Zoological Journal of the Linnean Society, 2023, XX, 1–15
https://doi.org/10.1093/zoolinnean/zlad178
Advance access publication 24 November 2023
Original Article

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Mitochondrial phylogenomics of bumblebees, *Bombus* (Hymenoptera: Apidae): a tale of structural variation, shifts in selection constraints, and tree discordance

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ABSTRACT

The mitochondrial DNA (mtDNA) of bumblebees (*Bombus*) has been widely used for phylogenetic studies, but its evolution is still underexplored. Here we report a comprehensive analysis of 60 bumblebee mitogenomes, including 40 newly assembled ones, to investigate bumblebee mtDNA structure, composition, and informativeness under a phylogenetic framework. Our mtDNA dataset supports the monophyly of *Bombus* and its subgenera, although we found a high degree of tree discordance in deeper nodes when using different inference methods or matrix composition. Concerning mitogenome structure, our results show that tRNA genes were often rearranged, with unique rearrangements indicating shared ancestry across bumblebee subgenera, illustrating their potential for subgeneric classification. Our results also challenge the notion that faster evolving mtDNA exhibits higher gene rearrangement rates. Finally, we explicitly assessed shifts in selection constraints of mtDNA genes in obligate social parasites of subgenus *Psithyrus* and found that their mtDNA evolved under relaxed selective constraints. Our findings show the utility of mtDNA in providing insights into bumblebee phylogenetics, evolution, and genome trait diversification. We also highlight the potential for comparative mitogenomics to uncover previously unknown aspects of bumblebee evolution, offering exciting opportunities for future research in this field.

Keywords: Apidae; evolutionary rate; Hymenoptera; mitochondrial genome; social parasitism

INTRODUCTION

Mitochondria play a pivotal role in providing energy for the cellular functions of eukaryotes. These organelles have their own genome, the mitogenome, which encodes proteins involved in ATP synthesis through oxidative phosphorylation. Insect mitogenomes are compact circular molecules that typically comprise 37 genes, including 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, and two ribosomal RNA (rRNA) genes (Wolstenholme 1992). Mitochondrial genes are widely used as markers in population genetics, phylogenetics, and taxonomic studies, particularly for insects (Cameron 2014). Apart from nucleotide polymorphisms, changes in mitochondrial gene order can also provide insights into phylogenetic relationships and genome evolution. Mitogenomes are conserved concerning gene order and content because they encode crucial proteins for eukaryotic life (Brown *et al.* 1979, Wolstenholme 1992). Thus, mitogenome rearrangements are considered rare genomic changes, which are often homoplasy-free and reflect shared ancestry (Rokas and Holland 2000). While rearrangements are typically characterized for major lineages, few studies have explored mitogenome evolution and rearrangements at lower taxonomic scopes, such as genus or subgenus.

Hymenopterans are exciting systems for studying mitochondrial DNA (mtDNA) evolution due to the high substitution and rearrangement rates observed in their mitogenomes (Dowton and Austin 1999, Dowton *et al.* 2003, Oliveira *et al.* 2008, Zheng *et al.* 2018, Françoso *et al.* 2023), particularly in tRNA gene clusters (Fig. 1) (Dowton and Austin 1999, Dowton *et al.* 2003, 2009, Françoso *et al.* 2020). Unlike other invertebrate groups, hymenopteran mitogenomes display rearrangements at lower

