# nature portfolio

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### **Reporting Summary**

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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St	at:	191	105

n/a	Confi	irmed
	X T	The exact sample size $(n)$ for each experimental group/condition, given as a discrete number and unit of measurement
x	A	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
	<b>x</b> <sup>T</sup> 0	The statistical test(s) used AND whether they are one- or two-sided Only common tests should be described solely by name; describe more complex techniques in the Methods section.
	<b>x</b> A	A description of all covariates tested
	<b>x</b> A	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
	X A	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
		For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i> ) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted Give <i>P</i> values as exact values whenever suitable.
	X F	or Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
	X F	or hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
	<b>X</b> E:	stimates of effect sizes (e.g. Cohen's $d$ , Pearson's $r$ ), indicating how they were calculated

### Our web collection on <u>statistics for biologists</u> contains articles on many of the points above.

### Software and code

Policy information about availability of computer code

Data collection

Features of all mitogenomes of bilaterian animals available in the NCBI's manually curated RefSeq database were extracted and comparative analyses conducted using PhyloSuite.

Data analysis

Features of all mitogenomes were extracted, duplicated genes resolved, and comparative analyses conducted using PhyloSuite. Phylogeny was inferred using IQ-TREE v2.2.0.7.mix. We conducted the ancestral mitogenomic architecture inference using TreeREx and MLGO. Mitogenomic gene order rearrangement rate (GORR) values were inferred using using the Breakpoint Distance measure in CREX. The Maximum Likelihood method in BayesTraits v4.0.1 was used to infer the ancestral states of strand distribution of genes. Pairwise comparisons of GORR values between different groups were conducted using Tukey HSD tests in the R package agricolae v1.3.5. Effect sizes were inferred using the R package effsize. Statistical power analyses were conducted for each effect size measure using the pwr.t2n.test method in the R package pwr. Bayesian hypothesis testing was conducted using BayesFactor. As the dataset violated the assumption of independence of data, two algorithms designed to account for phylogenetic relatedness were used to conduct multilevel linear regression analyses: linear fixed-effect models accounting for kinship implemented in the lmekin function in coxme v2.2.16, and phylogenetic multilevel Bayesian models implemented in brms v2.16.150. For these analyses, we used a matrix of phylogenetic distances extracted using the ape v5.5 package. The R2 value of lmekin models was calculated using the r.squaredLR method available in the MuMIn v1.46.0 package in R. The Bayesian R2 value was inferred using the bayes\_r2 function of brms package in R. Custom codes are available from Zhang, D. (2025). Source codes for "The evolution of mitogenomic architecture in Bilateria". Zenodo. https://doi.org/10.5281/zenodo.15447525

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

#### Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All data used in this study were retrieved from the NCBI's GenBank databases (RefSeq and Nucleotide). The full list of Accession Numbers used in the main dataset comprising 10,860 mitogenomes is provided in Dataset 1: Worksheet S1. Accession Numbers of additional (non-RefSeq) mitogenomes are provided in figures within the Supplementary file.

### Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	N.A.	
Reporting on race, ethnicity, or other socially relevant groupings	N.A.	
Population characteristics	N.A.	
Recruitment	N.A.	
Ethics oversight	N.A.	
Note that full information on the approval of the study protocol must also be provided in the manuscript.		
Field-specific reporting		
Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.		

# ☐ Behavioural & social sciences

For a reference copy of the document with all sections, see <a href="mailto:nature.com/documents/nr-reporting-summary-flat.pdf">nature.com/documents/nr-reporting-summary-flat.pdf</a>

### Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Describe how sample size was determined, detailing any statistical methods used to predetermine sample size OR if no sample-size calculation Sample size was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient.

Describe any data exclusions. If no data were excluded from the analyses, state so OR if data were excluded, describe the exclusions and the Data exclusions rationale behind them, indicating whether exclusion criteria were pre-established.

> Describe the measures taken to verify the reproducibility of the experimental findings. If all attempts at replication were successful, confirm this OR if there are any findings that were not replicated or cannot be reproduced, note this and describe why.

