffolds	43	
Longest scaffold (Mb)	22.95	
Assembly metrics	Measure	Benchmark
Contig N50 length	8.98 Mb	≥ 1 Mb
Scaffold N50 length	16.41 Mb	= chromosome N50
Consensus quality (QV)	Primary: 66.7; alternate: 66.4; combined 66.5	≥ 40
k-mer completeness	Primary: 68.01%; alternate: 68.02%; combined: 99.36%	$\geq 95\%$
BUSCO*	C:98.8%[S:98.0%,D:0.8%], F:0.2%,M:1.0%,n:5,286	$S > 90\%$, $D < 5\%$
Percentage of assembly mapped to chromosomes	99.92%	$\geq 90\%$
Sex chromosomes	Z	localised homologous pairs
Organelles	Mitochondrial genome: 15.38 kb	complete single alleles

* BUSCO scores based on the lepidoptera_odb10 BUSCO set using version 5.5.0. C = complete [S = single copy, D = duplicated], F = fragmented, M = missing, n = number of orthologues in comparison.

Hi-C sample preparation

Tissue from the head and thorax of the ilLitQuad1 sample was processed for Hi-C sequencing at the WSI Scientific Operations core, using the Arima-HiC v2 kit. In brief, 20–50 mg of frozen tissue (stored at -80 °C) was fixed, and the DNA crosslinked using a TC buffer with 22% formaldehyde concentration. After crosslinking, the tissue was homogenised using the Diagenode Power Masher-II and BioMasher-II tubes and pestles. Following the Arima-HiC v2 kit manufacturer's instructions, crosslinked DNA was digested using a restriction enzyme master mix. The 5'-overhangs were filled in and labelled with biotinylated nucleotides and proximally ligated. An overnight incubation was carried out for enzymes to digest remaining proteins and for crosslinks to reverse. A clean up was performed with SPRIselect beads prior to library preparation. Additionally, the biotinylation percentage was estimated using the Qubit Fluorometer v4.0 (Thermo Fisher

Scientific) and Qubit HS Assay Kit and Arima-HiC v2 QC beads.

Library preparation and sequencing

Library preparation and sequencing were performed at the WSI Scientific Operations core.

PacBio HiFi

At a minimum, samples were required to have an average fragment size exceeding 8 kb and a total mass over 400 ng to proceed to the low input SMRTbell Prep Kit 3.0 protocol (Pacific Biosciences, California, USA), depending on genome size and sequencing depth required. Libraries were prepared using the SMRTbell Prep Kit 3.0 (Pacific Biosciences, California, USA) as per the manufacturer's instructions. The kit includes the reagents required for end repair/A-tailing, adapter ligation, post-ligation SMRTbell bead cleanup, and nuclease treatment.