Received 8 June 2023; revised 6 September 2023; accepted 26 October 2023

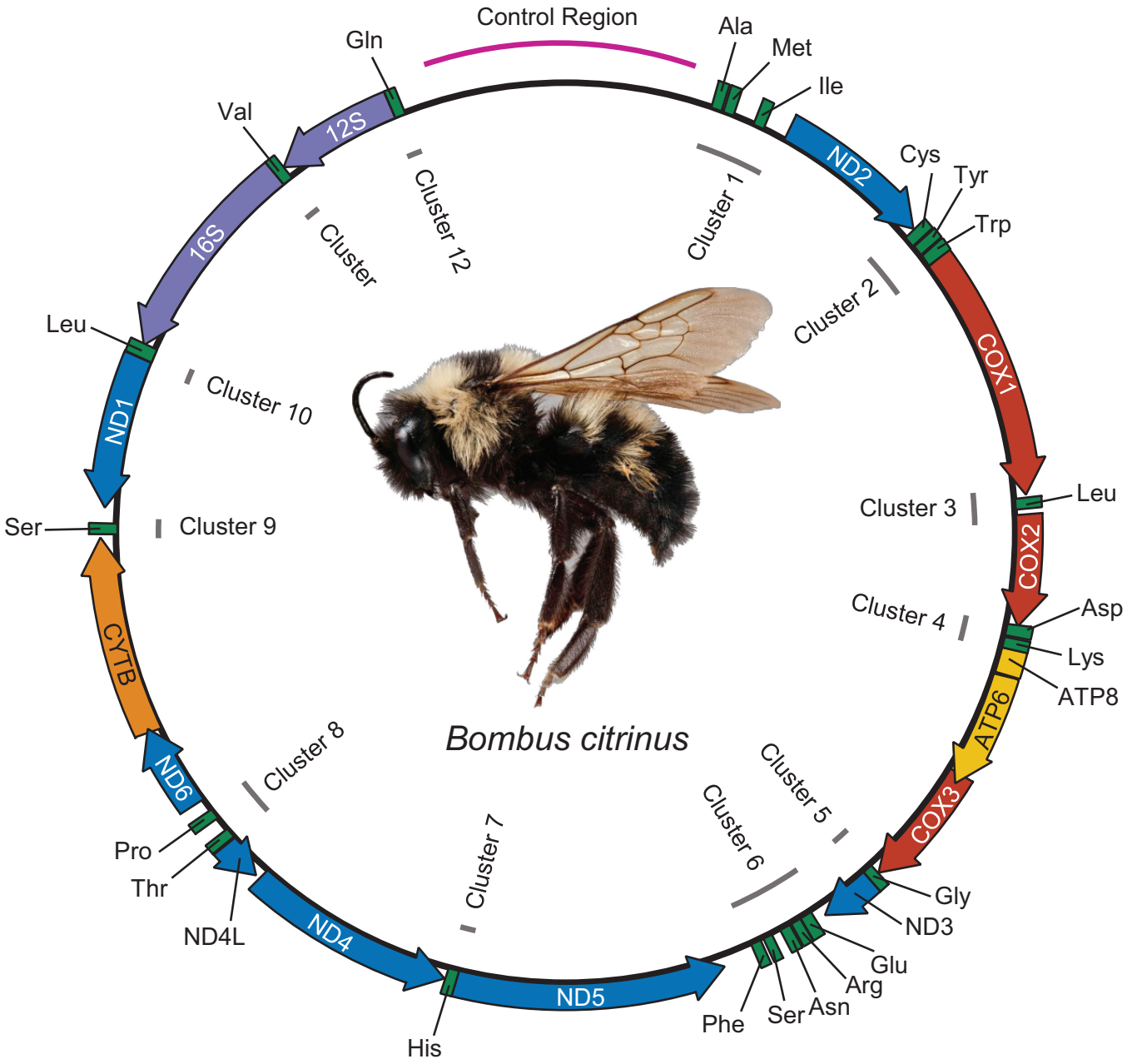

Figure 1. Typical bumblebee mitochondrial genome, using *Bombus (Psithyrus) citrinus* as an example. The arrowheads indicate the direction of protein-coding gene (PCG) transcription. Transfer RNA (tRNA) genes are represented by the three-letter IUPAC-IUB abbreviation for their corresponding amino acid. Gene sizes are proportional to their nucleotide length. Inner lines (in grey) refer to tRNA clusters, defined according to PCG junctions. Specimen image courtesy of Margarita Miklasevskaja at PCYU with funding from NSERC-CANPOLIN.

taxonomic levels, and synapomorphic rearrangements can be identified for species groups. In the case of bees, rearrangements in tRNA genes have been proposed as putative synapomorphies at the family or genus level (Françoso *et al.* 2020). For instance, a shuffle between $tRNA^{Lys}$ and $tRNA^{Asp}$ is present in all bee families when compared to a wasp outgroup; a translocation of $tRNA^{Ser1}$ and $tRNA^{Glu}$ from tRNA cluster 6 to cluster 1 was found in all analysed *Apis* species (details of taxon authors are in Table 1); and a translocation of the $tRNA^{Lys}$ from tRNA cluster 4 to cluster 1 has been identified in the stingless bee genus *Melipona* (Françoso *et al.* 2020).