Ecological, evolutionary & environmental sciences

Describe how samples/organisms/participants were allocated into experimental groups. If allocation was not random, describe how covariates were controlled OR if this is not relevant to your study, explain why.

Describe whether the investigators were blinded to group allocation during data collection and/or analysis. If blinding was not possible, describe why OR explain why blinding was not relevant to your study.

## Behavioural & social sciences study design

All studies must disclose on these points even when the disclosure is negative.

Briefly describe the study type including whether data are quantitative, qualitative, or mixed-methods (e.g. qualitative cross-sectional, quantitative experimental, mixed-methods case study).

Research sample

Study description

Replication

Blinding

Randomization

State the research sample (e.g. Harvard university undergraduates, villagers in rural India) and provide relevant demographic information (e.g. age, sex) and indicate whether the sample is representative. Provide a rationale for the study sample chosen. For (studies involving existing datasets, please describe the dataset and source.

Sampling strategy

Describe the sampling procedure (e.g. random, snowball, stratified, convenience). Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient. For qualitative data, please indicate whether data saturation was considered, and what criteria were used to decide that no further sampling was needed.

Data collection

Provide details about the data collection procedure, including the instruments or devices used to record the data (e.g. pen and paper, computer, eye tracker, video or audio equipment) whether anyone was present besides the participant(s) and the researcher, and whether the researcher was blind to experimental condition and/or the study hypothesis during data collection.

**Timing** 

Indicate the start and stop dates of data collection. If there is a gap between collection periods, state the dates for each sample cohort

Data exclusions

If no data were excluded from the analyses, state so OR if data were excluded, provide the exact number of exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.

Non-participation

State how many participants dropped out/declined participation and the reason(s) given OR provide response rate OR state that no participants dropped out/declined participation.

Randomization

If participants were not allocated into experimental groups, state so OR describe how participants were allocated to groups, and if allocation was not random, describe how covariates were controlled.

### Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description

Study description

We extracted mitogenomic gene orders and sizes for 10,860 bilaterian species, and conducted comparative analyses of mitogenomic evolutionary rates among major radiations. The dataset was categorised according to the lifestyle into endoparasites, ectoparasites, parasitoids, micropredators, and free-living species. According to the locomotory capacity, the dataset was divided into high, intermediate, and low categories. Further details and rationale are provided in our previous study (doi:10.1038/s41467-023-42095-8). First we inferred the ancestral bilaterian mitogenomic architecture, and then used it to infer gene order rearrangement rates (GORR) for all individual species. GORR and mitogenome sizes were then compared among all major bilaterian lineages (order and above) and among different lifestyle and locomotory capacity categories. Further tests were conducted to infer correlations between GORR, size, and branch length (used to represent sequence evolutionary rate). To infer branch lengths, we used the phylogeny from our previous study (doi:10.1038/s41467-023-42095-8), reconstructed using concatenated amino acid sequences of 12 mitochondrial protein-coding genes (atp8 removed) of all mitogenomes in the dataset, and mtZOA+G4+F+C50 model in IQ-TREE v2.2.0.7.mix. We constrained the phylum-level topology according to the bilaterian phylogeny in Laumer et al. (2019), with Xenacoelomorpha as the sister lineage to all other bilaterians. The molecular sequence evolution rate was defined as the root-to-tip branch lengths, which were extracted using the TreeSuite function in PhyloSuite.

In addition, all mitogenomes were classified into two categories according to the strand distribution of genes: double-stranded (genes encoded on both strands) and single-stranded (all genes encoded on a single strand); and then the Maximum Likelihood method in BayesTraits v4.0.1 was used to infer the ancestral states of strand distribution of genes. We then interred the evolutionary history of strandedness in Bilateria. To get a better resolution, for strandedness analyses, we further accessed complete GenBank mitogenomic datasets (as opposed to only RefSeq) for all poorly represented major lineages, and for all lineages with complex evolutionary histories.