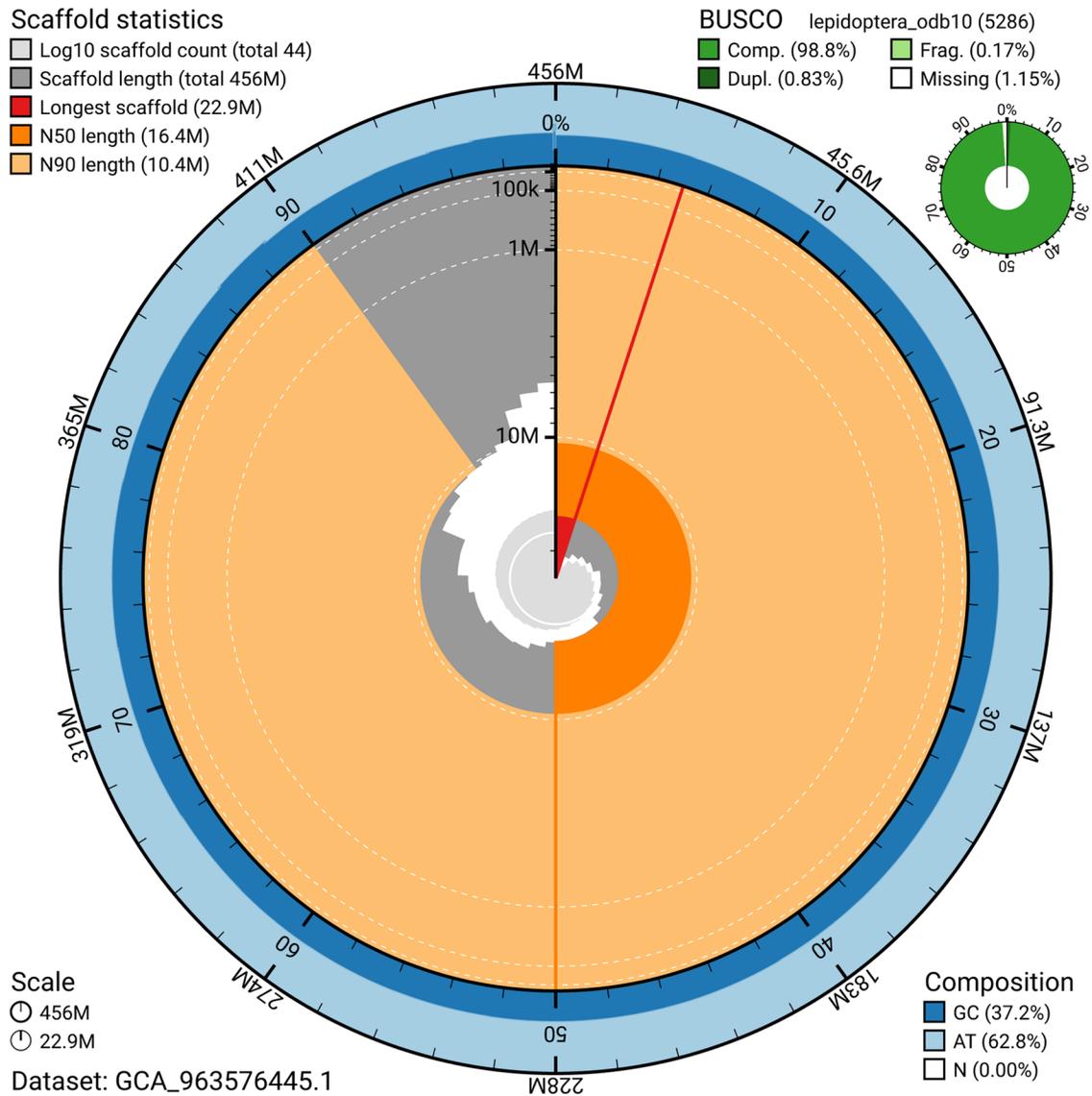


Figure 2. Genome assembly of *Lithosia quadra*, ilLitQuad1.1: metrics. The BlobToolKit snail plot provides an overview of assembly metrics and BUSCO gene completeness. The circumference represents the length of the whole genome sequence, and the main plot is divided into 1,000 bins around the circumference. The outermost blue tracks display the distribution of GC, AT, and N percentages across the bins. Scaffolds are arranged clockwise from longest to shortest and are depicted in dark grey. The longest scaffold is indicated by the red arc, and the deeper orange and pale orange arcs represent the N50 and N90 lengths. A light grey spiral at the centre shows the cumulative scaffold count on a logarithmic scale. A summary of complete, fragmented, duplicated, and missing BUSCO genes in the lepidoptera_odb10 set is presented at the top right. An interactive version of this figure is available at https://blobtoolkit.genomehubs.org/view/GCA_963576445.1/dataset/GCA_963576445.1/snail.

Following the manufacturer's instructions, size selection and clean up was carried out using diluted AMPure PB beads (Pacific Biosciences, California, USA). DNA concentration was quantified using the Qubit Fluorometer v4.0 (Thermo Fisher Scientific) with Qubit 1X dsDNA HS assay kit and the final library fragment size analysis was carried out using the

Agilent Femto Pulse Automated Pulsed Field CE Instrument (Agilent Technologies) and gDNA 55kb BAC analysis kit.

Samples were sequenced using the Sequel IIe system (Pacific Biosciences, California, USA). The concentration of the library loaded onto the Sequel IIe was in the range 40–135 pM.