Bumblebees (Hymenoptera: Apidae: *Bombus*) are major pollinators of wild flora and crops, comprising around ~280 species divided into 15 subgenera (Williams *et al.* 2022). The current phylogenetic hypothesis for *Bombus* is primarily based on five genes (four nuclear genes and one mitochondrial gene) and encompasses most known bumblebee species (Cameron *et al.* 2007, Santos Júnior *et al.* 2022). Recently, a genus-wide phylogenomic study utilizing nuclear data from 17 species supported previous topologies and refined subgeneric relationships (Sun *et al.* 2021). The majority of bumblebee species can be assigned to one of two major clades: a 'short-faced' clade (SF) and a 'long-faced' clade (LF), which broadly relate to differences in head morphology and tongue length (Cameron *et al.* 2007). While mitogenomes have been described for some bumblebee species, the evolution of mitogenomes in *Bombus* and its

Table 1. References and GenBank accession numbers for the mitochondrial genomes used in the analyses. Mitochondrial genomes that were assembled in this study are marked with an asterisk.

Species	Size (bp)	Dataset Reference	Acc. number	*
***Bombus (Alpigenobombus)* Skorikov, 1914**				
B. breviceps Smith, 1852	16 743	Zhao *et al.* (2017b)	MF478986	
B. kashmirensis Friese, 1909	16 793	Zhao *et al.* (2019)	MH998261	
***Bombus (Alpinobombus)* Skorikov, 1914**				
B. balteatus Dahlbom, 1832	18 099	Christmas *et al.* (2022)	BK063618	*
B. polaris Curtis, 1835	18 658	Sun *et al.* (2021)	BK063644	*
***Bombus (Bombias)* Robertson, 1903**				
B. confusus Schenck, 1859	16 832	Sun *et al.* (2021)	BK063651	*
B. nevadensis Cresson, 1874	18 238	Bossert *et al.* (2019)	BK063630	*
***Bombus (Bombus)* Latreille, 1802**				
B. cryptarum florilegus Panfilov, 1956	15 763	Takahashi *et al.* (2018b)	AP018158	
B. hypocrita sapporoensis Cockerell, 1911	15 468	Takahashi *et al.* (2016)	AP017370	
B. ignitus Smith, 1869	16 434	Cha *et al.* (2007)	DQ870926	
B. lantschouensis Vogt, 1908	16 153	Zhao *et al.* (2021)	BK063627	*
B. longipennis Friese, 1918	17 711	Zhou *et al.* (2021a)	MW741884	
B. lucorum (Linnaeus, 1761)	18 990	Lin *et al.* (2019a)	BK063625	*
B. terrestris (Linnaeus, 1758)	24 708	Crowley *et al.* (2023c)	OU342939	
B. terrestris canariensis Pérez, 1895	17 300	Ruiz *et al.* (2021)	MW959771	
B. terrestris lusitanicus Krüger, 1956	17 049	Cejas *et al.* (2020)	MK570128	
B. terrestris terrestris (Linnaeus, 1758)	17 232	Cejas *et al.* (2020)	MK570129	
B. terricola Kirby, 1837	20 452	Kent *et al.* (2018)	BK063649	*
***Bombus (Cullumanobombus)* Vogt, 1911**				
B. cullumanus (Kirby, 1802)	16 792	Sun *et al.* (2021)	BK063614	*
B. griseocollis (DeGeer, 1773)	17 587	Grab *et al.* (2019)	BK063647	*
***Bombus (Kallobombus)* Dalla Torre, 1880**				
B. soroeensis (Fabricius, 1777)	16 177	Sun *et al.* (2021)	BK063633	*
***Bombus (Megabombus)* Dalla Torre, 1880**				
B. consobrinus Dahlbom, 1832	17 966	Zhao *et al.* (2017a)	MF995069	
B. hortorum (Linnaeus, 1761)	21 620	Crowley *et al.* (2021)	BK063648	*
B. supremus Morawitz, 1887	19 280	Zhao *et al.* (2021)	BK063638	*
B. trifasciatus Smith, 1852	18 681	Lin *et al.* (2019a)	BK063641	*
B. ussurensis Radoszkowski, 1877	15 807	Yoon *et al.* (2020)	BK063640	*
***Bombus (Melanobombus)* Dalla Torre, 1880**				
B. ladakhensis Richards, 1928	15 877	Zhao *et al.* (2021)	BK063629	*
B. lapidarius (Linnaeus, 1758)	17 817	Tang *et al.* (2015)	KT164641	
B. pyrosoma Morawitz, 1890	18 897	Zhao *et al.* (2019)	MH998260	
B. sichelii Radoszkowski, 1860	17 165	Lin *et al.* (2019a)	BK063635	*
***Bombus (Mendacibombus)* Skorikov, 1914**				
B. convexus Wang, 1879	19 996	Lin *et al.* (2019a)	BK063615	*
B. superbus (Tkalcu, 1968)	16 855	Sun *et al.* (2021)	BK063632	*
B. waltoni Cockerell, 1910	19 349	Lin *et al.* (2019b)	MK252702	
***Bombus (Orientalibombus)* Richards, 1929**				
B. haemorrhoidalis Smith, 1852	16 595	Sun *et al.* (2021)	BK063620	*
***Bombus (Psithyrus)* Lepeletier, 1832**				
B. bohemicus Seidl, 1837	20 582	Lin *et al.* (2019a)	BK063613	*
B. campestris (Panzer, 1801)	24 740	Crowley *et al.* (2023e)	HG995151	
B. citrinus (Smith, 1854)	17 692	Bossert *et al.* (2019)	BK063616	*
B. skorikovi (Popov, 1927)	24 179	Sun *et al.* (2021)	BK063634	*
***Bombus (Pyrobombus)* Dalla Torre, 1880**				
B. bifarius Cresson, 1878	20 026	Heraghty *et al.* (2020)	BK063617	*
B. hypnorum (Linnaeus, 1758)	15 614	Crowley *et al.* (2023d)	OU427032	

