DATA NOTE

REVISED ERGA-BGE genome of the Spanish moon moth *Actias isabellae* Graells, 1849: a nocturnal lepidopteran protected by the Habitats Directive

[version 2; peer review: 2 approved]

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Abstract

High-quality reference genomes are critical resources for understanding biodiversity and supporting conservation efforts. We

present the chromosome-level assembly of the Spanish moon moth, *Actias isabellae* (Graells, 1849), a nocturnal lepidopteran protected under the EU Habitats Directive. The assembly spans 0.56 Gb across 31 pseudomolecules, including the Z chromosome, with a contig N50 of 18.9 Mb and a scaffold N50 of 20.4 Mb. The mitochondrial genome was also assembled into a 15,247 bp circular sequence. Annotation identified 11,805 protein-coding genes and 2,238 non-coding genes, with a BUSCO completeness of over 94%. Notably, no W chromosome was detected, suggesting a ZZ/Z0 sex determination system. This reference genome provides an essential foundation for studying sex chromosome evolution in Lepidoptera and enables advanced population genomics monitoring of this protected species. More broadly, it contributes to ongoing efforts within the European Reference Genome Atlas and the Earth BioGenome Project to harness genomics for biodiversity conservation.

Keywords
Graellsia isabellae, Actias isabellae, genome assembly, European Reference Genome Atlas, Biodiversity Genomics Europe, Earth Biogenome Project, Spanish moon moth, Saturniidae

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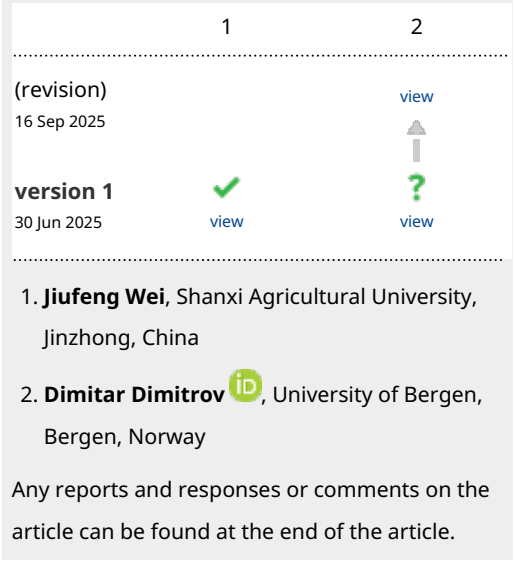

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REVISED **Amendments from Version 1**

In the new revised manuscript, we addressed the reviewers comments by modifying the abstract and the list of keywords. We have also reworded the introduction to better explain the role that recent phylogenomic analyses have played in the change in genus associated with *Actias isabellae*. We have also clarified the approach used to estimate the genome size based on ancestral taxa, as we realised our statement was misleading. Finally, we clarified the use of BioSample SAMEA114400479 for sequencing and assembly.

Any further responses from the reviewers can be found at the end of the article

Introduction

Recent phylogenomic analyses have supported have supported the transfer of the Spanish moon moth from the genus *Graellsia* (Graells, 1849) to genus *Actias* (Graells, 1849) (García-Souto *et al.*, 2025). This lepidopteran, belonging to the family Saturniidae, exhibits a diploid chromosome number of 2n = 62 (Templado *et al.*, 1973). This univoltine insect is primarily found in the mountainous regions of the Eastern Iberian Peninsula and the French Alps. The larvae predominantly feed on Scots pine (*Pinus sylvestris* L.), although some populations in Spain utilise Black pine (*P. nigra* Arnold). Using the nuclear microsatellite markers available for this species (Vila *et al.*, 2010), Marí-Mena *et al.* (2016) identified a strong phylogeographic pattern, revealing six genetic groups distributed mainly along distinct mountain ranges, though no host-associated differentiation was detected. More recent analyses using the same markers also revealed a west–east cline in allele frequencies along the Central Pyrenees that causes low overall genetic differentiation within and around the Ordesa y Monte Perdido National Park (González-Castellano *et al.*, 2023).