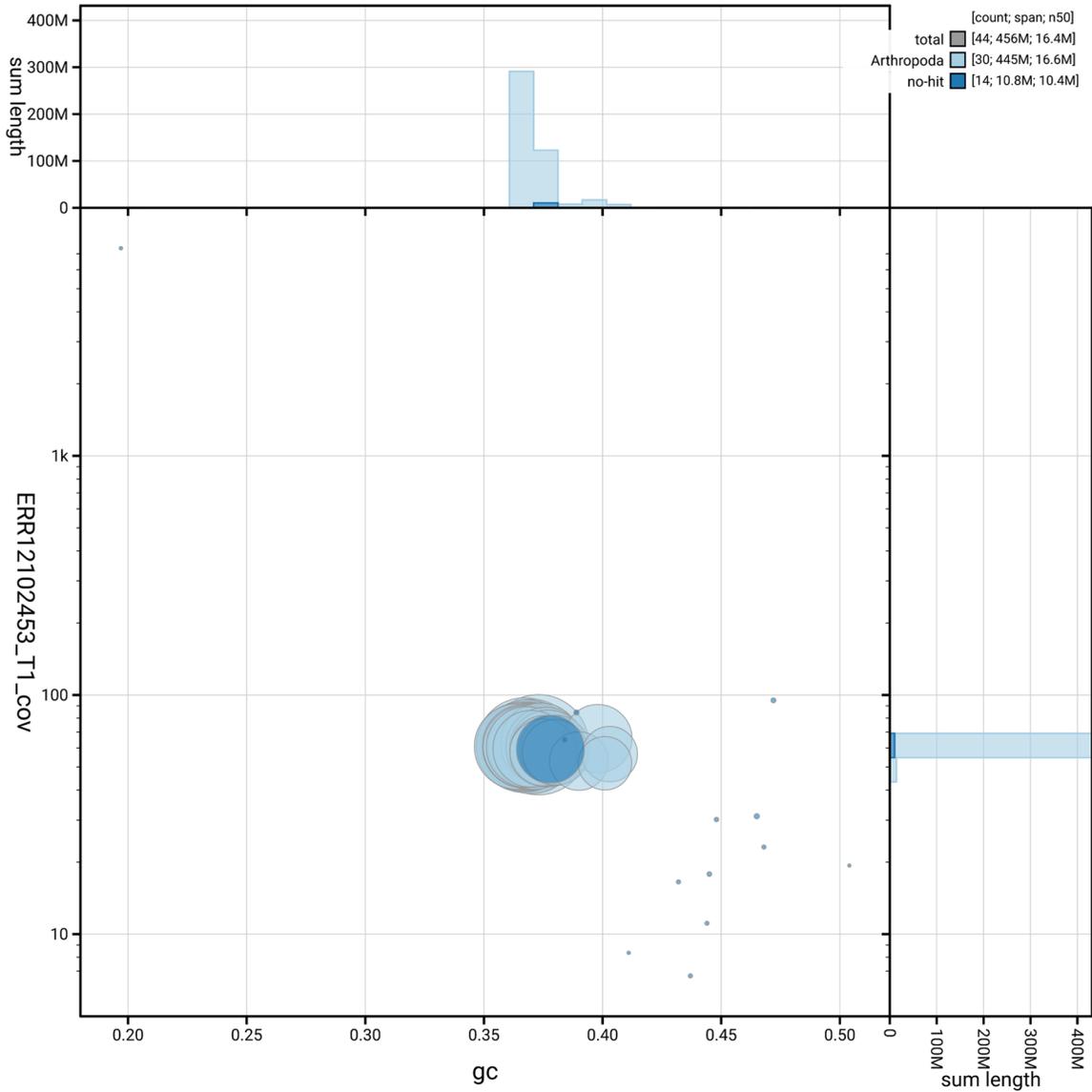


Figure 3. Genome assembly of *Lithosia quadra*, ilLitQuad1.1: BlobToolKit GC-coverage plot. Blob plot showing sequence coverage (vertical axis) and GC content (horizontal axis). The circles represent scaffolds, with the size proportional to scaffold length and the colour representing phylum membership. The histograms along the axes display the total length of sequences distributed across different levels of coverage and GC content. An interactive version of this figure is available at https://blobtoolkit.genomehubs.org/view/GCA_963576445.1/blob.

The SMRT link software, a PacBio web-based end-to-end workflow manager, was used to set-up and monitor the run, as well as perform primary and secondary analysis of the data upon completion.

Hi-C

For Hi-C library preparation, DNA was fragmented using the Covaris E220 sonicator (Covaris) and size selected using

SPRIselect beads to 400 to 600 bp. The DNA was then enriched using the Arima-HiC v2 kit Enrichment beads. Using the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs) for end repair, a-tailing, and adapter ligation. This uses a custom protocol which resembles the standard NEBNext Ultra II DNA Library Prep protocol but where library preparation occurs while DNA is bound to the Enrichment beads. For library amplification, 10 to 16 PCR cycles were required,