Table 1. Continued

Species	Size (bp)	Dataset Reference	Acc. number	*
B. impatiens Cresson, 1863	17 161	Sadd *et al.* (2015)	BK063623	*
B. lepidus Skorikov, 1912	19 530	Zhao *et al.* (2021)	BK063626	*
B. melanopygus Nylander, 1848	18 141	Tian *et al.* (2019)	BK063624	*
B. perplexus Cresson, 1863	17 226	Grab *et al.* (2019)	BK063646	*
B. picipes Richards, 1934	18 017	Sun *et al.* (2021)	BK063636	*
B. pratorum (Linnaeus, 1761)	21 229	Crowley *et al.* (2023b)	BK063650	*
B. sylvicola Kirby, 1837	20 535	Christmas *et al.* (2021)	BK063612	*
B. vancouverensis nearcticus Handlirsch, 1888	20 554	Heraghty *et al.* (2020)	BK063643	*
B. vancouverensis vancouverensis Cresson, 1878	17 062	Ghisbain *et al.* (2020)	BK063642	*
B. vosnesenskii Radoszkowski, 1862	18 652	Heraghty *et al.* (2020)	BK063639	*
***Bombus (Sibiricobombus)* Vogt, 1911**				
B. asiaticus Morawitz, 1875	19 752	Zhao *et al.* (2019)	MH998259	
B. sibiricus (Fabricius, 1781)	20 048	Zhao *et al.* (2019)	MH998258	
***Bombus (Subterraneobombus)* Vogt, 1911**				
B. difficillimus Skorikov, 1912	16 810	Sun *et al.* (2021)	BK063619	*
B. melanurus Lepeletier, 1835	17 173	Zhao *et al.* (2021)	BK063631	*
B. personatus Smith, 1879	15 892	Zhao *et al.* (2021)	BK063645	*
***Bombus (Thoracobombus)* Dalla Torre, 1880**				
B. fervidus (Fabricius, 1798)	16 440	Grab *et al.* (2019)	BK063621	*
B. filchnerae Vogt, 1908	16 804	Zhou *et al.* (2021b)	MW741886	
B. impetuosus Smith, 1871	16 973	Lin *et al.* (2019a)	BK063622	*
B. laesus Morawitz, 1875	15 712	Lin *et al.* (2019a)	BK063628	*
B. opulentus Smith, 1861	18 218	Sun *et al.* (2021)	BK063637	*
B. pascuorum (Scopoli, 1763)	21 904	Crowley *et al.* (2023a)	HG995285	
***Apis* Linnaeus, 1758**				
A. cerana Fabricius, 1793	15 895	Tan *et al.* (2011)	NC_014295	
A. dorsata Fabricius, 1793	15 892	Chhakchhuak *et al.* (2016)	NC_037709	
A. florea Fabricius, 1787	17 694	Wang *et al.* (2013)	NC_021401	
A. mellifera sahariensis Baldensperger, 1932	16 569	Haddad *et al.* (2017)	NC_035883	
A. nigrocincta Smith, 1860	15 855	Takahashi *et al.* (2018a)	NC_038114	
A. nuluensis Tingek, Koeniger and Koeniger, 1996	15 843	Eimanifar *et al.* (2017)	NC_036235	
***Melipona* Illiger, 1806**				
M. bicolor Lepeletier, 1836	15 001	Silvestre *et al.* (2008)	AF466146	
M. fasciculata Smith, 1854	14 753	Unpublished	MH680930	
M. scutellaris Latreille, 1811	14 862	Pereira *et al.* (2016)	NC_026198	