The Spanish moon moth is protected under the Bern Convention (ETS No. 104, Annex III), as well as the European Union's Habitats Directive (92/43/EEC, Annexes II and V). Consequently, Spain and France, where the species occurs naturally, have implemented conservation measures aimed at preventing any disturbance to the moth or its habitats. *Actias isabellae* is currently classified as "Data Deficient" on the IUCN Red List. This classification was assigned in 1996 and is due for an update. (Marí-Mena *et al.*, 2019) suggested reclassifying it as "Least Concern", reflecting a generally favorable conservation status. However, Marí-Mena *et al.*, 2019 also cautioned that some findings, such as a high temporal fragmentation index and very low values of N_e/N, suggest a potential risk of genetic erosion if populations become isolated due to habitat fragmentation.

The generation of this reference resource was coordinated by the European Reference Genome Atlas (ERGA) initiative's Biodiversity Genomics Europe (BGE) project, supporting ERGA's aims of promoting transnational cooperation to promote advances in the application of genomics technologies to protect and restore biodiversity (Mazzoni *et al.*, 2023).

Materials & methods

ERGA's sequencing strategy includes Oxford Nanopore Technology (ONT) and/or Pacific Biosciences (PacBio) for long-read sequencing, along with Hi-C sequencing for chromosomal architecture, Illumina Paired-End (PE) for polishing (i.e. recommended for ONT-only assemblies), and RNA sequencing for transcriptome profiling, to facilitate genome assembly and annotation.

Sample and sampling information

A female specimen of *Actias isabellae* was collected by Marta Vila and Neus Marí-Mena at the type locality (Peguerinos, Ávila, Castilla y León, Spain) on 25 June 2022. In addition, two male (homogametic) individuals of the same species were sampled. The first one was also collected on 25 June 2022 by Vila & Marí-Mena at Peguerinos. The second one was collected by Roger Vila in Casa de la Collada (Collsuspina, Catalunya, Spain) on 09 June 2023. The sampling of all specimens was performed under permission AUES/CYL/168/2022 issued by the Junta de Castilla y León, and SF/0187/23 issued by the Generalitat de Catalunya, Spain. The female was attracted to a light trap, and male attraction was additionally strengthened using synthetic female pheromones (Millar *et al.*, 2010). Specimens were subsequently collected using a butterfly net and identified based on external morphology.

Specimens were preserved in dry ice until arrival at the lab where the specimens were later preserved at -80°C in a freezer.

Vouchering information

Physical reference materials for the male sampled by Roger Vila have been deposited in the Museo Nacional de Ciencias Naturales (Entomology Collection) Madrid, Spain https://www.mncn.csic.es under the accession number MNCN:Ent:372276.

Frozen reference somatic tissue material is available from a proxy voucher at the Biobank of the Museo Nacional de Ciencias Naturales https://www.mncn.csic.es under the voucher ID MNCN:ADN:151.721. This specimen was non-lethally sampled in 2008 at the type locality by Marta Vila and Carlos Lopez-Vaamonde under permission of Junta de Castilla y León (EP/CYL/225/2008).

Genetic information

The estimated genome size, estimated by Genomes on a Tree (GoaT) (Challis *et al.*, 2023) by ancestral state reconstruction, is 0.65 Gb. This is a diploid genome with a haploid number of 31 chromosomes (2n = 62). Males are expected to be homogametic regarding sex chromosomes (ZZ), while females are yet to be confirmed as ZW or Z0 (ancestral W chromosome has been reported to fuse with an autosome in some *Saturniidae* species (Yoshido *et al.*, 2011). All information for this species was retrieved from GoaT.

DNA/RNA processing

DNA was extracted from the head and thorax using the Blood & Cell Culture DNA Mini Kit (Qiagen), following the

manufacturer's instructions. DNA quantification was performed using a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific) and DNA integrity was assessed using a Genomic DNA 165 Kb Kit (Agilent) on the Femto Pulse system (Agilent). The DNA was stored at +4°C until used.