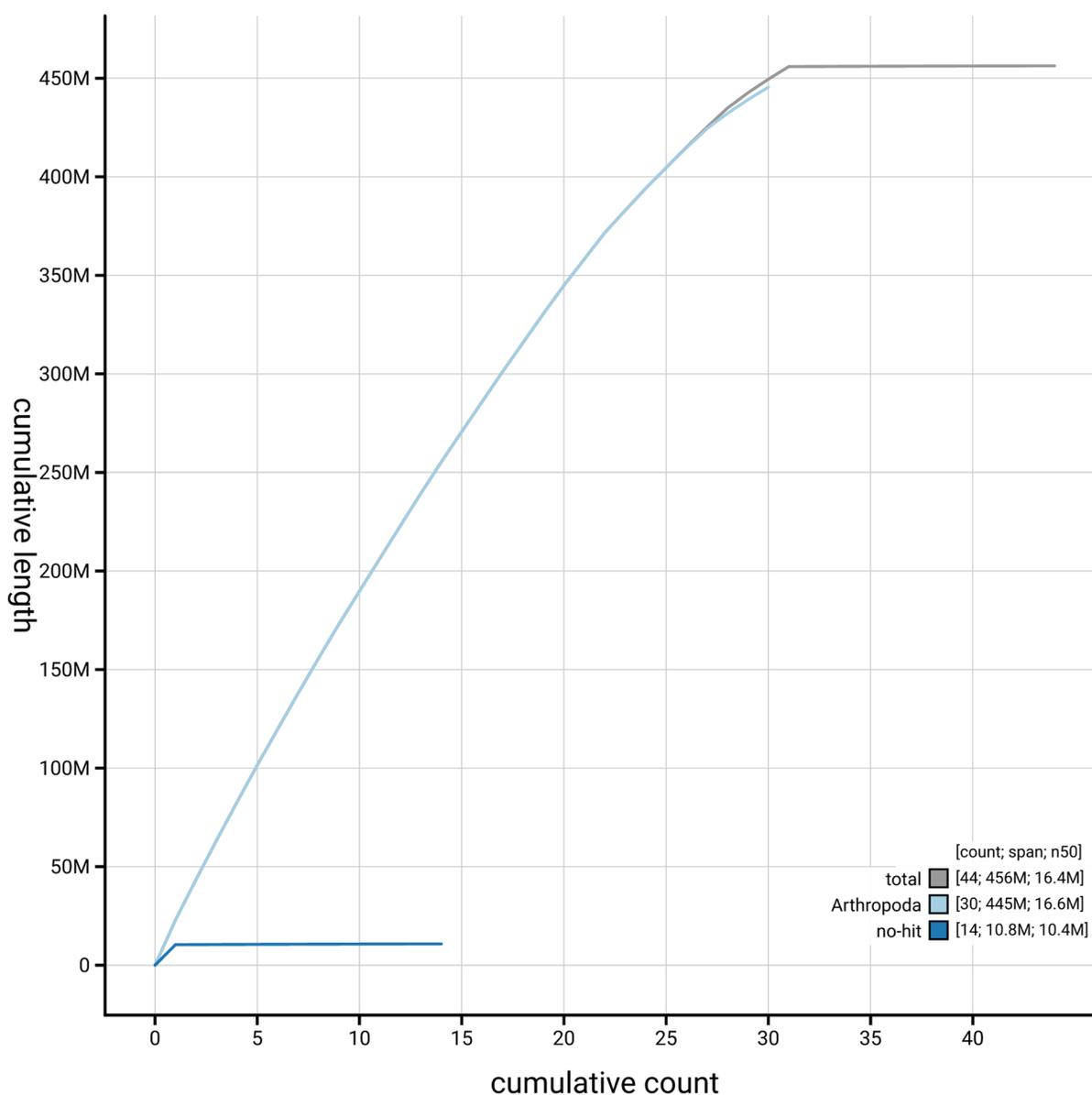


Figure 4. Genome assembly of *Lithosia quadra*, iLLitQuad1.1: BlobToolKit cumulative sequence plot. The grey line shows cumulative length for all scaffolds. Coloured lines show cumulative lengths of scaffolds assigned to each phylum using the buscogenes taxrule. An interactive version of this figure is available at https://blobtoolkit.genomehubs.org/view/GCA_963576445.1/dataset/GCA_963576445.1/cumulative.

determined by the sample biotinylation percentage. The Hi-C sequencing was performed using paired-end sequencing with a read length of 150 bp on an Illumina NovaSeq 6000 instrument.

Genome assembly, curation and evaluation

Assembly

Prior to assembly of the PacBio HiFi reads, a database of k -mer counts ($k = 31$) was generated from the filtered reads using FastK. GenomeScope2 (Ranallo-Benavidez *et al.*, 2020) was used to analyse the k -mer frequency distributions,

providing estimates of genome size, heterozygosity, and repeat content.