subgenera remains unexplored under a robust phylogenetic framework. Specifically, the phylogenetic utility of mitochondrial PCGs has not been thoroughly examined, and the potential phylogenetic informativeness of mitochondrial rearrangements has not been assessed.

Bumblebees exhibit a fascinating range of ecological diversity. Most bumblebee species form social colonies with dominance hierarchies and reproductive division of labour (Free 1955). However, cuckoo bumblebees (a group of 26 species) are obligate parasites of other bumblebee species, exploiting the social structure and food resources of their hosts to rear their own brood (Lhomme and Hines 2019). Cuckoo bumblebees are classified into their own subgenus, *Psithyrus*, which is sister to the non-parasitic subgenus *Thoracobombus*. Previous studies have suggested that elevated substitution and rearrangement rates in mtDNA may be associated with parasitic life histories in Hymenoptera, particularly in wasps (Xiao *et al.* 2011, Zhu *et al.* 2018). Furthermore, such significant shifts in life history often result in changes in the selection forces that act on both nuclear and mitochondrial genomes, primarily due to reduced effective population sizes (N_e) of these species. For instance, positive selection has been observed in the mtDNA of parasitoid wasps (Oliveira *et al.* 2008), while socially parasitic ants show consistent signs of relaxing purifying selection in their nuclear genomes (Schrader *et al.* 2021). Given the contrasting life histories of *Psithyrus* and *Thoracobombus*, these sister-subgenera provide a system to investigate if obligate social parasitism also shaped the selection regime of bumblebee mitogenomes.

In this study, we conducted a comprehensive analysis of bumblebee mtDNA evolution using a dataset of 60 mitogenomes, including 40 newly assembled sequences. We evaluated the structure and composition of the mitogenomes and assessed their informativeness under a phylogenomic framework. Furthermore, we explored shifts in selection constraints in *Bombus* mitogenomes, focusing on the obligate social parasites of subgenus *Psithyrus*. Our study aimed to test the following hypotheses: (i) bumblebee mitogenomes resolve previous uncertainties while largely aligning with prior phylogenetic hypotheses, such as the monophyly of LF and SF groups; (ii) mitochondrial gene order and content are highly conserved, and gene rearrangement events are rare; (iii) unique gene rearrangements can serve as synapomorphies across bumblebee subgenera; (iv) faster-evolving mitogenomes exhibit higher rates of gene rearrangement; (v) the mitochondrial genes of *Psithyrus* exhibit positive or relaxed purifying selection. Our results provide valuable insights into bumblebee phylogenetics, evolution, and the diversification of genome traits, highlighting the utility of mitogenomes as a valuable resource in these research areas.

MATERIALS AND METHODS

Data retrieval, assembly, and annotation

We retrieved 40 publicly available Illumina paired-end datasets from the NCBI Sequence Read Archive (SRA) database, covering 39 *Bombus* species (Supporting Information, Table S1). Most of these libraries were originally prepared for whole-genome sequencing or to obtain ultra-conserved element loci (Supporting Information, Table S1). Datasets were converted to FASTQ using fastq-dump of the SRA TOOLKIT v.2.11.0 (https://trace.ncbi.nlm.nih.gov/Traces/sra/). Read quality was assessed using FastQC v.0.11.9 (Andrews 2010), and sequence adapters were trimmed with TRIMMOMATIC 0.39 (Bolger *et al.* 2014) with default parameters for Illumina data processing. The resulting reads were utilized as input for MitoFinder v.1.4 (Allio *et al.* 2020), a specialized pipeline for mitochondrial genome assembly and annotation. We employed the RefSeq mitogenomes of *B. waltoni* (NC_045283), *B. hypocrita sapporensis* (NC_011923), *B. terrestris lusitanicus* (NC_045178), and *B. terrestris terrestris* (NC_045179) as reference sequences. To ensure the quality of the assemblies and downstream analyses, we manually inspected each generated contig for all species and retained only the longest contig that contained all PCGs and rRNA genes. We also double-checked for evidence of pseudogenization or nuclear mitochondrial DNA (numt) contamination, such as premature stop codons. Annotations provided by MitoFinder were cross-checked with ARWEN (Laslett and Canbäck 2008) and MITOS2 WebServer (Bernt *et al.* 2013b).