RNA was extracted using a RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was extracted from three different specimen parts: legs, head, and abdomen. RNA quantification was performed using the Qubit RNA BR kit and RNA integrity was assessed using a Fragment Analyzer system (RNA 15nt Kit, Agilent). The RNA was pooled in a 1:2:2 ratio (leg:head:abdomen) for the library preparation and stored at -80°C until used.

Library preparation and sequencing

For long-read whole genome sequencing, a library was prepared using the SQK-LSK114 Kit (Oxford Nanopore Technologies, ONT), which was then sequenced on a PromethION 24 A Series instrument (ONT) producing good quality raw data (mean read length = 36,434.9, mean read quality = 21.3). A short-read whole genome sequencing library was prepared using the KAPA Hyper Prep Kit (Roche). The short-read libraries were sequenced 2*150bp. Insert size was 450bp (WGS), 550bp (HiC), 230bp (RNA). A Hi-C library was prepared from head and thorax using the ARIMA High Coverage Hi-C Kit (ARIMA), followed by the KAPA Hyper Prep Kit for Illumina sequencing (Roche). The RNA library was prepared from the pooled sample using the KAPA mRNA Hyper prep kit (Roche). All the short-read libraries were sequenced on a NovaSeq 6000 instrument (Illumina).

In total 265x Oxford Nanopore, 78x Illumina WGS shotgun, and 63x HiC data were sequenced to generate the assembly.

Genome assembly methods

The genome was assembled using the CNAG CLAWS pipeline (Gomez-Garrido, 2024). Briefly, Illumina and ONT reads were pre-processed for quality and length using Trim Galore v0.6.7 and Filtlong v0.2.1, respectively. The filtered subset of 70 Gbp or 125x coverage of ONT reads with a read N50 of 39.6 kb were assembled into contigs using NextDenovo v2.5.0, followed by polishing of the assembled contigs using HyPo v1.0.3, removal of retained haplotigs using purge-dups v1.2.6 and scaffolding with YaHS v1.2a. Finally, assembled scaffolds were curated via manual inspection using Pretext v0.2.5 with the Rapid Curation Toolkit (https://gitlab.com/wtsi-grit/rapid-curation) to remove any false joins and incorporate any sequences not automatically scaffolded into their respective locations in the chromosomal pseudomolecules (or super-scaffolds). Finally, the mitochondrial genome was assembled as a single circular contig of 15,247 bp using the FOAM pipeline (https://github.com/cnag-aat/FOAM) and included in the released assembly (GCA_964265105.1). Summary analysis of the released assembly was performed using the ERGA-BGE Genome Report ASM Galaxy workflow (10.48546/workflowhub.workflow.1103.2).

Genome annotation methods

A gene set was generated using the Ensembl Gene Annotation system (Aken *et al.*, 2016), primarily by aligning publicly available short-read RNA-seq data from BioSample SAMEA116288378 to the genome. Gaps in the annotation were filled via protein-to-genome alignments of a select set of clade-specific proteins from (UniProt Consortium, 2019), which had experimental evidence at the protein or transcript level. At each locus, data were aggregated and consolidated, prioritising models derived from RNA-seq data, resulting in a final set of gene models and associated non-redundant transcript sets. To distinguish true isoforms from fragments, the likelihood of each open reading frame (ORF) was evaluated against known metazoan proteins. Low-quality transcript models, such as those showing evidence of fragmented ORFs, were removed. In cases where RNA-seq data were fragmented or absent, homology data were prioritised, favouring longer transcripts with strong intron support from short-read data. The resulting gene models were classified into two categories: protein-coding, and long non-coding. Models that did not overlap protein-coding genes, and were constructed from transcriptomic data were considered potential lncRNAs. Potential lncRNAs were further filtered to remove single-exon loci due to their unreliability. Putative miRNAs were predicted by performing a BLAST search of miRBase (Kozomara *et al.*, 2019) against the genome, followed by RNAfold analysis (Gruber *et al.*, 2008). Other small non-coding loci were identified by scanning the genome with Rfam (Kalvari *et al.*, 2018) and passing the results through Infernal (Nawrocki & Eddy, 2013). Summary analysis of the released annotation was carried out using the ERGA-BGE Genome Report ANNOT Galaxy workflow (10.48546/workflowhub.workflow.1096.1).