The HiFi reads were first assembled using Hifiasm (Cheng *et al.*, 2021) with the --primary option. Haplotypic duplications were identified and removed using purge_dups (Guan *et al.*, 2020). The Hi-C reads were mapped to the primary contigs using bwa-mem2 (Vasimuddin *et al.*, 2019). The contigs were further scaffolded using the provided Hi-C data (Rao *et al.*, 2014) in YaHS (Zhou *et al.*, 2023) using the --break

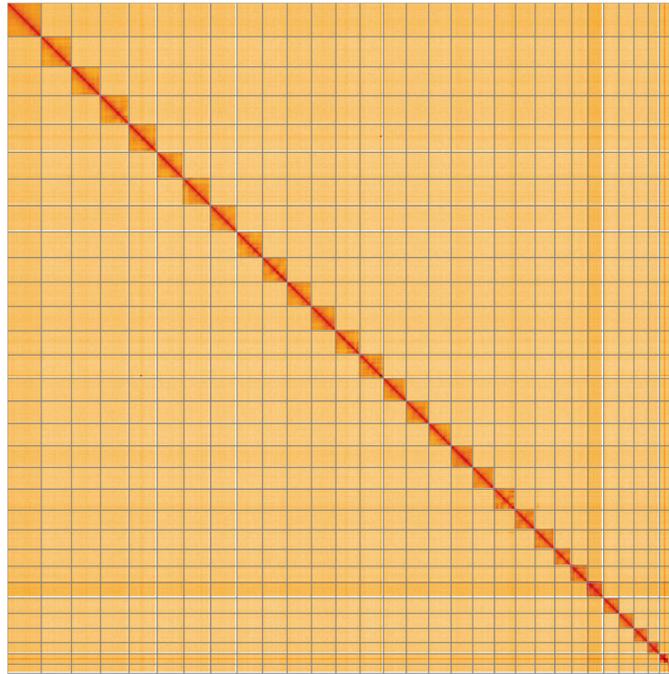


Figure 5. Genome assembly of *Lithosia quadra*: Hi-C contact map of the iLitQuad1.1 assembly, visualised using HiGlass. Chromosomes are shown in order of size from left to right and top to bottom. An interactive version of this figure may be viewed at <https://genome-note-higlass.tol.sanger.ac.uk/?d=SbppNic8RpuV088Ms069EQ>.

Table 3. Chromosomal pseudomolecules in the genome assembly of *Lithosia quadra*, iLitQuad1.

INSDC accession	Name	Length (Mb)	GC%
OY754874.1	1	20.43	36.5
OY754875.1	2	19.83	37
OY754876.1	3	19.35	37
OY754877.1	4	18.95	37
OY754878.1	5	18.27	37
OY754879.1	6	18.2	36.5
OY754880.1	7	17.79	36.5
OY754881.1	8	17.38	36.5
OY754882.1	9	16.64	36.5
OY754883.1	10	16.6	37
OY754884.1	11	16.56	37
OY754885.1	12	16.41	37
OY754886.1	13	15.92	36.5
OY754887.1	14	15.38	37
OY754888.1	15	15.24	37

INSDC accession	Name	Length (Mb)	GC%
OY754889.1	16	15.21	36.5
OY754890.1	17	14.75	37.5
OY754891.1	18	14.73	37
OY754892.1	19	14.28	37.5
OY754893.1	20	13.38	37.5
OY754894.1	21	13.36	37
OY754895.1	22	11.35	37.5
OY754896.1	23	11.15	38
OY754897.1	24	10.54	40
OY754898.1	25	10.42	38
OY754899.1	26	10.13	37.5
OY754900.1	27	9.66	38
OY754901.1	28	7.72	39
OY754902.1	29	6.92	40.5
OY754903.1	30	6.41	40
OY754873.1	Z	22.95	37.5
OY754904.1	MT	0.02	20

option for handling potential misassemblies. The scaffolded assemblies were evaluated using Gfastats (Formenti *et al.*, 2022), BUSCO (Manni *et al.*, 2021) and MERQURY.FK (Rhie *et al.*, 2020).

The mitochondrial genome was assembled using MitoHiFi (Uliano-Silva *et al.*, 2023), which runs MitoFinder (Allio *et al.*, 2020) and uses these annotations to select the final mitochondrial contig and to ensure the general quality of the sequence.

Assembly curation

The assembly was decontaminated using the Assembly Screen for Cobionts and Contaminants (ASCC) pipeline (article in preparation). Flat files and maps used in curation were generated in TreeVal (Pointon *et al.*, 2023). Manual curation was primarily conducted using PretextView (Harry, 2022), with additional insights provided by JBrowse2 (Diesh *et al.*, 2023) and HiGlass (Kerpedjiev *et al.*, 2018). Scaffolds were visually inspected and corrected as described by Howe *et al.* (2021). Any identified contamination, missed joins, and misjoins were corrected, and duplicate sequences were tagged and removed. The sex chromosome was identified based on synteny analysis. The curation process is documented at <https://gitlab.com/wtsi-grit/rapid-curation> (article in preparation).

