

Mitonuclear Discordance of Beetles Shaped by Incomplete Lineage Sorting and Introgression Under Loose Interaction Mechanism

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Abstract

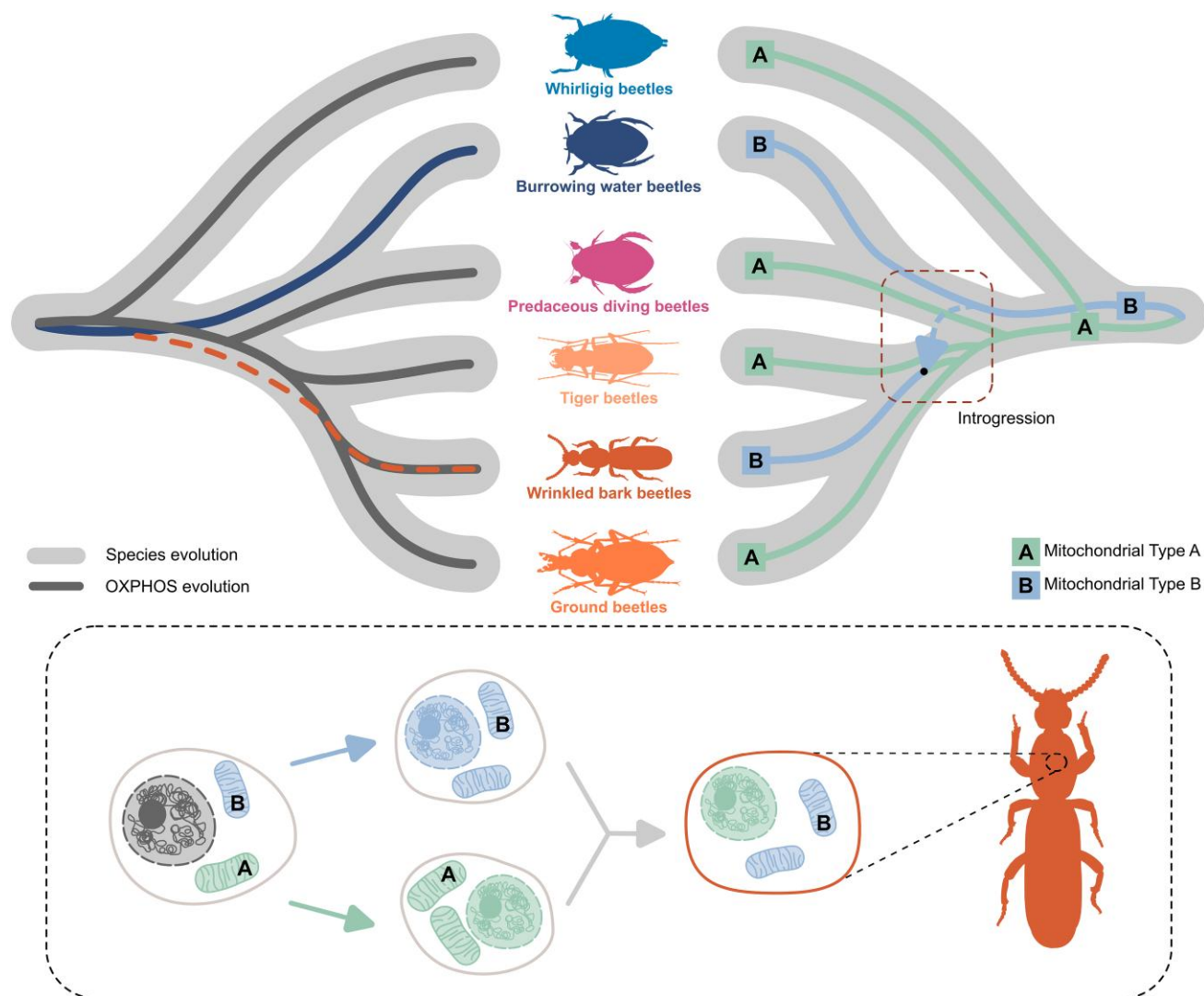
Metazoan oxidative phosphorylation (OXPHOS) complexes are composed of subunits encoded by mitochondrial and nuclear genes, requiring continuous mitonuclear coevolution to ensure functional compatibility. However, mitochondrial and nuclear genomes exhibit separate inheritance patterns, leading to their distinct or even conflicting evolutionary histories. This study aimed to analyse phylogenetic signals among mitochondrial genes, nuclear-encoded OXPHOS genes, and general nuclear genes across 53 beetle species. Two major cases of mitonuclear discordance were detected. The nuclear-encoded OXPHOS genes supported mitochondrial phylogenetic signals in noterids, indicating that in noterids the evolutionary history of OXPHOS complexes diverged from the phylogenetic history. Conversely, nuclear-encoded OXPHOS genes aligned with the phylogenetic history of rhyssodines, and this mitonuclear discordance suggests that mitochondrial genomes exhibited clear signatures of genetic introgression. By integrating phylogenetic reconstructions and reticulate evolutionary network analyses, we attributed the mitonuclear discordance in noterids to incomplete lineage sorting. In contrast, the mitochondrial genomes of rhyssodines underwent introgressive hybridization events. Although mitonuclear incompatibility is typically resolved by nuclear compensatory mechanisms, our findings indicate that nuclear compensation exhibits limited efficacy at the gene level, yet locally adaptive residues persist. This was further supported by the weak correlation between nuclear-encoded OXPHOS genes and mitochondrial genes, with no robust mitonuclear coevolutionary signals detected. These findings collectively suggest a loose mitonuclear interaction in beetles. The decoupling of mitochondrial and nuclear evolutionary trajectories may serve as an evolutionary “buffer” to accommodate genomic conflicts while maintaining essential OXPHOS systems.

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Graphical abstract



Keywords: mitonuclear discordance, nuclear compensation, OXPHOS, mitochondrial introgression, adephaga

Introduction

Mitochondria originated from an α -proteobacterial ancestor, as evidenced by phylogenetic analyses demonstrating shared genomic and metabolic features between extant mitochondria and α -proteobacteria (Wang and Wu 2015; Fan et al. 2020; Vosseberg et al. 2024). The evolution of mitochondrial genomes involved extensive gene loss coupled with nuclear relocation through endosymbiotic gene transfer (Roger et al. 2017). This genomic streamlining shaped the formation of the semi-autonomous mitochondria. Across eukaryotic lineages, the retention of divergent mitochondrial genes, varying in both quantity and functional categories, reflects distinct cooperation models between mitochondrial and nuclear genomes (Gabaldón and Huynen 2004; Gray 2015; Stairs et al. 2015; Johnston and Williams 2016; Roger et al. 2017). Maternal mitochondrial inheritance is a unique phenomenon in genetics characterized by the exclusive transmission of the mitochondrial genome from the mother to offspring. During fertilization, paternal mitochondria carried by the

sperm are selectively degraded through autophagy due to their encapsulation within specialized membrane structures, ensuring that only the maternal mitochondrial genomes persists in the zygote (Sato and Sato 2013; Lee et al. 2023). This mechanism is in stark contrast to the biparental inheritance of nuclear DNA, which relies on chromosomal recombination from both parents. The semi-autonomy of mitochondria and their distinct maternal inheritance patterns create an evolutionary basis for mitonuclear discordance.

Mitonuclear discordance refers to the incongruence observed when evolutionary relationships reconstructed using mitochondrial genomic data diverge from those reconstructed using nuclear genomic markers (Toews and Brelsford 2012). This phenomenon has been widely observed in a broad spectrum of taxa from mammals to insects, particularly among lineages characterized by complex evolutionary trajectories (Pagès et al. 2013; Cairns et al. 2021; Sarver et al. 2021; Xu et al. 2021; Kao et al. 2022; DeRaad et al. 2023). The pervasive nuclear mitochondrial-plastid genomic discordance observed in

Emilia–*Gephyrocapsa* likely arises from a complex interplay of evolutionary mechanisms, such as recombination, introgression, and incomplete lineage sorting (ILS) (Kao et al. 2022). Multiple mitochondrial introgressions across *Drosophila paulistorum* semispecies, revealed through nuclear mitochondrial insertions (NUMTs), provide novel insights into the species' evolutionary dynamics (Baião et al. 2023). Multiple sunflower species exhibit two phylogenetically divergent cytoplasmic lineages resulting from historical interspecific hybridization (Owens et al. 2025). This mitonuclear discordance underlies phenotypic divergence in seed morphology and contributes to potentially adaptive variation across environmental gradients. This finding underscores that hybridization and the subsequent genomic introgression serve as critical drivers of genetic diversity across biological lineages, a phenomenon particularly pronounced in some clades where interspecific genomic mixing (Figueiró et al. 2017; Li et al. 2022; Combrink et al. 2025). Mitonuclear discordance often reflects complex evolutionary histories within lineages, highlighting that phylogenetic relationships may deviate from a simple bifurcating phylogenetic tree. Furthermore, reconstructing accurate evolutionary history of mitochondrial genome remains challenging, particularly due to the confounding effects of ILS and introgression.