Results

Genome assembly

The genome assembly has a total length of 560,920,942 bp in 32 scaffolds (Z chromosome included) and the mitogenome (Figure 1 & Figure 2), with a GC content of 35.6%. No W chromosomal sequence was found, consistent with the existence of an X0 sex determination system. The assembly has a contig N50 of 18,864,205 bp and L50 of 14 and a scaffold N50 of 20,417,927 bp and L50 of 13. The assembly has a total of 6 gaps, totalling 1.2 kb in cumulative size. The single-copy gene content analysis using the Lepidoptera database with BUSCO v5.5.0 (Manni *et al.*, 2021) resulted in 98.2% completeness (97.9% single and 0.3% duplicated). 91.9% of reads k-mers were present in the assembly and the assembly has a base accuracy Quality Value (QV) of 48.5 as calculated by Merqury (Rhie *et al.*, 2020).

Genome annotation

The genome annotation consists of 11,805 protein-coding genes with associated 22,873 transcripts, in addition to 2,238 non-coding genes (Table 1). Using the longest isoform per transcript, the single-copy gene content analysis using the Lepidoptera odb10 database with BUSCO resulted in 94.2% completeness. Using the OMAmer Metazoa-v2.0.0.h5 database for OMArk (Nevers *et al.*, 2025) resulted in 93.2% completeness and 91.4% consistency (Table 2).

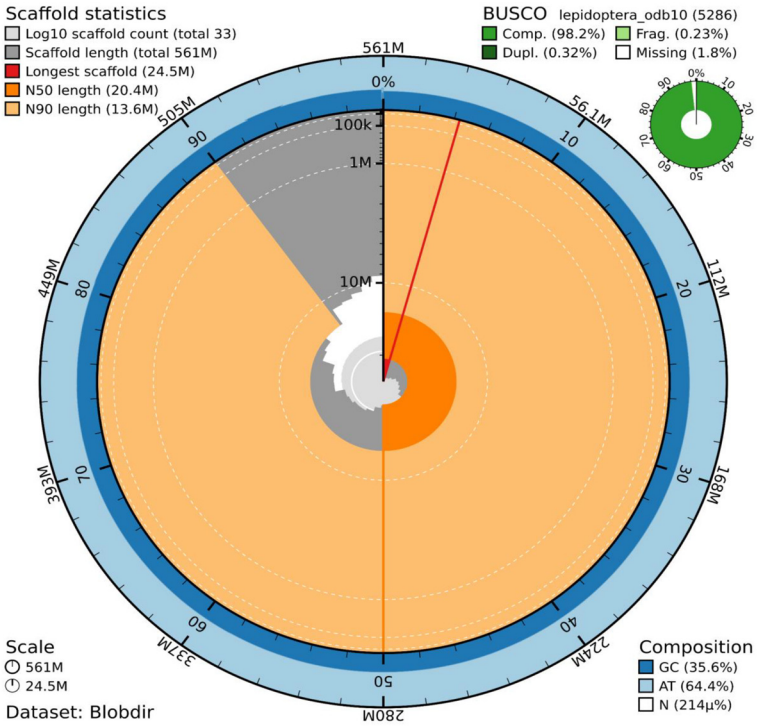

Figure 1. Snail plot summary of assembly statistics. The main plot is divided into 1,000 size-ordered bins around the circumference, with each bin representing 0.1% of the 560,920,942 bp assembly including the mitochondrial genome. The distribution of sequence lengths is shown in dark grey, with the plot radius scaled to the longest sequence present in the assembly (24.5 Mb, shown in red). Orange and pale-orange arcs show the scaffold N50 and N90 sequence lengths (20,417,927 bp and 9,853,470 bp), respectively. The pale grey spiral shows the cumulative sequence count on a log-scale, with white scale lines showing successive orders of magnitude. The blue and pale-blue area around the outside of the plot shows the distribution of GC, AT, and N percentages in the same bins as the inner plot. A summary of complete, fragmented, duplicated, and missing BUSCO genes found in the assembled genome from the Lepidoptera database (odb10) is shown in the top right.