Comparative analyses

The base composition of the mitogenomes and the pairwise *p*-distances for each gene were assessed using the base.freq() and dist.dna() functions of R package ape v.5.6-2 (Paradis and Schliep 2019), respectively. Strand asymmetry was calculated using the formulas AT skew = (A – T)/(A + T) and GC skew = (G – C)/(G + C) (Perna and Kocher 1995). Sequence divergence heterogeneity was assessed with AliGROOVE v.1.08 (Kück *et al.* 2014) with the default sliding window size.

Phylogenetic inference

Phylogenetic analyses were based on the 40 assembled mitogenomes and 20 bumblebee mitogenomes available on GenBank (Table 1), covering all 15 recognized *Bombus* subgenera (Williams *et al.* 2008). We used as outgroups the mitogenomes of six honey bee species (*Apis*) and three stingless bee species of the genus *Melipona* (Table 1). The GenBank mitogenomes were re-annotated following the procedures described above. The 13 mitochondrial PCGs and the two rRNA genes were extracted and processed separately. Stop codons were removed from the PCGs before subsequent analyses. The PCG sequences were aligned using the codon-aware program MACSE v.2.03 (Ranwez *et al.* 2018), which preserves the reading frame and prohibits indels within codons. The rRNA gene sequences were aligned using MAFFT v.7 (Katoh *et al.* 2019) with the Q-INS-i iterative refinement algorithm, which accounts for secondary RNA structure. The resulting alignments were concatenated, and ambiguously aligned fragments were removed with GBlocks v.0.91b (Talavera and Castresana 2007) using the default settings.

Three data matrices were prepared for phylogenetic analyses: PCG12RNA (first and second codon positions of the PCGs and the two rRNA genes), PCG123RNA (all codon positions combined), and a dataset of translated amino acids (AA). PartitionFinder v.2.1.1 (Lanfear *et al.* 2016) was employed to determine the optimal model for the partitioned alignments using a greedy search algorithm and Bayesian information criterion (BIC). Phylogenetic analyses were performed using the partitioned alignments and two different algorithms: Bayesian inference (BI) and maximum likelihood (ML). We performed the BI in MrBayes v.3.2.7a (Ronquist *et al.* 2012) through the CIPRES Science Gateway (Miller *et al.* 2010) with two simultaneous runs of 50 million generations, sampling trees every 5000 generations and a burn-in fraction of 0.25. We confirmed the convergence of BI runs using TRACER v.1.7.1 (Rambaut *et al.* 2018). ML trees were constructed using RAxML v.8.2.12 (Stamatakis 2014) through an ML + rapid bootstrap (BS) algorithm with 1000 replicates. Due to computational limitations, the AA dataset was exclusively analysed using the ML approach. We used the PCG123 + RNA BI tree for subsequent analyses because of the higher support values and similarity with the nuclear genome-wide phylogeny (Sun *et al.* 2021).

Gene order analysis

To investigate gene rearrangements, we registered the order of PCGs, tRNA, and rRNA genes and mapped the rearrangements on to the obtained PCG123 + RNA BI tree to visualize shared gene orders among species. Additionally, we employed qMGR (Zhang *et al.* 2020) to calculate the rearrangement score for each mitogenome, with the ancestral pancrustacean gene order serving as a reference (Lavrov *et al.* 2004). In brief, qMGR quantifies the extent of rearrangement in mitogenomes by measuring accumulated neighbour changes for each rearranged gene (Zhang *et al.* 2020). To examine whether species with fast-evolving mitogenomes exhibit higher rates of gene rearrangement, we compared the rearrangement scores of each

species with their respective root-to-tip distances. We extracted the root-to-tip distances for each species using Newick Utilities (Junier and Zdobnov 2010), serving as proxies for mitogenome evolutionary rates (Bernt *et al.* 2013a). Root-to-tip distances and rearrangement scores were standardized for comparative purposes using the scale() function available in base R (R Core Team 2021).