Mitonuclear coevolution is the core mechanism underlying the maintenance of energy homeostasis in eukaryotes (Bar-Yaacov et al. 2012; Quirós et al. 2016; Boscenco and Reznik 2023). Research into mitochondrial mutational dynamics has led to a well-accepted model of nuclear compensatory mechanism (Lynch 1996, 1997; Goldberg et al. 2003; Rand et al. 2004; Sloan et al. 2014). When mitochondrial genomes acquire deleterious mutations, the nuclear genome can restore their functional compatibility through the nuclear compensatory mechanism (Oliveira et al. 2008; Osada and Akashi 2012; Hill 2020). Deleterious mutations in mitochondrial genes impose a strong positive selection pressure on corresponding nuclear-encoded subunits of oxidative phosphorylation (OXPHOS) complexes. This drives adaptive amino acid (AA) substitutions at protein-protein interaction interfaces to rescue structural compatibility, thereby preserving the biochemical functionality of OXPHOS complexes through compensatory mutations in nuclear genes (Goldberg et al. 2003). However, many studies across diverse taxonomic groups have failed to substantiate nuclear compensation as the dominant mechanism of mitonuclear coevolution (Zhang and Broughton 2013; Adrion et al. 2016; Piccinini et al. 2021; Weaver et al. 2022). Compensatory evolution shows limited evidence as a predominant driver in mitonuclear coevolution. Instead, the relative contributions of compensatory mechanisms versus evolutionary constraints are expected to vary across phylogenetic contexts, shaping taxon-specific evolutionary trajectories in mitochondrial-associated nuclear genes (Adrion et al. 2016). Furthermore, while nuclear compensatory mechanisms may theoretically counteract mutations occurring at limited sites, they are likely insufficient to address mismatches resulting from mitochondrial introgression events, as such challenges may require more comprehensive adaptive responses than localized compensatory mutations alone. *Drosophila simulans* possesses two mitochondrial haplogroups within the same nuclear background (Pichaud et al. 2012). In sunflowers, the trans-species cytoplasmic polymorphism reflects a lack of cytonuclear interactions, indicating weak cytonuclear coevolutionary constraints (Owens et al. 2025). These findings support

that a loose interaction mechanism can accommodate mitochondrial introgression. We posit that a loose interaction mechanism may represent a more robust evolutionary strategy to accommodate both deleterious mutations and exogenous mitochondrial introgression. Ancestral populations harbouring polymorphic mitochondrial genomes may retain pre-adapted OXPHOS genes that confer compatibility with diverse mitochondrial genotypes. This mechanism accommodates substantial mitonuclear genotypic mismatches, simultaneously buffering the fitness costs associated with exogenous mitochondrial introgression or maintaining intra-population mitochondrial polymorphism. Nuclear compensation and loose interaction mechanism collaboratively shape the genomic architecture of mitonuclear coevolution.

Mitonuclear coevolution in beetles is an example of a loose interaction mechanism. Adephaga comprising species-rich and globally distributed ground beetles and predaceous diving beetles, represents a monophyletic lineage that underwent a dual ecological evolution into terrestrial and aquatic niches. Phylogenomic analyses based on single-copy nuclear genes and morphological analyses have established a robust evolutionary history for adephagans (Beutel et al. 2020; Baca et al. 2021; Vasilikopoulos et al. 2021). Notably, Adephaga exhibits pronounced mitonuclear discordance, with mitochondrial and nuclear gene trees displaying topological conflicts across critical phylogenetic nodes (López-López and Vogler 2017; Vasilikopoulos et al. 2021). These studies attribute topological conflicts to data artefacts arising from limited sampling bias; however, they may have overlooked the organelles' distinct evolutionary trajectories. We investigated the mechanisms underlying mitonuclear discordance in Adephaga by integrating mitochondrial, single-copy nuclear, and nuclear-encoded OXPHOS gene datasets. The recent expansion of DNA sequencing data for Noteridae has provided a robust foundation for further resolving this phylogenetic incongruence (Baca et al. 2025). We aimed to investigate mitonuclear discordance from three key perspectives: (i) assessing the prevalence of mitonuclear discordance across Adephaga; (ii) identifying the evolutionary drivers underlying its emergence; and (iii) investigating the mechanisms employed by OXPHOS genes to mitigate mitonuclear incompatibilities.

Results

Datasets and Gene Classification

We conducted phylogenetic analyses based on nuclear genome and mitochondrial genome sequences of 53 samples (details in Table S1). Based on the gene annotation information of FlyBase (Öztürk-Çolak et al. 2024), we collected and utilized 745 genes related to mitochondrial function (Table S2). To perform the diverse types of analyses, we constructed the following three workflows.

The first workflow was designed to reconstruct the phylogenetic relationships among different genes. It included 53 samples and three types of genes: single-copy genes (AA sequences of 2,123 single-copy nuclear genes), mitochondrial genes (13 protein-coding genes [PCGs] and two rRNA genes), and nuclear genes related to OXPHOS (nucl-OXPHOS: AA sequences of 156 genes). Additionally, a dataset of 195 samples was constructed using only the mitochondrial genes (Table S3). The AA sequences of the PCGs that excluded termination codons constituted the AA dataset. The P123R dataset was composed of nucleotide sequences of PCGs and rRNAs. The P12R dataset was composed of PCG sequences with the third codon position

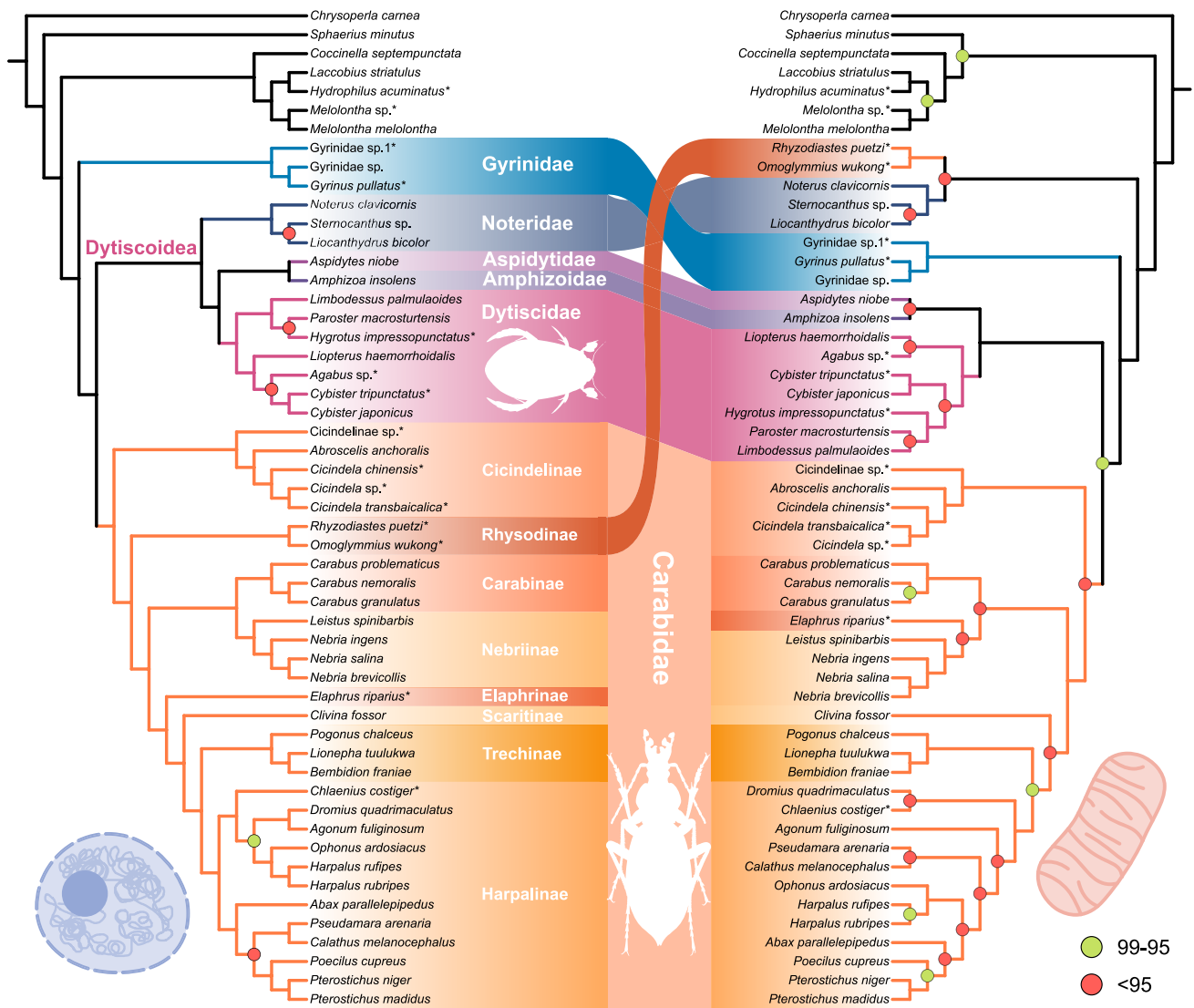


Fig. 1. Mitonuclear phylogenetic relationships within Adephaga. Left panel: ML phylogeny based on AA sequences of single-copy nuclear gene. Right panel: ML phylogeny reconstructed from mitochondrial AA sequences. Nodes with full bootstrap support (100) are unlabelled. Support values <95 highlighted in red, 95 to 99 in green. Species marked with an asterisk (*) represent novel sequences generated in this study.

excluded and rRNA sequences. Both the 53-sample and 195-sample datasets comprised the AA, P12R, and P123R datasets for mitochondrial phylogenetic analyses. The second workflow was designed for the detection of ILS and introgression. Due to the analytical constraints of requiring datasets with fewer than ten samples, we conducted genetic introgression analyses using 2,018 single-copy genes from eight representative species. The third workflow was designed for evolutionary rate and selection pressure analyses. To ensure that these genes present in all the 53 samples, we constructed four independent datasets: (i) a mitochondrial genes dataset (mito-genes) containing 12 genes (excluding *ND2*); (ii) a nucl-OXPPOS genes dataset containing 52 genes; (iii) a mitochondria-related nuclear genes dataset excluding OXPPOS genes (nucl-noOXPPOS) containing 588 genes; and (iv) a nuclear gene dataset (nucl-genes) unrelated to mitochondrial function, containing 3,818 genes.

Mitonuclear Discordance of Noteridae and Rhysodinae

To reconstruct the phylogenetic trees, we conducted maximum likelihood (ML) analyses based on the single-copy

nuclear gene, mitochondrial gene, and the nucl-OXPPOS datasets (see Materials and Methods for details). Phylogenetic trees based on the single-copy nuclear genes were constructed using the ML method implemented in IQTREE, with the optimal substitution model (JTT + F + I + R8) selected under the Bayesian Information Criterion (BIC). We also reconstructed a species tree using ASTRAL and compared it to the ML tree, revealing congruent topologies. Furthermore, the results based on different rates of evolution among the single-copy genes showed topological consistency (Fig. S1). All trees derived from the single-copy gene dataset aligned with the evolutionary relationships reported in previous studies (Beutel et al. 2020; Vasilikopoulos et al. 2021). This ML tree was subsequently adopted as a reference species tree for downstream evolutionary analyses (Fig. 1).