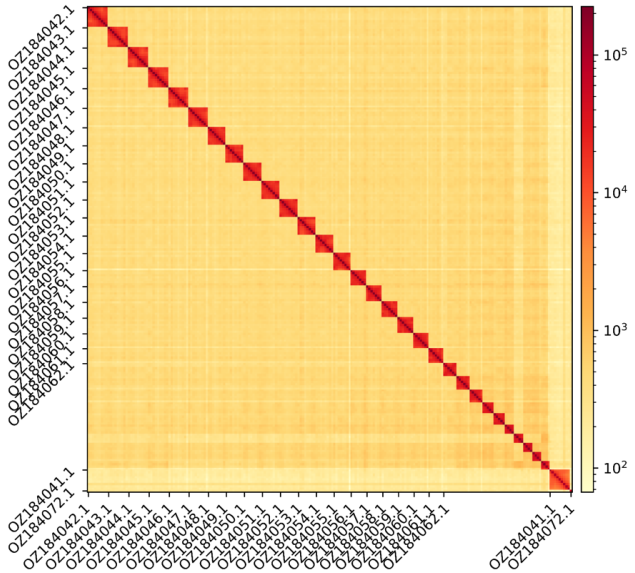

Figure 2. Hi-C contact map showing spatial interactions between regions of the genome. The diagonal in the Hi-C contact map generated with HiCExplorer corresponds to the intra-chromosomal contacts, depicting chromosome boundaries. The frequency of contacts is shown on a logarithmic heatmap scale. Hi-C matrix bins were merged into a 25 kb bin size for plotting. Due to space constraints on the axes, only the GenBank names of the 21st largest autosomes, the Z chromosome (GenBank name: OZ184041.1), and the mitochondrial genome (GenBank name: OZ184072.1) are shown.

Table 1. Statistics from assembled gene models.

	No. genes	No. transcripts	Mean gene length (bp)	No. single-exon genes	Mean exons per transcript
mRNA	11,805	22,873	21,248	415	8.2
pseudogene	0.0	0.0	0.0	0.0	0.0
snoRNA	17	17	132	17	1.0
lncRNA	1,484	1,762	9,221	4.0	2.4
miRNA	0.0	0.0	0.0	0.0	0.0
snRNA	96	96	125	96	1.0
rRNA	153	153	119	153	1.0
scRNA	0.0	0.0	0.0	0.0	0.0
tRNA	485	485	75	485	1.0
Other ncRNA	3	3	297	3	1.0

Table 2. Annotation completeness and consistency scores calculated by BUSCO run in protein mode (lepidoptera_odb10) and OMArk (Metazoa-v2.0.0.h5).

	Complete	Single copy	Duplicated	Fragmented	Missing
BUSCO	4,978 (94.2%)	4,948 (93.6%)	30 (0.6%)	59 (1.1%)	249 (4.7%)
OMArk	6,329 (93.2%)	6,046 (89.1%)	283 (4.1%)	-	452 (6.7%)
	Consistent	**Inconsistent**	**Contaminants**	**Unknown**	
OMArk	10,793 (91.4%)	201 (1.7%)	0.0 (0.0%)	811 (6.8%)	

Data availability

Actias isabellae and the related genomic study were assigned to Tree of Life ID (ToLID) 'ilGraIsab1' and all sample, sequence, and assembly information are available under the umbrella BioProject PRJEB77895. The sample information is available at the following BioSample accessions: SAMEA114400479 and SAMEA116288378. The specimen with BioSample SAMEA114400479 was the one sequenced and assembled. This was a female and was selected because of its heterogametic sex (W and Z chromosomes in many Lepidoptera). The genome assembly is accessible from ENA under accession number GCA_964265105.1 and the annotated genome is available through the Ensembl website (https://projects.ensembl.org/erga-bge/). Sequencing data produced as part of this project are available from ENA at the following accessions: ERX12756251, ERX13166513, ERX13166514, and ERX13166515. Documentation related to the genome assembly and curation can be found in the ERGA Assembly Report (EAR) document available at https://github.com/ERGA-consortium/EARs/blob/main/Assembly_Reports/Graellsia_isabellae/ilGraIsab1/. Further details and data about the project are hosted on the ERGA portal at https://www.ebi.ac.uk/biodiversity/data_portal/63975.