Research sample

Bilateria: 10,860 mitogenomes from the RefSeq database, supplemented by additional mitogenomes from the Nucleotide database for strandedness analyses.

Sampling strategy

All data used in this study were retrieved from the NCBI's GenBank databases (RefSeq and Nucleotide). The main dataset (10,860) mitogenomes comprised all mitogenomes from the curated RefSeq database. To obtain a better resolution for strandedness analyses, we further downloaded complete mitogenomic datasets from the NCBI's Nucleotide database (as opposed to only RefSeq) for all poorly represented major lineages, and for all lineages with complex evolutionary histories.

Data collection

Life history and locomotory capacity data were collected from a wide range of internet sources by Ivan Jakovlic, Dong Zhang, and Chuan-Yu Xiang.

Timing and spatial scale

Data were collected from NCBI's databases, RefSeq and Nucleotide, between March 2022 and May 2025.

Data exclusions

Mitogenomes with missing key genes (protein-coding or rRNA) were excluded from the dataset used for CREX analyses (used to calculate mitogenomic GORR values using the Breakpoint Distance measure) as this algorithm cannot handle missing genes. This included the entire phylum Chaetognatha, because all available species are missing the atp6.

Reproducibility

We tested all major hypotheses using multiple statistical tests. For example, for pairwise comparisons of GORR between lifestyle and locomotory capacity, we relied on Tukey HSD pairwise tests, phylogenetic generalised least squares (PGLS) ANOVA tests, Effect Sizes, Statistical power analyses, Bayesian hypothesis testing, and two algorithms designed to account for phylogenetic relatedness were used to conduct multilevel linear regression analyses: linear fixed-effect models accounting for kinship implemented in the lmekin function in coxme v2.2.16, and phylogenetic multilevel Bayesian models implemented in brms v2.16.150. In this way we ensured that our findings are not statistical flukes.

Randomization

We subdivided the dataset according to major taxonomic categories (superphyla, phyla, classes, and orders), and repeated all key statistical analyses using different taxonomic levels in order to identify any putative outliers at lower taxonomic levels.

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Blinding	As lifestyle and locomotory capacity categorisations were conducted before we inferred GORR, size, and branch length values, blinding was not relevant for the design of this study.	
Did the study involve field work? Yes X No		
ield work, collec	tion and transport	
Field conditions	Describe the study conditions for field work, providing relevant parameters (e.g. temperature, rainfall).	
Location	State the location of the sampling or experiment, providing relevant parameters (e.g. latitude and longitude, elevation, water depth).	
Access & import/export	Describe the efforts you have made to access habitats and to collect and import/export your samples in a responsible manner and in ompliance with local, national and international laws, noting any permits that were obtained (give the name of the issuing authority, the date of issue, and any identifying information).	
Disturbance	Describe any disturbance caused by the study and how it was minimized.	
Materials & experimental systems  N/a Involved in the study Antibodies  Eukaryotic cell lines  Animals and other organisms  Animals and other organisms  Animals and other organisms  Dual use research of concern  Plants  Methods  N/a Involved in the study  N/a Involved in the study		
Antibodies  Antibodies used	Describe all antibodies used in the study; as applicable, provide supplier name, catalog number, clone name, and lot number.	
Validation	Describe the validation of each primary antibody for the species and application, noting any validation statements on the manufacturer's website, relevant citations, antibody profiles in online databases, or data provided in the manuscript.	
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Cell line source(s)	State the source of each cell line used and the sex of all primary cell lines and cells derived from human participants or vertebrate models.	
Authentication	Describe the authentication procedures for each cell line used OR declare that none of the cell lines used were authenticated.	
Mycoplasma contaminati	Confirm that all cell lines tested negative for mycoplasma contamination OR describe the results of the testing for mycoplasma contamination OR declare that the cell lines were not tested for mycoplasma contamination.	
Commonly misidentified (See <u>ICLAC</u> register)	monly misidentified lines   Name any commonly misidentified cell lines used in the study and provide a rationale for their use.	