The topology inferred from the mitochondrial genes conflicted with that based on the single-copy genes regarding the placement of critical lineages (Fig. 1). First, the mitochondrial genes robustly supported the exclusion of Noteridae from Dytiscoidea, contrasting with its traditional phylogenetic placement as a branch within Dytiscoidea. Second, although the single-copy nuclear genes confirmed Rhysodinae as a

Table 1. Detailed information on the alignment and corresponding trees for three datasets of 53 samples

Dataset	Partition scheme (t)	ln(Lik)	BIC	Sites	PIS	RCV
AA dataset	MP (4)	−105,312.03	212248.20	3806	2010	0.0579
AA dataset	FP (13)	−105,431.82	212998.92	3806	2010	0.0579
AA dataset	NP (1)	−106,230.51	213557.52	3806	2010	0.0579
P12R dataset	MP (5)	−147,344.33	296289.78	9915	4527	0.0576
P12R dataset	FP (15)	−147,149.19	296994.51	9915	4527	0.0576
P12R dataset	NP (1)	−150,564.97	302224.95	9915	4527	0.0576
P123R dataset	FP (15)	−284,344.50	571585.10	13720	7956	0.0425
P123R dataset	MP (5)	−284,516.81	570757.94	13720	7956	0.0425
P123R dataset	NP (1)	−289,851.61	580865.46	13720	7956	0.0425

In partition scheme (t), the number in parentheses indicates the number of partitions. NP, unpartitioned model; FP, edge-unlinked full partition model; MP, merged and edge-unlinked partition model. PIS, Number of parsimony-informative sites in the alignment; RCV, Relative composition variability of the alignment; Sites: Total number of sites in the alignment; ln(Lik): Log-likelihood.

Table 2. Results of likelihood mapping for the hypotheses based on four datasets

Hypothesis and group	Nucl-OXPHOS dataset	Mitochondrial genes		
		AA dataset	P12R dataset	P123R dataset
H1 Q1:(Noteridae, Carabidae)–(Gyrinidae, Dytiscoidea)	19.16	3.57	8.09	8.23
H1 Q2:(Noteridae, Gyrinidae)–(Carabidae, Dytiscoidea)	53.77	56.47	51.49	48.51
H1 Q3:(Noteridae, Dytiscoidea)–(Carabidae, Gyrinidae)	25.63	34.71	35.99	37.72
H2 Q1:(Rhysodinae, Carabidae)–(Gyrinidae, Dytiscoidea)	63.86	23.59	26.95	31.62
H2 Q2:(Rhysodinae, Gyrinidae)–(Carabidae, Dytiscoidea)	14.11	30.11	37.31	41.43
H2 Q3:(Rhysodinae, Dytiscoidea)–(Carabidae, Gyrinidae)	20.43	34.50	19.82	17.70

Bold numbers represent resolved quartets that are strong phylogenetic signals in the hypothesis.

subfamily of Carabidae, the mitochondrial genes suggested to place it outside Carabidae. Notably, the mitochondrial genes reconstructed Noteridae and Rhysodinae as sister groups, a topology potentially influenced by long-branch attraction artefacts, as they are distantly related to Adephaga.

For mitochondrial phylogenetic reconstruction, we generated three mitochondrial datasets (AA, P12R, and P123R), and optimal partition schemes were selected based on BIC (Table 1) and phylogenetic trees were constructed (Figs. S2–S4). The ML trees of 53 and 195 samples demonstrated that the phylogenetic placement of Noteridae and Rhysodinae outside Adephaga remained stable (Figs. S2–S7). Bayesian inference under the CAT-GTR models failed to provide reliable resolution for the placements of Noteridae and Rhysodinae (Figs. S8–S10). The ML tree under the C60 model consistently supported the phylogenetic placement of Noteridae and Rhysodinae outside Dytiscoidea and Carabidae, respectively (Fig. S11). Genetic distance matrices derived from mitochondrial AA alignments revealed a significant divergence of Rhysodinae and Noteridae from other Adephaga lineages (Fig. S12). It is noteworthy that only the AA dataset in both the 53 and 195 samples restored the phylogenetic position of Cicindelinae as a member of Carabidae. The observed discrepancies between the AA and P12R/P123R datasets may be attributed to AA sequences containing less noise signal relative to nucleotide sequences. The phylogenetic placement of Cicindelinae was recalibrated through phylogenomic analyses using the AA dataset coupled with expanded sample sizes (Figs. S2–S7). The positions of Rhysodinae and Noteridae discordant with the phylogeny constructed with the single-copy nuclear genes and reported in previous studies appear not to be influenced by differing sample sizes or dataset types. Mitochondrial phylogenetic reconstruction and genetic distance matrices consistently demonstrated mitonuclear discordance, placing Rhysodinae and Noteridae outside of their

conventionally recognised families (Carabidae and Dytiscidae, respectively).

The phylogenetic tree based on 156 nucl-OXPHOS genes exhibited significant discrepancies compared with those based on the single-copy gene and mitochondrial gene datasets (Fig. S13). Specifically, the phylogenetic placement of Noteridae was consistent with that based on mitochondrial gene results, whereas the position of Rhysodinae aligned with that in the single-copy gene tree. Furthermore, we incorporated analyses based on the likelihood mapping approach to validate the key phylogenetic relationships (Table 2). These analyses revealed that the mitochondrial genes of Noteridae were congruent with the nucl-OXPHOS genes in terms of the phylogenetic signal. Moreover, the nucl-OXPHOS genes of Rhysodinae demonstrated phylogenetic consistency with the single-copy gene results.

ILS and Introgression of Carabidae and Dytiscoidea

Topological analyses of 2,018 single-gene trees from eight taxonomically representative species revealed signals of ILS and introgression events in the evolutionary trajectories of Dytiscoidea and Carabidae (see Materials and Methods). The ancestral node (Node 1 in Fig. 2) shared by Carabidae and Dytiscoidea exhibited prominent signatures of ILS (ILS index = 81.6%) coupled with introgression/hybridization (IH) signals (IH index = 32.4%). Subsequent divergence events maintained substantial ILS persistence (Fig. 2: ILS index = 61.8% in Node 2 and 81.8% in Node 3) but showed a striking contrast in IH intensity (IH index = 9.8% vs 39.8%, respectively). These findings demonstrate that the ILS predominantly governed the divergence process, and the hybridization events occurred during specific evolutionary phases. A consistent signal pattern was observed in the 53 samples (Fig. S14). The IH index suggested that multiple hybridization and introgression

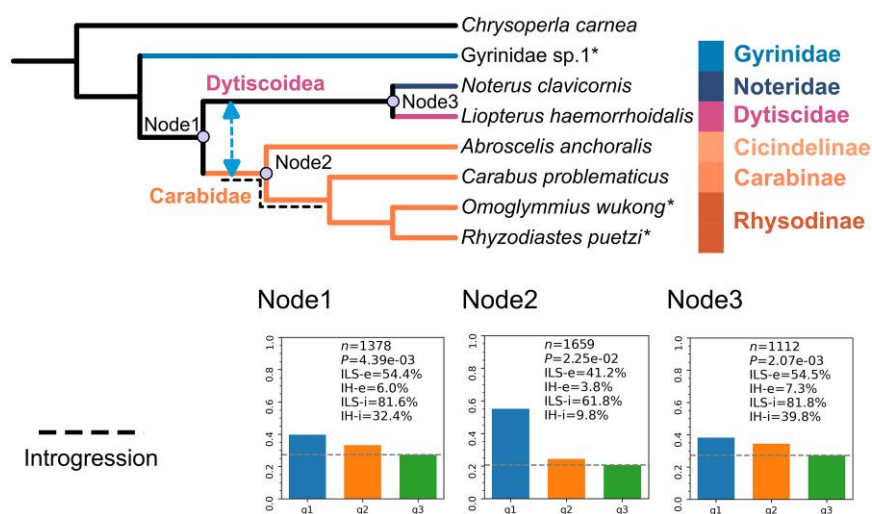


Fig. 2. Signals of incomplete lineage sorting and introgression in eight representative samples of Adephaga. The arrow-headed dashed lines depict predominant genetic introgression directions (inferred using PhyloNET). Signals of ILS and introgression at Nodes 1, 2, and 3 were detected through PhyTop. The q1, q2, and q3 represent the proportions of three topological structures of gene trees present in the nodes. n represents the number of simulated gene trees, P is the P -value of the Chi-Squared test to check whether the number of topologies q2 and q3 are equal, ILS-i and IH-i represent the calculated ILS index and IH index respectively. ILS index and IH index assessing ILS and IH signals in a species tree. ILS-e and IH-e represent the proportion of gene tree topological incongruence that can be explained by ILS and IH, respectively.

events likely occurred within Dytiscoidea. Reticulate evolutionary network analyses consistently identified three scenarios of ancient genetic introgression between Carabidae and Dytiscoidea. These introgression events were localized to the basal nodes within the Carabidae stem lineage (Fig. S15). This pattern implies that critical genetic exchanges likely occurred during the early divergence stages, with introgressive processes potentially coinciding with the adaptive events that shaped diversity of Carabidae and Dytiscoidea. Our findings demonstrate that the early divergence stages between Carabidae and Dytiscoidea were marked by hybrid of introgression and ILS.

Based on analyses of 53 samples with 2,123 single-copy genes and five fossil calibrations, the divergence time estimation of Adephaga revealed that Carabidae and Dytiscoidea diverged during the Late Triassic period (Fig. 3). We concurrently quantified the proportion of nuclear genes (total 3,818 genes) under positive selection across different phylogenetic nodes using the aBSREL model. Major cladogenesis occurred during the Jurassic and Cretaceous periods. Notably, the divergence between Rhysodinae and Noteridae spanned the Late Jurassic to the Cretaceous periods, suggesting a protracted diversification process. Early-divergence nodes in Carabidae and Dytiscoidea demonstrated significantly elevated proportions of positively selected genes compared to those in other nodes. Functional annotation of these positively selected genes revealed convergent enrichment in iron ion binding, heme binding, oxidoreductase activity and ATPase-coupled transmembrane transport (Table S4). Nuclear genes with different functions exhibit a higher proportion of positive selection at early-divergence nodes compared to nodes lacking hybridization signals (Fig. S16). This suggests that gene flow through hybridization provides positive selective pressure for adaptive evolution.