Author contributions

MV coordinated the project; MV, NM-N, and RV collected the species; MV, NM-N, and RV identified the species; DG-S, CL-V, and AR obtained the barcode sequences; MV and RV sampled and preserved biological material and provided metadata; AsB, RM, TM, RO, and THS provided support in sampling, shipping of biological material, metadata collection, and management; MLAG and MG extracted DNA, prepared libraries, and performed sequencing; FCF, FCR, and JGG performed genome assembly and curation under the supervision of TSA; LH and FM performed genome annotation; CB generated the analysis and report. All authors contributed to the writing, review, and editing of this genome note and read and approved the final version.

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Vila M, Marí-Mena N, Yen SH, *et al.*: **Characterization of ten polymorphic microsatellite markers for the protected Spanish moon moth *Graellsia isabelae* (Lepidoptera: Saturniidae).** *Conserv Genet.* 2010; **11**: 1151–1154.
Publisher Full Text

Yoshido A, Yasukochi Y, Sahara K: ***Samia cynthia* versus *Bombyx mori*: comparative gene mapping between a species with a low-number karyotype and the model species of Lepidoptera.** *Insect Biochem Mol Biol.* 2011; **41**(6): 370–377.
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Version 2

Reviewer Report 23 September 2025

https://doi.org/10.21956/openreseurope.23139.r60461

✔ **Dimitar Dimitrov**
University of Bergen, Bergen, Norway

I think that the authors have addressed satisfactory my comments on the previous version of the manuscript.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: molecular systematics, phylogenetics, phylogenomics, macroecology, macroevolution

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.

Version 1

Reviewer Report 15 August 2025

https://doi.org/10.21956/openreseurope.22348.r56370

? **Dimitar Dimitrov**
University of Bergen, Bergen, Norway

In the present article, Vila et al present a reference genome assembly of the Spanish moon moth. This species has been included as protected in the Bern convention and the EU habitat directive,

hence it is of interest for conservation biologists. In that respect generating a high-quality reference genome can help future population genomics work. The present genome also adds to our knowledge of lepidopteran genomes and improves taxon samples of potential comparative genomics studies focused on Lepidoptera.

The present work is part of the ERGA effort to compile a reference genomes atlas of European species, and the authors follow the protocols established by the ERGA consortium. In that respect, protocols are well established and information about sequencing and bioinformatics pipelines is available. Overall, the article is concise and contains all necessary information. However, I think that in few places it could benefit from additional work to improve the flow and style. In that respect here are few suggestions to the authors:

Abstract – I would suggest that the authors consider reworking a bit the abstract. For example, they can start with a general introductory sentence, then present their findings and finally speculate about the broader impact.

Key words – some of the key words are already present in the title.

“Recent phylogenomic analyses have supported a change in the scientific name of the Spanish moon moth, ..” that is odd wording. The analyses have supported a transfer to other genus, hence the change of name.

“The estimated genome size, based on ancestral taxa, is “ – that is strange wording. Did you have direct information about ancestral taxa genome size? I guess you mean, that information comes from ancestral state reconstruction analyses ... or similar.

“The nuclear microsatellite markers used in that work (Vila *et al.*, 2010) also revealed a west–east cline in allele frequencies along the Central Pyrenees that causes low overall genetic differentiation within and around the Ordesa y Monte Perdido National Park (González-Castellano *et al.*, 2023).” – reading this sentence I get the impression that “that work” refers to Marí-Mena *et al.* (2016) mentioned in the previous sentence. However, here Ville et al., 2010 is cited which is confusing. Further, still talking about different aspects of “that work” two other different papers are cited. Maybe all these works are relevant and thus it is probably better to add few sentences here to make that clear and avoid confusion.