Assembly quality assessment

The Merqury.FK tool (Rhie *et al.*, 2020), run in a Singularity container (Kurtzer *et al.*, 2017), was used to evaluate k -mer completeness and assembly quality for the primary and alternate haplotypes using the k -mer databases ($k = 31$) that were computed prior to genome assembly. The analysis outputs included assembly QV scores and completeness statistics.

A Hi-C contact map was produced for the final version of the assembly. The Hi-C reads were aligned using bwa-mem2

(Vasimuddin *et al.*, 2019) and the alignment files were combined using SAMtools (Danecek *et al.*, 2021). The Hi-C alignments were converted into a contact map using BEDTools (Quinlan & Hall, 2010) and the Cooler tool suite (Abdennur & Mirny, 2020). The contact map was visualised in HiGlass (Kerpedjiev *et al.*, 2018).

The blobtoolkit pipeline is a Nextflow port of the previous Snakemake Blobtoolkit pipeline (Challis *et al.*, 2020). It aligns the PacBio reads in SAMtools and minimap2 (Li, 2018) and generates coverage tracks for regions of fixed size. In parallel, it queries the GoAT database (Challis *et al.*, 2023) to identify all matching BUSCO lineages to run BUSCO (Manni *et al.*, 2021). For the three domain-level BUSCO lineages, the pipeline aligns the BUSCO genes to the UniProt Reference Proteomes database (Bateman *et al.*, 2023) with DIAMOND blastp (Buchfink *et al.*, 2021). The genome is also divided into chunks according to the density of the BUSCO genes from the closest taxonomic lineage, and each chunk is aligned to the UniProt Reference Proteomes database using DIAMOND blastx. Genome sequences without a hit are chunked using seqtk and aligned to the NT database with blastn (Altschul *et al.*, 1990). The blobtools suite combines all these outputs into a blobdir for visualisation.

The blobtoolkit pipeline was developed using nf-core tooling (Ewels *et al.*, 2020) and MultiQC (Ewels *et al.*, 2016), relying on the Conda package manager, the Bioconda initiative (Grüning *et al.*, 2018), the Biocontainers infrastructure (da Veiga Leprevost *et al.*, 2017), as well as the Docker (Merkel, 2014) and Singularity (Kurtzer *et al.*, 2017) containerisation solutions.

Table 4 contains a list of relevant software tool versions and sources.

Table 4. Software tools: versions and sources.

Software tool	Version	Source
BEDTools	2.30.0	https://github.com/arq5x/bedtools2
BLAST	2.14.0	ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/ /
BlobToolKit	4.3.9	https://github.com/blobtoolkit/blobtoolkit
BUSCO	5.5.0	https://gitlab.com/ezlab/busco
bwa-mem2	2.2.1	https://github.com/bwa-mem2/bwa-mem2
Cooler	0.8.11	https://github.com/open2c/cooler
DIAMOND	2.1.8	https://github.com/bbuchfink/diamond
fasta_windows	0.2.4	https://github.com/tolkit/fasta_windows
FastK	427104ea91c78c3b8b8b49f1a7d6bbeaa869ba1c	https://github.com/thegenemyers/FASTK
Gfastats	1.3.6	https://github.com/vgl-hub/gfastats

Software tool	Version	Source
GoaT CLI	0.2.5	https://github.com/genomehubs/goat-cli
Hifiasm	0.19.5-r587	https://github.com/chhyllp123/hifiasm
HiGlass	44086069ee7d4d3f6f3f0012569789ec138f42b84aa44357826c0b6753eb28de	https://github.com/higlass/higlass
Mercury.FK	d00d98157618f4e8d1a9190026b19b471055b22e	https://github.com/thegenemyers/MERQURY.FK
Minimap2	2.24-r1122	https://github.com/lh3/minimap2
MitoHiFi	3	https://github.com/marcelauliano/MitoHiFi
MultiQC	1.14, 1.17, and 1.18	https://github.com/MultiQC/MultiQC
NCBI Datasets	15.12.0	https://github.com/ncbi/datasets
Nextflow	23.04.1	https://github.com/nextflow-io/nextflow
PretextView	0.2	https://github.com/sanger-tol/PretextView
purge_dups	1.2.5	https://github.com/dfguan/purge_dups
samtools	1.19.2	https://github.com/samtools/samtools
sanger-tol/ascc	-	https://github.com/sanger-tol/ascc
sanger-tol/blobtoolkit	0.5.1	https://github.com/sanger-tol/blobtoolkit
Seqtk	1.3	https://github.com/lh3/seqtk
Singularity	3.9.0	https://github.com/sylabs/singularity
TreeVal	1.2.0	https://github.com/sanger-tol/treeval
YaHS	1.2a.2	https://github.com/c-zhou/yahs