Limited Nuclear Compensatory Phenomenon in Adephaga

The nuclear compensatory mechanism hypothesis postulates that mitochondria-interacting genes exhibit elevated positive

selection pressure due to the necessity to compensate for the high mutation rates characteristic of mitochondrial genes. Under the aBSREL model, we calculated the proportion of sites under selective pressure for each gene across all samples. We observed a heterogeneous distribution pattern of a nuclear compensatory phenomenon across Adephaga. By comparing the proportions of sites under selective pressure among nucl-OXPHOS, nucl-noOXPHOS, and all nuclear genes, we found that nuclear compensation specifically affected nucl-OXPHOS genes in Adephaga by the Mann-Whitney test (Fig. S17). However, this nuclear compensatory phenomenon was not detected in Cicindelineae and Gyrinidae.

We further delineated the nuclear compensatory phenomenon associated with complexes I–V within nuclear-encoded OXPHOS genes (Fig. 4). The number of genes and aligned codon counts for each gene are provided in the Table S5. In the Rhysodinae and Dytiscidae lineages, Complex I genes exhibited a significantly higher number of positively selected sites than nucl-noOXPHOS genes. Nuclear compensatory effects were observed for both Complex I and Complex IV in Noteridae and Carabidae. Interestingly, although components of Complex II are encoded in only nuclear genes, members of Cicindelineae, Carabidae, and Dytiscidae also demonstrated more positively selected sites in Complex II genes than in nucl-noOXPHOS genes. No significantly nuclear compensatory phenomenon was detected for complexes III and V in any adephagan lineages. The heterogeneous distribution of nuclear compensation suggests that this process is not universally required for all complexes. Nucl-OXPHOS genes exhibit a higher proportion of sites under positive selection compared to other nuclear genes (Fig. S17). However, positive selection pressure in Complex II genes complicates the assessment of nuclear compensation. The limited nuclear compensatory phenomenon is unaffected by positive selection pressure in Complex II genes only in Rhysodinae and Noteridae (Fig. 4).

If nuclear compensatory phenomenon for mitochondrial introgression occurred, we would expect convergent AA

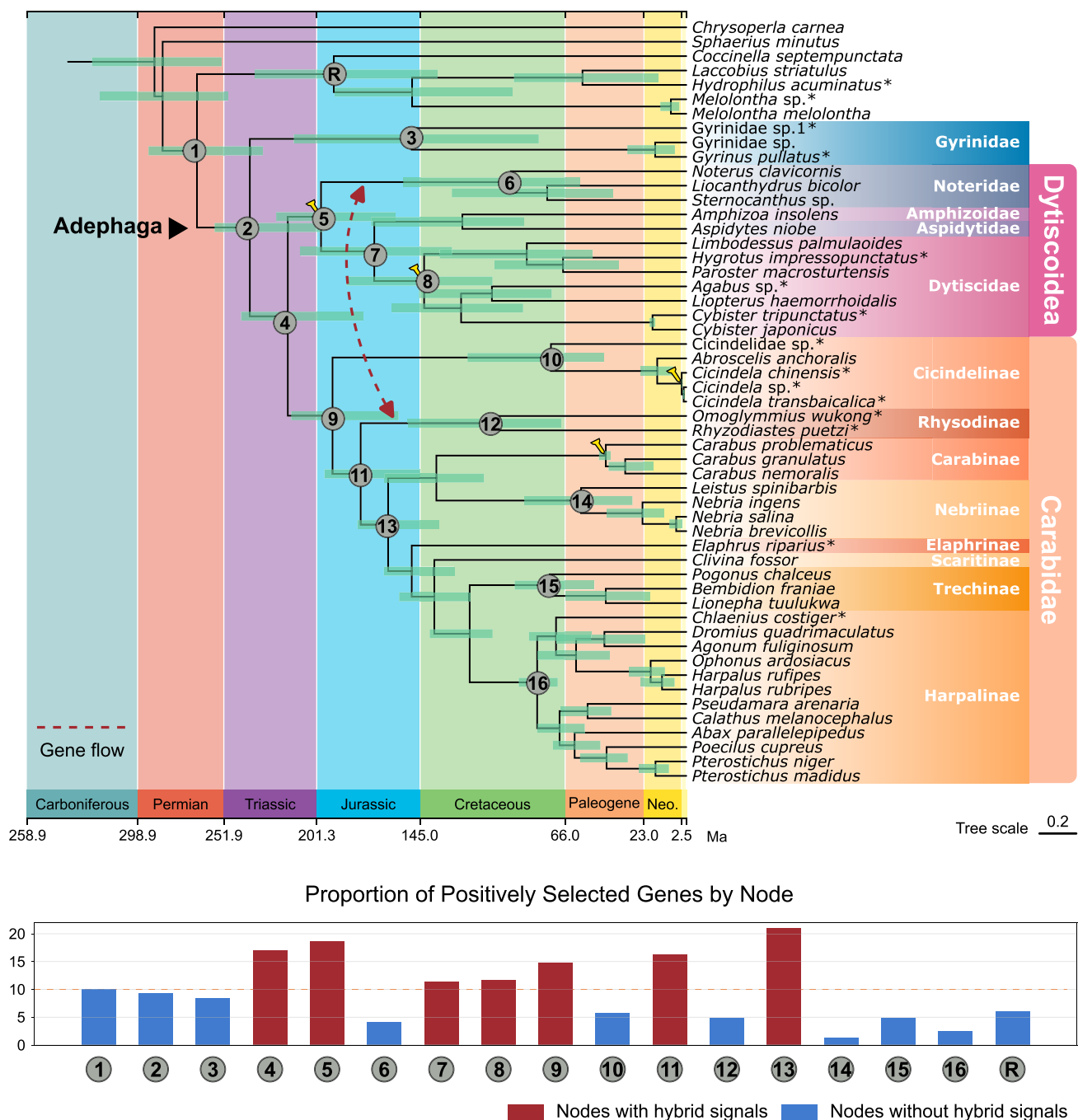


Fig. 3. Dated phylogeny of Adephaga and proportion of genes under positive selection. Green horizontal bars on the nodes represent 95% credibility intervals. The fossil calibrations employed in this study are depicted as orange triangles. The scale axis of the tree is expressed in millions of years. The Quaternary is represented by purple rectangles, the Neogene is denoted by Neo., and other periods are not abbreviated. The bar plot at the bottom displays the percentage of positively selected genes (total 3,818 nuclear genes) at each major node. The node of Polyphaga during the Jurassic period is used as a reference value. Genes with >90% positively selected sites are operationally classified under strong positive selection.

substitutions in mitochondria-related genes of Rhysodinae resembling Noteridae but diverging from other Adephaga. A few candidate residues were identified across 745 mitochondria-related genes (Table S6). Three significant residues localized to conserved domains (Fig. S18) likely represent mutations arising independently in Rhysodinae and Noteridae under the same selective pressures. While nuclear compensatory phenomenon is not pervasive in mitochondria-related genes globally, sporadic manifestations in some loci of nucl-OXPHOS remain plausible. This supports our identification of a limited nuclear compensatory phenomenon specific to Adephaga. Nuclear compensation exhibits intrinsic limitations: absent

from many complexes and taxa at macroevolutionary scales, yet demonstrably present in localized microevolutionary contexts.

Coevolutionary Signals Between Mitochondrial and nucl-OXPHOS Genes

To investigate the intensity of coevolutionary signals between mitochondrial and nucl-OXPHOS genes, we employed evolutionary rate covariation (ERC) and linear regression analyses. The phylogenetic tree constructed from single-copy genes of 53 samples was used as a reference species tree (Fig. 1). For