# Palaeontology and Archaeology

Specimen provenance

Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information). Permits should encompass collection and, where applicable, export.

Specimen deposition	
specimen deposition	Indicate where the specimens have been deposited to permit free access by other researchers.
Dating methods	If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.
Tick this box to confire	m that the raw and calibrated dates are available in the paper or in Supplementary Information.
Ethics oversight	Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not.
	r research organisms
	udies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in
Laboratory animals	For laboratory animals, report species, strain and age OR state that the study did not involve laboratory animals.
Wild animals	Provide details on animals observed in or captured in the field; report species and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.
Reporting on sex	Indicate if findings apply to only one sex; describe whether sex was considered in study design, methods used for assigning sex. Provide data disaggregated for sex where this information has been collected in the source data as appropriate; provide overall numbers in this Reporting Summary. Please state if this information has not been collected. Report sex-based analyses where performed, justify reasons for lack of sex-based analysis.
Field-collected samples	For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.
Ethics oversight	Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not.
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#### Experiments of concern

Does the work involve any of these experiments of concern:		
No	Yes	
	Demonstrate how to render a vaccine ineffective	
	Confer resistance to therapeutically useful antibiotics or antiviral agents	
	Enhance the virulence of a pathogen or render a nonpathogen virulent	
	Increase transmissibility of a pathogen	
	Alter the host range of a pathogen	
	Enable evasion of diagnostic/detection modalities	
	Enable the weaponization of a biological agent or toxin	
	Any other potentially harmful combination of experiments and agents	

### Plants

Seed stocks

Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.

Novel plant genotypes

Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor

Authentication

Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosiacism, off-target gene editing) were examined.

### ChIP-seq

#### Data deposition

Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks. Data access links For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, May remain private before publication. provide a link to the deposited data.

Files in database submission

Provide a list of all files available in the database submission.

Genome browser session (e.g. UCSC)

Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

#### Methodology

Replicates Describe the experimental replicates, specifying number, type and replicate agreement.

Confirm that both raw and final processed data have been deposited in a public database such as GEO.

Sequencing depth Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

Antibodies Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and

lot number.

Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files Peak calling parameters

Data quality Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community Software repository, provide accession details.

### Flow Cytometry

Plots			
Confirm that:			
The axis labels state the mark	The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).		
The axis scales are clearly visi	ble. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).		
All plots are contour plots wit	h outliers or pseudocolor plots.		
A numerical value for number	r of cells or percentage (with statistics) is provided.		
Methodology			
Sample preparation	Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.		
Instrument	Identify the instrument used for data collection, specifying make and model number.		
Software	Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details.		
Cell population abundance	Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.		
Gating strategy	Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between "positive" and "negative" staining cell populations are defined.		
Tick this box to confirm that a	figure exemplifying the gating strategy is provided in the Supplementary Information.		
Magnetic resonance in	naging		
Experimental design			
Design type	Indicate task or resting state; event-related or block design.		
Design specifications	Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.		
Behavioral performance measure	State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).		
Acquisition			
Imaging type(s)	Specify: functional, structural, diffusion, perfusion.		
Field strength	Specify in Tesla		
Sequence & imaging parameters	Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.		
Area of acquisition	State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.		
Diffusion MRI Used	☐ Not used		
Preprocessing			
Preprocessing software	Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).		
Normalization	If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.		
Normalization template	Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.		
Noise and artifact removal	Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).		
Volume censoring	Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.		

### Statistical modeling & inference Model type and settings Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation). Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA Effect(s) tested or factorial designs were used. Specify type of analysis: ROI-based ☐ Whole brain \_ Both Statistic type for inference Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods. (See Eklund et al. 2016) Correction Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo). Models & analysis Involved in the study Functional and/or effective connectivity Graph analysis Multivariate modeling or predictive analysis Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, Functional and/or effective connectivity mutual information). Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, Graph analysis subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency,

etc.).