Wellcome Sanger Institute – Legal and Governance

The materials that have contributed to this genome note have been supplied by a Darwin Tree of Life Partner. The submission of materials by a Darwin Tree of Life Partner is subject to the **‘Darwin Tree of Life Project Sampling Code of Practice’**, which can be found in full on the Darwin Tree of Life website [here](#). By agreeing with and signing up to the Sampling Code of Practice, the Darwin Tree of Life Partner agrees they will meet the legal and ethical requirements and standards set out within this document in respect of all samples acquired for, and supplied to, the Darwin Tree of Life Project.

Further, the Wellcome Sanger Institute employs a process whereby due diligence is carried out proportionate to the nature of the materials themselves, and the circumstances under which they have been/are to be collected and provided for use. The purpose of this is to address and mitigate any potential legal and/or ethical implications of receipt and use of the materials as part of the research project, and to ensure that in doing so

we align with best practice wherever possible. The overarching areas of consideration are:

- Ethical review of provenance and sourcing of the material
- Legality of collection, transfer and use (national and international)

Each transfer of samples is further undertaken according to a Research Collaboration Agreement or Material Transfer Agreement entered into by the Darwin Tree of Life Partner, Genome Research Limited (operating as the Wellcome Sanger Institute), and in some circumstances other Darwin Tree of Life collaborators.

Data availability

European Nucleotide Archive: *Lithosia quadra* (four-spotting footman). Accession number PRJEB66748; <https://identifiers.org/ena.embl/PRJEB66748>. The genome sequence is released

openly for reuse. The *Lithosia quadra* genome sequencing initiative is part of the Darwin Tree of Life (DToL) project. All raw sequence data and the assembly have been deposited in INSDC databases. The genome will be annotated using available RNA-Seq data and presented through the **Ensembl** pipeline at the European Bioinformatics Institute. Raw data and assembly accession identifiers are reported in **Table 1** and **Table 2**.

Author information

Members of the University of Oxford and Wytham Woods Genome Acquisition Lab are listed here: <https://doi.org/10.5281/zenodo.12157525>.

Members of the Darwin Tree of Life Barcoding collective are listed here: <https://doi.org/10.5281/zenodo.12158331>.

Members of the Wellcome Sanger Institute Tree of Life Management, Samples and Laboratory team are listed here: <https://doi.org/10.5281/zenodo.12162482>.

Members of Wellcome Sanger Institute Scientific Operations: Sequencing Operations are listed here: <https://doi.org/10.5281/zenodo.12165051>.

Members of the Wellcome Sanger Institute Tree of Life Core Informatics team are listed here: <https://doi.org/10.5281/zenodo.12160324>.

Members of the Tree of Life Core Informatics collective are listed here: <https://doi.org/10.5281/zenodo.12205391>.

Members of the Darwin Tree of Life Consortium are listed here: <https://doi.org/10.5281/zenodo.4783558>.

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 **Sivasankaran Kuppusamy** 
Loyola College, Chennai, India

The authors have done the chromosome-level genome assembly of *Lithosia quadra* (Linnaeus, 1758). Authors have used the appropriate methods for the DNA isolation, purification and library preparation for sequencing. Appropriate software's were used for assembly and sequence annotation. They observed a 456.17 Mb of genome sequence of the species. They have assigned synteny Z chromosome with the genome of *Nudaria mundana*.

Comments on the manuscript

The first sentence of the Background can be modified as "*Lithosia quadra* (Linnaeus, 1758) or the Four-spotted Footman moth belonging to the family Erebidae".

The last sentence of the first paragraph "With a wingspan of 35-55 mm...." can be deleted.

Last paragraph of the Background can be modified as "Here we present a chromosome-level genome assembly of *L. quadra*....."

Throughout the manuscript, the authors consistently used the genus name *Lithosia quadra* in its full form. If possible, use the full genus name at first, and later to shorten it to *L. quadra*

Authors have sequenced the genome's total length of 112.29 Gb using three different sequencing platforms. Though authors assembled and received 456.27 Mb genome in size but haven't observed the protein-coding genes, non-coding genes and gene transcripts. What is the reason?

Authors have mentioned the sequence was done based on a male specimen in the Abstract, Background and Genome sequence report. But in the Methods authors have mentioned "An adult female *Lithosia quadra* was collected from Wytham Woods Oxfordshire". I think there is an error. Authors can change it.

Above all, I confirm that the manuscript meets the necessary scientific standard and is suitable for indexing

Is the rationale for creating the dataset(s) clearly described?