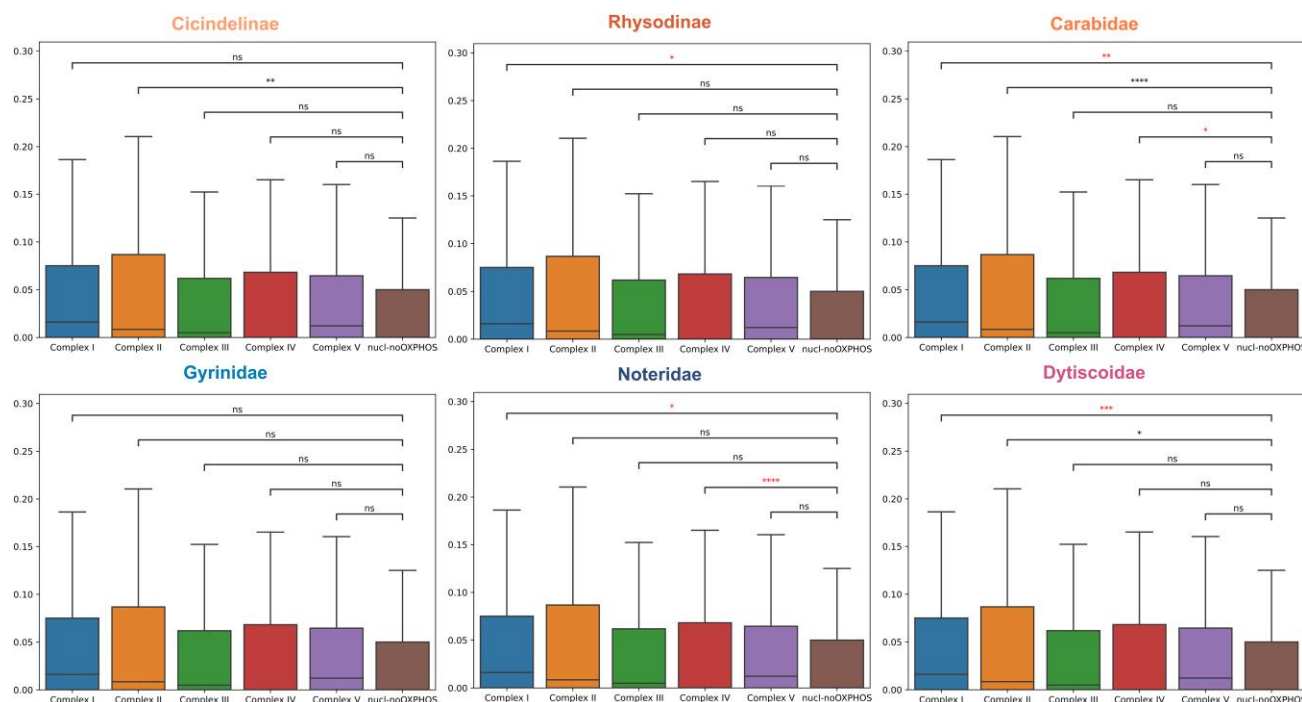


Fig. 4. The proportions of codons under positive selection pressure. Selection pressures on codons in nuclear genes encoding Complexes I–V and nucl-noOXPHOS genes within Adephaga were analysed using the aBSREL method in HyPhy. Mann-Whitney analyses were conducted between the genes encoding Complex I–V and nucl-noOXPHOS genes. Asterisks denote statistical significance of evolutionary pressure associations (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns stands for non-significant). Red asterisks indicate nuclear compensation. Box boundaries represent the interquartile range (IQR: 25th to 75th percentiles), with the internal line indicating the median (50th percentile). Whiskers extend to the minimum and maximum values within 1.5xIQR from the lower and upper quartiles (outliers not shown). IQR is the abbreviation for interquartile range.

dataset construction, we selected genes present in all samples and constructed four independent datasets (see Materials and Methods for details). Subsequently, we performed ERC analyses on the gene pairs between the mitochondrial gene dataset and the other three datasets.

The intensity of coevolutionary signals was assessed using ERC coefficients. The ERC coefficients of the 12 mitochondrial genes averaged 0.45. In contrast, the ERC coefficients of the 52 nucl-OXPHOS genes averaged 0.28, while those of the 588 nucl-noOXPHOS genes and 3,818 nuclear genes unrelated to mitochondrial function averaged 0.21. The overall distribution of ERC coefficients in the nuclear datasets was significantly lower than that observed for mitochondrial genes (Fig. 5a). Our analysis was restricted to gene pairs showing significant positive correlations ($P < 0.05$), yielding 110 mitogene pairs (12 genes), 127 nucl-OXPHOS pairs (52 genes), 771 nucl-noOXPHOS pairs (588 genes), and 4,837 nuclear gene pairs (3,818 genes). We examined the potential variation in ERC distributions at different scales of data quantity. To evaluate scale effects, we performed random sampling of the complete nuclear gene set (4,837 pairs) to generate subsets matching three scales: 110 pairs (comparable to the 127 pair scale), 700 pairs, and the full 4,837 pairs. Mann-Whitney tests across three independent replicates confirmed no significant differences ($\alpha = 0.05$) in ERC distributions between these scales of the complete nuclear gene pairs. Importantly, triple replicate random sampling analyses based on 110 gene pairs revealed no significant ERC distribution differences between nucl-OXPHOS versus nucl-noOXPHOS genes or between nucl-OXPHOS and nucl-genes. These comprehensive analyses demonstrate that our conclusions are robust to dataset size variations and that nucl-OXPHOS genes do not exhibit

stronger constraints on co-evolutionary signals than other nuclear genes. The average R-squared values between mitochondrial genes and each of the other three datasets were 0.21 for nucl-OXPHOS genes, 0.22 for nucl-noOXPHOS genes, and 0.19 for the nuclear dataset. Linear regression analyses of branch lengths revealed weak correlations between mitochondrial and nuclear genes related to mitochondrial function (Fig. 5b). The Pearson correlation coefficient (r) between nucl-OXPHOS and mitochondrial genes was 0.46, which was not significantly higher than that between nucl-noOXPHOS and mitochondrial genes ($r = 0.47$). Through ERC and linear regression analyses, a weak correlation was observed between mitochondrial and nucl-OXPHOS genes compared to other nuclear genes.

Discussion

Mitonuclear Discordance Shaped by ILS and Introgression

Based on current phylogenomic and morphological analyses, the phylogenetic relationship that Rhysodinae belongs to Carabidae and Noteridae to Dytiscoidae is now widely accepted (Beutel et al. 2020; Gustafson et al. 2020; Baca et al. 2021; Vasilikopoulos et al. 2021). Our results consistently revealed mitonuclear discordance in Rhysodinae and Noteridae (Fig. 1). We initially hypothesized that the mitochondrial sequences of Rhysodinae and Noteridae underwent extensive AA substitutions (López-López and Vogler 2017), leading to their distant positions from those of Carabidae and Dytiscoidae in the AA distance matrices (Fig. S12). The elevated mitochondrial substitution rate in Hymenoptera supports this evolutionary scenario (Li et al. 2017, 2024). Both

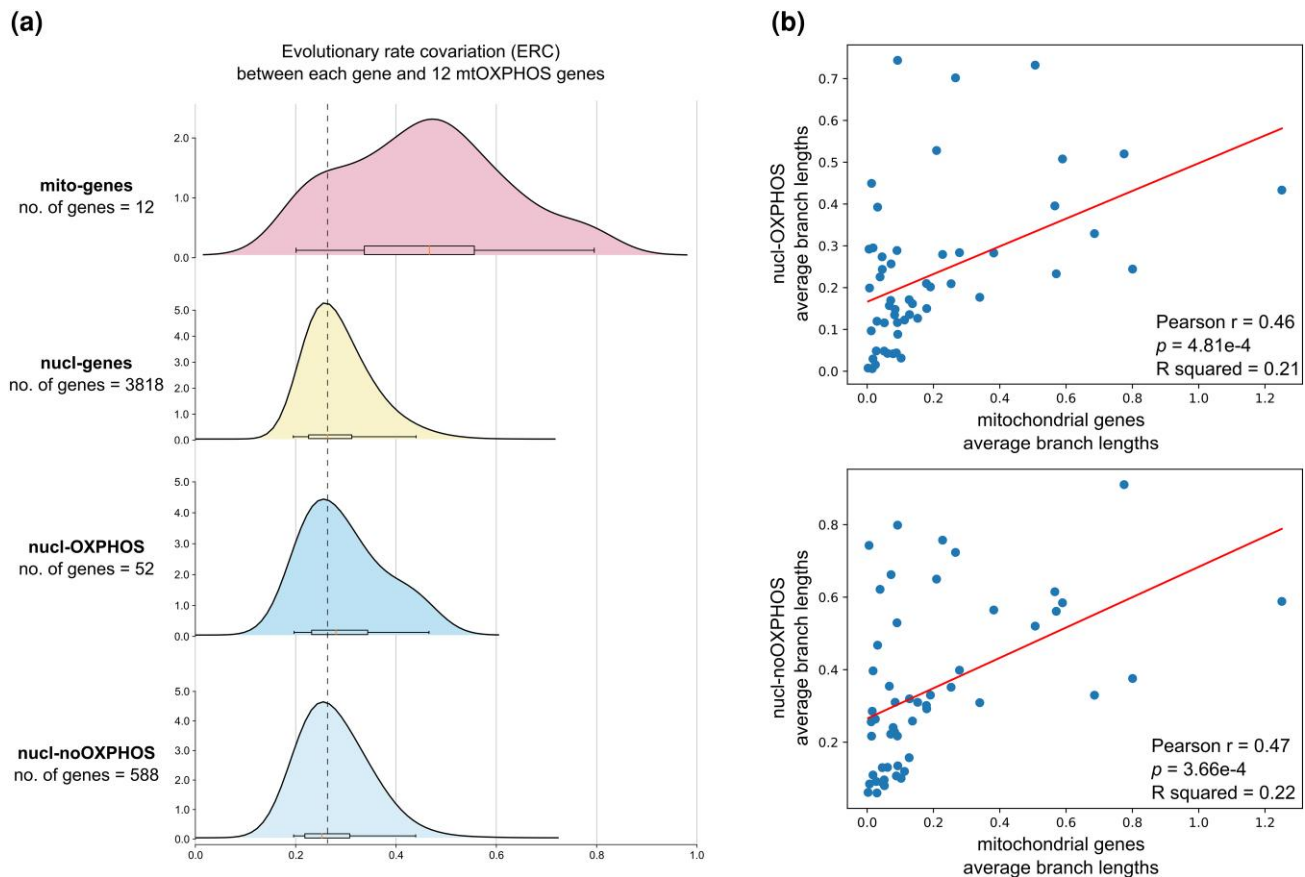


Fig. 5. ERC landscape and linear correlation profiles between nuclear and mitochondrial genes. a) The distribution of ERC across mitochondrial genes (mito-genes), nucl-OXPHOS genes, nucl-noOXPHOS genes, and nuclear genes (nucl-genes) with boxplot elements (25th, 50th, and 75th percentiles) explicitly labelled. b) Correlation graphs demonstrate mitonuclear evolutionary rate correlations through pairwise comparisons: mitochondrial versus nucl-OXPHOS genes and mitochondrial versus nucl-noOXPHOS genes, with Pearson's correlation coefficients annotated. Each point represents an individual sample, and the XY-axis values correspond to the average branch lengths.

of the Adephaga lineages showed a lower proportion of positively selected codons in mitochondrial genes (mean: 0.043%) relative to nucl-OXPHOS genes (mean: 0.075%) in Fig. S17. This suggests that Rhysodinae and Noteridae likely retain synonymous mutations rather than accumulating AA substitutions that diverge from ancestral states. This observation potentially elucidates why the AA dataset offered phylogenetic resolution in the placement of Cicindelinae, due to synonymous mutations accumulating in the nucleotide sequence whereas ancestral sequence information is retained at the AA dataset. AAs often provide more reliable phylogenetic signal than nucleotides at specific phylogenetic nodes, as evidenced across diverse lineages (Plotkin and Kudla 2011; Yuan et al. 2016; Liu et al. 2018; Nie et al. 2021).