Can you explain a bit why BioSample SAMEA116288378 was used as reference.

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: molecular systematics, phylogenetics, phylogenomics, macroecology, macroevolution

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.

Author Response 09 Sep 2025

Chiara Bortoluzzi

The abstract was changed as suggested. We have changed the wording to: “High-quality reference genomes are critical resources for understanding biodiversity and supporting conservation efforts. We present the chromosome-level assembly of the Spanish moon moth, Actias isabellae (Graells, 1849), a nocturnal lepidopteran protected under the EU Habitats Directive. The assembly spans 0.56 Gb across 31 pseudomolecules, including the Z chromosome, with a contig N50 of 18.9 Mb and a scaffold N50 of 20.4 Mb. The mitochondrial genome was also assembled into a 15,247 bp circular sequence. Annotation identified 11,805 protein-coding genes and 2,238 non-coding genes, with a BUSCO completeness of over 94%. Notably, no W chromosome was detected, suggesting a ZZ/Z0 sex determination system. This reference genome provides an essential foundation for studying sex chromosome evolution in Lepidoptera and enables advanced population genomics monitoring of this protected species. More broadly, it contributes to ongoing efforts within the European Reference Genome Atlas and the Earth BioGenome Project to harness genomics for biodiversity conservation”. Keywords were changed as suggested. These are the key words in the revised version: “Graellsia isabellae, Biodiversity Genomics Europe, Insecta, Lepidoptera, Saturniidae”. We agree that the wording regarding the recent phylogenetic analyses was misleading. Accordingly, we have changed the text to: “Recent phylogenomic analyses have supported the transfer of the Spanish moth from the genus Graellsia (Graells, 1849) to genus Actias (Graells, 1849).” The reviewer is correct that we do not have information about ancestral taxa - this was a mistake. We have changed the wording to: “The estimated genome size, estimated by Genomes on a Tree (GoaT) by ancestral state reconstruction, is”. The estimated genome size reported in the main text is the one reported by GoaT (https://goat.genomehubs.org/), which is used prior to sequencing to help calculate sequencing effort (e.g. library prep reagents and number of Revio flow cells). We are aware that the value reported in GoaT might not be exact, but it is an educated guess especially for taxa and lineages with no genome size available. The assumption that a species might have its genome size within the range described for its taxonomic group is the basis for GoaT estimates: the median of genome sizes are computed from taxa with direct measurements and propagated to the closest common ancestor, then back to fill the missing data. More information on how GoaT calculates ancestral values to propagate can be found in https://wellcomeopenresearch.org/articles/8-24/v1. We modified the text accordingly to state this more clearly, specifying the source of the markers (Vila et al. 2010) used for the 2016 and 2023 studies.

The specimen used for the assembly is the one with BioSample SAMEA114400479. This specimen is a female and was selected because it is the heterogametic sex. However our analyses revealed an X0 determination system and therefore is 'hemizygous' for the sex chromosome. We changed the main text accordingly to clarify this.

Competing Interests: No competing interests were disclosed.

Reviewer Report 15 August 2025

https://doi.org/10.21956/openreseurope.22348.r56364

Jiufeng Wei
Shanxi Agricultural University, Jinzhong, Shanxi, China

The manuscript, titled "ERGA-BGE genome of the Spanish moon moth Actias isabellae Graells, 1849: a nocturnal lepidopteran protected by the Habitats Directive," reports the sequencing and assembly of a lepidopteran species. The chromosome-level genome assembly spans approximately 0.56 Gb in length. Both the sequencing methods and analyses were adequate. The results are reliable, and I recommend to index the manuscript.

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: Phylogenomic analysis of Insect.

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.

Author Response 09 Sep 2025

Chiara Bortoluzzi

We thank the reviewer for their positive response and for approving our Genome Report.

Competing Interests: No competing interests were disclosed.