Yes

Are the protocols appropriate and is the work technically sound?

Yes

Are sufficient details of methods and materials provided to allow replication by others?

Yes

Are the datasets clearly presented in a useable and accessible format?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Phylogenetic analysis of Moths using mitogenome sequence

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 19 April 2025

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Yu-Feng Huang 

National Chung Hsing University, Taichung, Taiwan

This manuscript presents a high-quality, chromosomal-level genome assembly of *Lithosia quadra*, the Four-spotted Footman moth, a lichen-feeding Erebiidae species with widespread distribution across the Palearctic region. Generated as part of the Darwin Tree of Life (DTOL) initiative, the assembly is based on PacBio HiFi long-read sequencing and Hi-C scaffolding. The final genome spans 456.27 Mb, with 99.92% of the assembly placed on 31 chromosomal pseudomolecules, including the Z chromosome. The authors also assembled the 15.38 kb mitochondrial genome. Quality metrics are excellent, with a primary haplotype consensus QV of 66.7, a scaffold N50 of 16.41 Mb, and a BUSCO completeness score of 98.8% (based on the lepidoptera_odb10 dataset). The assembly meets the Earth BioGenome Project's reference standard (6.C.Q66).

The technical and methodological execution of the project is of high standard. Sequencing, assembly, curation, and evaluation are thoroughly documented, supported by open-access tools and interactive visualizations. The manuscript's value lies in contributing to the broader effort of cataloguing biodiversity genomics within Lepidoptera, and Erebiidae in particular. However, several areas of improvement are necessary to elevate the manuscript's scientific utility and clarity.

Major Concerns

1. Haplotype Representation and Diversity

While both primary and alternate assemblies are deposited, the manuscript lacks discussion on phased regions or heterozygosity. With an estimated 2.02% heterozygosity, the authors should clarify whether any haplotype-resolved analysis was attempted or if structural differences were observed.

2. Unplaced Scaffolds

The assembly claims 99.92% chromosomal placement, yet there is no description of the remaining 0.08%. Are these repeats, contamination, or unresolved genomic regions? A short explanation would improve transparency.

3. Mitochondrial Genome Representation

The mitochondrial genome is included alongside nuclear chromosomes in Table 3, which could be confusing due to its much smaller size. It should be reported separately, as is standard in genomic publications. Also, the mitochondrial genome length is given in floating-point (15.38 kb) in multiple places and should be reported as the full integer value for accuracy.

Minor Concerns

- A comparison of the presented assembly quality (e.g., QV, k-mer completeness) with other Lepidoptera genomes would provide helpful context.
- The Methods section is highly technical; a concise overview or flow diagram could help general readers.

Is the rationale for creating the dataset(s) clearly described?

Yes

Are the protocols appropriate and is the work technically sound?

Partly

Are sufficient details of methods and materials provided to allow replication by others?

Yes

Are the datasets clearly presented in a useable and accessible format?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Bioinformatics, Genome assembly and annotation, Structural bioinformatics, Cancer biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 08 April 2025

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Axel Künstner 

University of Lübeck, Lübeck, Germany

The submitted manuscript describes the genome assembly of the Four-spotted Footman moth (*Lithosia quadra*). The genome was assembled using a single male individual, and the assembly comprises a total length of 456.27 MB. As males are the homogametic sex in *Lithosia quadra*, the assembly lacks the W sex chromosome. The mitochondrial genome is provided as well with the nuclear genome assembly.

The methods section thoroughly describes sample collection, DNA extraction, sequencing (using PacBio HiFi long reads and Hi-C), assembly approach, and quality assessment. They used appropriate tools for genome assembly (Hifiasm), scaffolding (YaHS), and quality evaluation (BUSCO, Merqury.FK).

The paper follows the standard format for Darwin Tree of Life genome notes, including appropriate figures showing assembly metrics, GC-coverage distributions, and Hi-C contact maps that validate the chromosomal-level scaffolding.

Overall, the paper presents a technically sound chromosome-level genome assembly that meets the Earth BioGenome Project reference standard (6.C.Q66). The genome should be valuable for future studies on lepidopteran genomics and evolution.

The data is available via the European Nucleotide Archive (Accession number PRJEB66748).

Is the rationale for creating the dataset(s) clearly described?

Yes

Are the protocols appropriate and is the work technically sound?

Yes

Are sufficient details of methods and materials provided to allow replication by others?

Yes

Are the datasets clearly presented in a useable and accessible format?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cancer genomics, Microbiome, Genome assembly

I confirm that I have read this submission and believe that I have an appropriate level of

expertise to confirm that it is of an acceptable scientific standard.