Remarkably, we detected strong ILS and introgression signals at the ancestral nodes of Carabidae and Dytiscoidea, which coincided with the mitonuclear discordance of Rhysodinae and Noteridae. Mitochondrial introgression has been demonstrated as a driver of mitonuclear discordance across multiple lineages (Wood and Duda 2021; Baião et al. 2023; Baltazar-Soares et al. 2023; Dong et al. 2023; Shi et al. 2025). The mitochondrial genes of Rhysodinae exhibit significant phylogenetic discordance relative to nucl-OXPHOS genes (Table 2). Integrating evidence of ancestral hybridization and introgression events at the Carabidae-Dytiscoidea divergence node during this evolutionary period (Fig. 2), these collective results support a hybrid

origin for the Rhysodinae mitogenome through mitochondrial introgression. In Noteridae, both mitochondrial genes and nucl-OXPHOS exhibit phylogenetic signals discordant with the species' evolutionary history. This indicates that the entire OXPHOS system underwent independent evolution, typically resulting from ancestral ILS. Such ILS-mediated mitonuclear discordance has been widely reported across some clades (Piccinini et al. 2021; Kao et al. 2022; DeRaad et al. 2023). While current nuclear data for Meruidae are lacking, mitochondrial evidence suggests possible parallels in mitonuclear discordance between Meruidae and Noteridae (Figs. S5–S7). Therefore, we conclude that the ILS interacted with mitochondrial introgression events to shape the observed mitonuclear discordance in beetles.

Based on current evidence, we propose that the mitochondrial genotypes of Rhysodinae and Noteridae could potentially originate either from a shared ancestral genotype or from distinct genotype. We provisionally hypothesize a shared common ancestral origin for mitogenomes in Rhysodinae and Noteridae to facilitate interpretation of this evolutionary process. The ancestral Adephaga likely harbored at least two distinct mitochondrial genotypes (Fig. 6). During speciation of ancestral Adephaga, the ancestor of Noteridae retained one mitochondrial genotype through ILS. However, in the early stages of ancestral lineage formation for either Rhysodinae or Carabidae, a hybridization event occurred between closely related ancestral species, accompanied by

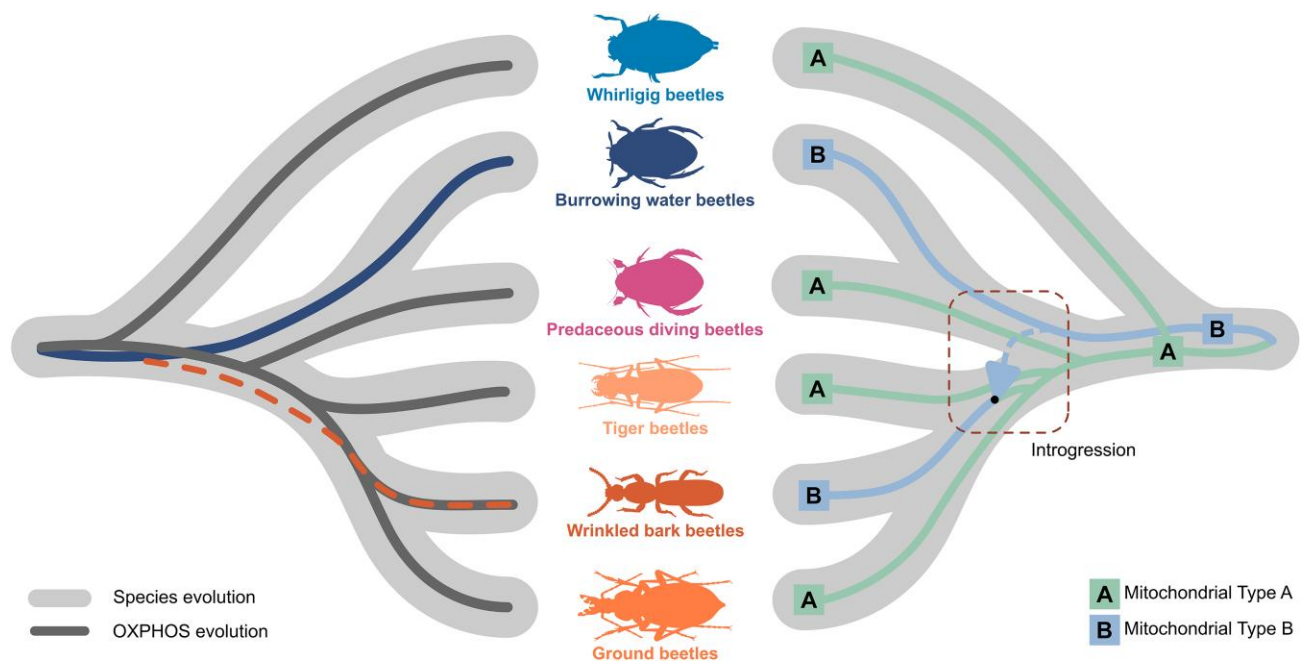


Fig. 6. Schematic representation of mitochondrial evolutionary patterns in Adephaga. Gray-shaded lines in the background depict the species divergence history. In the left panel, thin lines represent mitochondrial phylogenetic signals: solid lines indicate concordance between mitochondrial genes and nucl-OXPPOS genes signals, while dashed lines denote discordance. In the right panel, thin lines illustrate hypothesized evolutionary trajectories of two mitochondrial genotypes.

mitochondrial introgression. This evolutionary event resulted in Rhysodinae possessing mitochondrial genes compatible with their non-homologous nuclear OXPPOS gene system. While most cases of mitonuclear discordance often exhibit conflated signals of ILS and introgression that obscure mitochondrial evolutionary pathways (Hawlitschek et al. 2022; Prediger et al. 2024; van der Heijden et al. 2025), the case of Adephaga demonstrates clear evidence shaped by ILS and introgression.

Hybridization-Driven Adaptive Evolution

Hybridization-mediated introgression, characterized by inter-specific genetic exchanges between closely related taxa, serves as a key driver of evolutionary novelty through the transfer of adaptive genetic material (Edelman and Mallet 2021; Moran et al. 2021; Fu et al. 2022; Li et al. 2022; Suvorov et al. 2022). Emerging experimental evidence has revealed that mitonuclear interactions during interspecific hybridization may not only accelerate speciation processes but also drive diverse phenotypes (Nguyen et al. 2020; Owens et al. 2025; Shi et al. 2025). The adaptive potential of hybridization extends beyond mitochondrial introgression to encompass substantial contributions from nuclear genome introgression (Gaczorek et al. 2024). This mechanism may explain the terrestrial adaptation observed in ancestral Carabidae. The temporal correlation between hybridization events and speciation timelines in Carabidae and Dytiscoidea was hypothesized to be more than a coincidence (Figs. 2–3). During the Jurassic period, ancestral nodes of Dytiscoidea (nodes 5, 7, and 8) and Carabidae (nodes 9, 11, 13) exhibiting signals of ILS and hybridization and possess elevated proportions of positively selected genes, unlike other nodes which show lower levels of these signatures (Fig. 3). In contrast, no above-average positive selection was detected in nodes of Polyphaga dating back to the Jurassic period. Based on a sustained positive selection pressure, the

genetic exchange between Carabidae and Dytiscoidea may have also persisted for a considerable period during the Jurassic period. Hybridization events between Carabidae and Dytiscoidea may have served as one of the key drivers facilitating adaptive evolution. Moreover, similar cases have been documented across diverse taxonomic groups (Everson et al. 2025; Massatti et al. 2025; Owens et al. 2025). Our findings suggest that genetic introgression during the early speciation phases may have enhanced adaptive genetic variation for environmental divergence.

Nuclear Compensation Under Mitonuclear Discordance

In mitonuclear discordance, increased selection pressure on incompatible nuclear genes promotes nuclear compensation to preserve mitonuclear compatibility (Princepe and de Aguiar 2024). It is generally posited that mitonuclear interaction mechanisms demand stringent compatibility, as mutations in either mitochondrial or nuclear genomes often result in severe functional consequences (Han et al. 2023; Zheng et al. 2023; Moran et al. 2024). Mitonuclear incompatibility drives a nuclear compensatory phenomenon, wherein deleterious mitochondrial alleles induce adaptive changes in nuclear genes to preserve mitonuclear coadaptation and mitochondrial function, thereby generating signatures of positive selection (Adrian et al. 2016; Havird and Sloan 2016; Zwonitzer et al. 2023). Across all taxa of beetles, the proportions of positively selected codons in genes encoding Complexes I–V consistently exceed those observed in nuclear non-OXPPOS genes, although statistical significance remains challenging to establish (Fig. 4). Moreover, Complex II, whose components are not encoded by mitochondrial genes, exhibited notable positive selection pressure. This indicates that positive selection pressure in some clades is unlikely to result from physical contact with mitochondrial genes. Collectively, these

observations suggested that nuclear compensation was not universally required for all nuclear-encoded complexes in Adephaga. The ancestral Adephagan lineage likely possessed a loose interaction mechanism, as evidenced by the traits preserved in Gyrinidae, the earliest diverging lineage within this suborder. None of the genes encoding complexes I–V in Gyrinidae exhibited significant positive selection pressure. It is generally hypothesized that the dynamics of mitonuclear incompatibility caused by mitochondrial introgression resemble those of compensatory coevolution (Princepe and de Aguiar 2024). In Rhysodinae mitochondrial introgression events, we initially hypothesized significant selection pressure on nucl-OXPHOS genes. Nevertheless, Complex III–V genes maintain functional integrity with intrusive mitochondrial variants without requiring extensive adaptive AA substitutions (Fig. 4). Based on some adaptive residues (Fig. S18), we propose that nuclear genes related to mitochondria have not abandoned the nuclear compensatory strategy; it is merely challenging to achieve statistical significance. Moreover, genes harbouring adaptive residues are not enriched in nucl-OXPHOS genes (Table S6), indicating that compensatory mutations in nuclear genes are not limited to protein-protein interactions. Notably, nuclear compensation likely extends beyond purely physical interactions, whether analysed at the level of OXPHOS complexes or individual residues.

In summary, these findings demonstrate that the positive selection pressure exhibits restricted occurrence across OXPHOS complexes and taxonomic groups, indicating its demonstrably limited impact at the macroscale. This limited nuclear compensatory phenomenon fundamentally reflects the evolutionary constraints of the loose interaction mechanism. This establishes a novel paradigm for explaining intrusive mitochondrial survival: structural conservation reduces the demand for nucl-OXPHOS genes, enabling the formation of functionally equivalent OXPHOS complexes from divergent mitonuclear gene combinations. The loose mitonuclear interaction mechanism, coupled with nuclear compensation, may represent an evolutionary strategy for mitigating the effects of mitonuclear incompatibility.

The Loose Interaction Mechanism of Mitonuclear Coevolution

The ERC analyses revealed correlations in evolutionary rates among genes during evolution, reflecting their functional cooperativity. Although ERC signals co-functional interactions, it does not consistently predict physical interactions (Little et al. 2024). Revealing longevity-regulating genes by ERC analyses in insect mitonuclear genes interactions (Tao et al. 2024). The direct interaction of nucl-OXPHOS genes with mitochondrial genes results in a stronger ERC (Yan et al. 2019). However, such strong ERC is unlikely to be universally conserved across all insect taxa. Our analyses revealed that nucl-OXPHOS genes do not exhibit stronger co-evolutionary constraints than other nuclear genes. Comparative results of ERC between mitochondrial and nucl-OXPHOS genes across insects revealed a clade-specific reduction in ERC signals within Adephaga (Tao et al. 2024). Mitochondrial genes exhibit stronger coevolutionary signals, presumably attributable to their elevated substitution rates relative to nuclear genes. Despite the evident functional relationship between mitochondrial and nuclear OXPHOS genes, the ERC analysis revealed no significant co-evolutionary signals. Although localized coevolutionary loci remain plausible, we propose that the

globally attenuated ERC may reflect reduced mitonuclear co-evolution signals. The limited nuclear compensatory phenomenon also suggested that mitonuclear interaction mechanisms may not necessitate tight coordination, with nuclear OXPHOS genes potentially conferring more error tolerance capacity.

Given the observed mitochondrial introgression, attenuated ERC, and limited nuclear compensation in Rhysodinae, we considered the existence of a loose mitonuclear interaction mechanism. In this mechanism, mitochondrial and nuclear OXPHOS genes from divergent sources can assemble into properly functioning OXPHOS complexes. The occurrence of cytonuclear incompatibility in sunflowers, genomic scans revealed no evidence of cytonuclear interactions, suggesting the absence of mitonuclear coadaptation (Owens et al. 2025). This case suggests that the relaxed mitonuclear interaction mechanism in Adephaga likely originated from ancestral mitochondrial polymorphisms, which enabled nuclear-encoded OXPHOS genes to accommodate divergent mitochondrial lineages. Rhysodinae may have acquired mitochondrial introgression from a lineage sharing a common ancestor with Noteridae. Crucially, the introgressed mitochondrial genotype remained within the accommodation range of the ancestral nuclear-encoded OXPHOS system. The mitochondrial introgression events observed in Adephaga were unlikely to represent isolated occurrences. Mitochondrial evolutionary trajectories shaped by introgression in fruit flies and some bird lineages have demonstrated that mitonuclear interaction mechanisms can integrate mitochondrial and nucl-OXPHOS genes from different sources (Morales et al. 2018; Baião et al. 2023; Baltazar-Soares et al. 2023). This plasticity suggests that loose mitonuclear coevolution may act as an evolutionary “buffer”, enabling hybridization-driven adaptive evolution. Importantly, such genomic flexibility may explain the recurrent emergence of mitonuclear discordance among divergent lineages. We propose that a loose mitonuclear interaction mechanism may operate broadly across diverse lineages.

Materials and Methods

Sample Collection and DNA Sequence Generation Workflow

We collected 15 beetle specimens encompassing the major clades of the suborder Adephaga, including Carabidae, Dytiscidae, and Gyrinidae, with a particular focus on supplementing the underrepresented data from Rhysodinae (Table S7). The specimens used for DNA extraction and sequencing were collected in the field and preserved in a dry ice storage bucket and were subsequently stored in a -80 °C freezer. Total genomic DNA was extracted from cephalothoracic tissue using a DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA, USA) according to the manufacturer’s instructions. A genomic DNA library was constructed, and a total of 40 GB of clean data were generated using the paired-end 150 sequencing method on the Illumina Hiseq 6000 platform (Berry Genomics, Beijing, China).

Raw sequencing data were subjected to quality control and normalization using Fastp 0.24.0 (Chen et al. 2018). The quality control criteria included: a Phred quality score of $\geq Q30$ for qualified bases, a maximum of 10% unqualified bases per read, and a minimum read length of 100 bp after trimming. All other parameters were set to default. *De novo* genome assembly was subsequently performed using SPAdes 3.15.5 (Prjibelski et al. 2020) with default settings.

Mitochondrial sequences were iteratively assembled using GetOrganelle v1.7.2a (Jin et al. 2020), default parameters were used.

Gene Annotation

Universal single-copy orthologues (USCOs) were extracted from genomes using BUSCO v5.8.2 (Manni et al. 2021) with the odb10 Endopterygota dataset (Kriventseva et al. 2019). We modified the default standard deviations (σ) of the mean USCO length to 2σ to classify more USCOs as “complete” (Du et al. 2023, 2024), because the BUSCO pipeline did not output incomplete “fragmented” USCO sequences. The USCO AA sequences were used for subsequent analyses. PCGs, rRNAs, and tRNAs of mitochondrial genes were uniformly annotated using MitoZ v3.3 (Meng et al. 2019), with the genetic code selected as No. 5 (The Invertebrate Mitochondrial Code) and the clade chosen as Arthropoda. Nuclear PCGs were annotated and retrieved using MetaEuk v7.bba0d80 (Levy Karin et al. 2020) in the easy-predict mode. The Insecta non-redundant (NR) database downloaded from the NCBI was used as a reference database. Both AA and nucleotide sequences were used for subsequent analyses.

Nuclear genes associated with mitochondrial functions and common nuclear genes in *Drosophila melanogaster* were obtained from FlyBase and DroID (Yu et al. 2008; Öztürk-Çolak et al. 2024). These AA sequences were compiled into a mitochondria-related nuclear gene reference library (details in Table S2). Iterative searches by Diamond v2.1.11 (Buchfink et al. 2021), as described in previous study (Liu et al. 2024), were then performed against this library to comprehensively capture mitochondria-related nuclear genes in each Adephaga species sample.

Phylogenetic Inference and Divergence Time Estimation

The 53 genome sequences included 19 pre-assembled sequences, 19 sequences derived from Sequence Read Archive (SRA) datasets, and 15 self-assembled genome sequences (details in Table S1). The 53 mitochondrial genome sequences included 15 self-assembled sequences, and 11 sequences derived from SRA datasets.

The AA sequences of the PCGs were aligned using the default strategy in MAFFT v7.310 (Katoh and Standley 2013). The nucleotide sequences of PCGs based on aligned AA sequences in the previous step were aligned using TranslatorX v1.1 (Abascal et al. 2010). Throughout this study, this alignment method is applied to all multiple sequence alignments (MSAs) generation. The mitochondrial rRNA was aligned by MAFFT under the L-INS-I method. Ambiguous sites and poorly aligned positions were pruned using ClipKIT v2.3.0 (Steenwyk et al. 2020) with smart-gap mode for mitochondrial datasets and kpic mode for nuclear datasets. The aligned and pruned sequences were concatenated into a matrix by PhyKIT v2.0.1 (Steenwyk et al. 2021).

ML inference was conducted in IQTREE v2.1.2 (Minh et al. 2020). Substitution models were compared and selected according to the BIC using ModelFinder (Kalyaanamoorthy et al. 2017). The reliability of the branching pattern was evaluated using bootstrap support (BS). A total of 1,000 ultrafast bootstraps were used to evaluate the nodal support of the ML tree (Hoang et al. 2018). For mitochondrial genes an edge-unlinked model (each partition has its own branch lengths) was specified for both the full partition (FP) and merged

partition (MP) schemes. For each matrix, three partition schemes were applied for ML: (i) no partition (NP); (ii) FP that provides the best-fitting model for each individual gene; and (iii) MP that implements a greedy strategy starting with the FP model and subsequently merging pairs of genes until the model fit does not improve any further. We selected the best partition scheme according to the BIC.

In the Bayesian analysis using the PhyloBayes-MPI v1.8 (Lartillot et al. 2013), the CAT-GTR model consistently showed the best fit among all models implemented for datasets exceeding 1,000 aligned positions. Additionally, the CAT-GTR model is likely a highly effective general-purpose model for AA, DNA and RNA. Bayesian inference was performed using the CAT-GTR model (Lartillot and Philippe 2004; Le et al. 2008; Lartillot et al. 2009, 2013). Two independent Markov Chain Monte Carlo (MCMC) runs of 5,000 generations each were executed. Convergence was evaluated with the “bpcomp” and “tracecomp” procedure in the PhyloBayes package with a burn-in of first 20% by the recommended criterion of maximum discrepancy <0.1 . A consensus tree was simultaneously built by pooling the remaining MCMC trees of both runs in Figs. S8–S10.

A coalescent-based species tree was reconstructed using a multi-locus approach. Initially, ML trees for individual genes were inferred with IQ-TREE v2.1.2 (Minh et al. 2020), employing the ModelFinder module to automatically select optimal substitution models through BIC. Gene trees were then aggregated using ASTRAL v3.2 (Zhang et al. 2018), a weighted statistical framework that resolves topological incongruence among loci by optimizing quartet-based likelihood scores (Fig. S19).

The following metrics were computed by the Phylogenomics Toolkit v1.20.0 (Steenwyk et al. 2021). Relative composition variability (RCV) was calculated to assess the compositional differences. Parsimony informative sites (PIS) were identified to see the extent of phylogenetic signal contained. Phylogenetic trees were visualized in iTOL v 6.8.1 (Letunic and Bork 2021).

Based on the phylogenetic tree obtained in the present study, we used five fossil calibration nodes to estimate the divergence time of Adephaga (Table S8). The upper- and lower-bound values (Ma) of fossil records were uniformly provided by the Paleobiology Database (<https://paleobiodb.org/>). Single-copy AA sequences and their reference species tree were used to estimate divergence time. Divergence time estimation was carried out using MCMCTree in PAML 4.9j (Yang 2007), which performs Bayesian estimation of species divergence times using soft fossil constraints with the molecular clock under auto-correlated rates model. The Dirichlet-gamma prior for overall substitution rate (rgene gamma) was set to G (1 15.97), which was calculated by codeml. The Dirichlet-gamma prior for the rate-drift parameter (sigma2 gamma) was set to G (1, 4.5) based on empirical values (Tong et al. 2015). The root age of the tree was calibrated at 251.878 to 307.10 Ma, reflecting the estimated origin time of Coleoptera based on the fossil *Ponomarenkium belmontheense* (Yan et al. 2018; Cai et al. 2022). The first 100,000 cycles were discarded as burn-in, before we drew samples every 10 cycles over 500,000 cycles. Two independent runs reached stable and similar results in all analyses. TVBOT v2.5.378 (Xie et al. 2023) was used for visualizing images of divergence time trees generated by MCMCTree. The geologic timescale was based on international chronostratigraphic chart v2023/06 (<https://stratigraphy.org/chart#latest-version>).

Hypothesis Testing With Likelihood Mapping and Distance Calculation

The phylogenetic information of the datasets was evaluated using quartet likelihood mapping in IQTREE v2.1.2 (Minh et al. 2020) to assess the informative resolution. This method allowed us to visualize the tree-likeness of all quartets in a single graph and therefore provided a robust interpretation of the phylogenetic content of a dataset. The Four-cluster Likelihood Mapping (FcLM) method was used to assess the relationship between nucl-OXPHOS and mitochondrial divergence in Adephaga. Four distinct datasets were analysed: (i) nucl-OXPHOS AA alignments, (ii) mitochondrial AA alignments (AA), (iii) mitochondrial nucleotide alignments incorporating all three codon positions (P123R), and (iv) mitochondrial nucleotide alignments restricted to the first and second codon positions (P12R).

For all FcLM analyses the following topologies and groups were defined:

Hypothesis 1: Noteridae and Dytiscoidea represent the closest phylogenetic relationship.

Groups: G1: Noteridae; G2: Carabidae (excluding Rhysodinae); G3: Dytiscoidea (including Aspidytidae, Amphizoidae, and Dytiscidae); G4: Gyrinidae.

Hypothesis 2: Rhysodinae and Carabidae share the closest phylogenetic relationship.

Groups: G1: Rhysodinae; G2: Carabidae (excluding Rhysodinae); G3: Dytiscoidea (including Aspidytidae, Amphizoidae, and Dytiscidae); G4: Gyrinidae.

Pairwise distances were calculated for 53 species within the suborder Adephaga using the aligned AA sequences with the Poisson correction model (Zuckerandl and Pauling 1965). All ambiguous positions were removed from each sequence pair (pairwise deletion option). There was a total of 3,806 positions in the final dataset. The pairwise distance analysis was calculated using the distance function in MEGA11 (Tamura et al. 2021). All pairwise distance values are provided in Table S9 and Fig. S12.

Detection of Nuclear Compensation across Lineages and Genes

The nonsynonymous (dN) and synonymous (dS) substitution rates were used to infer purifying selection ($dN/dS < 1$) and positive selection ($dN/dS > 1$). The adaptive Branch-Site Random Effects Likelihood (aBSREL) allows either a priori specification of branches to test for selection or can test each lineage for selection in an exploratory fashion (Smith et al. 2015). Selection pressures on site were calculated using the aBSREL method in Hyphy v2.5.62 (Kosakovsky Pond et al. 2020). A reference species tree was reconstructed using single-copy orthologs. MSAs for each individual gene were used in subsequent phylogenetic analyses. The aBSREL method infers the optimal number of dN/dS classes for each branch. Each node and samples typically comprised two or three categories ($dN/dS > 1$, $dN/dS < 1$ and $dN/dS = 1$), and we quantified the proportion of sites falling into each category within each gene. Genes with >90% positively selected sites are operationally classified under strong positive selection. We established a conservatively high threshold (90%) to rigorously identify strong positive selection (Liu et al. 2023; Zhao et al. 2025). We plotted a bar chart of the proportion of genes under positive selection pressure at different nodes (Fig. 3 and Fig. S16).

We grouped samples by the family or subfamily that it belongs to and plotted the proportion of positively selected sites as box plots (Fig. 4 and Fig. S17). The Mann-Whitney test is used to assess the significance of differences between different groups of genes.

To determine evidence for nuclear compensation at the residue scale, putative nuclear compensatory candidate residues were identified through analysis of 745 mitochondrial-related genes. These residues were characterized by a specific evolutionary pattern: conservation across the majority of examined Adephaga lineages, but divergence within Rhysodinae and Noteridae. MSAs of 745 mitochondrial-related genes scanning utilized the discovery module of CAAStools 1.0 (Barteri et al. 2023), designating Rhysodinae and Noteridae as the foreground lineages and other Adephaga species as the background. Remaining parameters used default values. We manually verified the candidate residues and recorded them in Table S6. Visualizations of Fig. S18 employed iTOL v6.8.1 (Letunic and Bork 2021).

Evolutionary Rate Correlation and Branch Statistics

By choosing genes present in all samples, we constructed a dataset of four gene categories as follows: (i) 12 mitochondrial PCGs (excluding ND2); (ii) 3,818 nuclear genes; (iii) 52 nucl-OXPHOS genes; (iv) 588 nucl-noOXPHOS genes. Branch lengths (root to terminal tip distances) in the gene trees computed using the terminal branch statistics module Phylogenomic Toolkit v1.20.0 (Steenwyk et al. 2021) and were normalized to the corresponding background branch lengths of the reference species phylogeny to account for lineage-specific evolutionary rate heterogeneity and independent branch length contrasts.

The workflow for the ERC calculation was adapted from existing methodologies (Steenwyk et al. 2021; Tao et al. 2024). Specifically, we employed a two-step workflow for the computation of ERC, following established methodologies. First, ML trees were inferred for each gene using IQTREE v2.1.2 (Minh et al. 2020), with the optimization process constrained by a reference species tree as the fixed topological framework. The model automatically selected the best-fit substitution model for each gene based on the BIC. Second, pairwise correlations between genes were assessed using the Evaluate gene-gene covariation module in Phylogenomics Toolkit v1.20.0 (Steenwyk et al. 2021), which computes Pearson correlation coefficients and their associated *P*-values as final outputs. This module derives correlations by leveraging the branch lengths of the parameterized species tree to evaluate dependencies in evolutionary rates across gene pairs. The method normalized gene tree branch lengths to relative evolutionary rates by dividing them by corresponding branches in the reference species tree. During the computation, we only used correlation coefficients with *P*-values less than 0.05 to ensure statistical reliability of the results.

To analyse the evolutionary rates of individual genes, branch lengths for each sample were calculated. For each sample, we computed the average branch length across the four gene categories. Subsequently, linear regression models were constructed to evaluate the correlations between the mitochondrial gene branch lengths and those of the other three nuclear gene categories. Pearson's correlation coefficients were calculated to quantify the strength of associations. Scatter plots were generated to visually represent these relationships, with mitochondrial branch lengths plotted against the branch lengths of nucl-OXPHOS genes and nucl-noOXPHOS genes (Fig. 5).

Detection of ILS and Introgression

To quantitatively assess the signal intensities of ILS and introgression events at key nodes, we conducted a multi-genes tree topology analysis. We constructed a nuclear genome dataset comprising 53 species integrated AA sequences of 2,123 single-copy orthologous genes. Phylogenetic reconstruction was performed using IQTREE v2.1.2 (Minh et al. 2020) without topological constraints. The optimal substitution models were automatically selected based on BIC for individual gene tree construction.

All generated gene trees were subsequently integrated through ASTRAL v3.2 (Zhang et al. 2018) to build the species tree with detailed node topological conflict information, using *Chrysoperla carnea* (GCF_905475395.1) as the designated outgroup for phylogenetic rooting. We employed PhyTop v0.3 (Shang et al. 2025) to quantify the signals of ILS and IH, using the species tree generated in the previous step from ASTRAL. PhyTop enabled systematic quantification of topology distribution frequency characteristics at each node, effectively distinguishing between ILS signatures and recent IH events.

The construction of reticulate evolutionary networks facilitates the identification of genetic introgression between species and enables the detection of introgression events. We employed PhyloNET v 3.8.4 (Than et al. 2008) to infer species networks through ML analyses, accounting for both ILS and introgression. Due to the computational constraints of PhyloNET, eight representative species were selected for subsequent analyses. The small-scale dataset, comprising samples from these eight species, provided 2,018 single-copy gene trees with *Chrysoperla carnea* (GCF_905475395.1) as the outgroup. The parameter configurations were set as follows: number of reticulations to add=1, number of optimal networks to return=3, and number of independent search runs=20. The resulting phylogenetic network, encoded in the Rich Newick format, was visualized using IcyTree (Vaughan 2017). The small-scale dataset also quantifies signals of ILS and introgression using PhyTop.

Supplementary material

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

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Author Contributions

Hu Li, Wanzhi Cai (Conceptualization and supervision), Tianyou Zhao, Pingzhou Zhu, Liang Lü (Material preparation), Tianyou Zhao, Ling Ma, Ye Xu, Qiaoqiao Liu, and Yuange Duan (Data analyses), Tianyou Zhao (Writing—original draft), Liang Lü, Yuange Duan, Fan Song, Li Tian, Hu Li, and Wanzhi Cai (Writing—review & editing)

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Conflict of Interest

The authors declare no competing interests.

Data Availability

The sequence and tree files have been deposited in Zenodo (<https://doi.org/10.5281/zenodo.17265979>). This study established a Bioproject (PRJNA1267476) on NCBI. The accession numbers for biosample and SRA corresponding to the genomic data of 15 beetle specimens have been submitted to NCBI (see Table S7). All Supplementary Data are available.

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