Selection tests

Because of the unique social parasitism of cuckoo bumblebees (subgenus *Psithyrus*), we tested whether a proportion of sites (i.e. codons) in *Psithyrus* (test branch) underwent positive selection compared with non-parasitic lineages (background branches) using the branch-site model in CODEML (Yang and Nielsen 2002, Zhang *et al.* 2005), implemented in PAML (Yang 2007). This method assesses the selective forces in the dataset through the ω value, the ratio of nonsynonymous (dN) to synonymous (dS) substitution rates, assuming that ω varies among sites in the alignments throughout the test branches of a phylogeny. We compared the alternative model (model A, which allows a subset of sites to have $\omega > 1$ in the test branch) with the null model (which applies a restriction to $\omega \leq 1$ to detect positively selected sites). Statistical significance between models was assessed using a likelihood ratio test (LRT). When the LRT was significant, we used the Bayes empirical Bayes (BEB) approach to calculate the posterior probability (PP) that individual codon sites are putatively under positive selection. We corrected significant *P*-values using a false discovery rate analysis (FDR) (Benjamini and Yekutieli 2001) implemented in base R as the function p.adjust() (R Core Team 2021), and q-values represent corrected *P*-values. We also independently ran these positive selection tests setting the non-parasitic *Thoracobombus* as the foreground to assess the extent of selection in other bumblebee lineages.

To distinguish between positive selection and relaxed purifying selection, we utilized the RELAX branch method (Wertheim *et al.* 2015) implemented in HyPhy (Pond *et al.* 2005) through the Datamonkey Adaptive Evolution Server (https://www.datamonkey.org/). RELAX compares the ω values between the background phylogeny and the lineages of interest, testing for relaxed or intensified selection using the selection intensity parameter k, where $k > 1$ indicates intensified/positive selection and $k < 1$ indicates relatively relaxed selection constraints in the test branches (Wertheim *et al.* 2015). Then, RELAX conducts an LRT to compare the alternative and null models. To confirm that selection relaxation is restricted to *Psithyrus*, we again independently assessed shifts in selection constraints setting *Thoracobombus* as a test branch in RELAX.

RESULTS

General features of novel bumblebee mitogenomes

A total of 40 bumblebee mitogenomes were assembled and annotated, representing 13 subgenera of *Bombus* (Table 1). All newly assembled mitogenomes were obtained as single contigs, with sizes ranging from 15 712 bp in *B. laesus* to 24 179 bp in *B. skorikovi*, and an average size of 18 200 bp (Table 1). These mitogenomes contained the standard set of 13 protein-coding genes (PCGs), two rRNA genes, and a control region (Fig. 1). However, eight mitogenomes lacked up to three tRNAs in cluster 1, probably due to challenges in assembling the repetitive regions flanking the control region.

Consistent with previously reported bumblebee mitogenomes, the novel sequences exhibited an AT-biased composition, with AT content ranging from 79.83% (*B. convexus*) to 89.07% (*B. pratorum*). AT-skew values were predominantly positive, indicating an excess of A over T, although 12 out of the 40 newly assembled mitogenomes showed negative AT values (Supporting Information, Table S2). GC-skew values were uniformly negative, reflecting the typical pattern observed in insect mitogenomes (Wei *et al.* 2010) (Table S2). Among the PCGs, *ATP8*, *NAD2*, and *NAD6* exhibited the highest levels of divergence, while *COI*, *COII*, and *Cytb* showed the highest conservation across the mitogenomes (Supporting Information, Fig. S1). AliGROOVE results indicated an overall absence of significant compositional heterogeneity within our dataset (Supporting Information, Fig. S2).

Phylogenetic tree

Bombus was recovered as a monophyletic group with robust support in all analyses (PP = 1.0; BS = 100; Fig. 2; Supporting Information, Figs S3–S8). Similarly, all subgenera of *Bombus* were resolved as monophyletic with strong support (PP ≥ 0.9; BS ≥ 80), except for *Cullumanobombus* in the ML trees (Supporting Information, Figs S4–S6) and the PCG12 + RNA BI tree (Supporting Information, Fig. S8). Subgenera were mainly subdivided into the two large clades, LF and SF, across all trees, confirming our initial hypothesis and the results of previous studies (Fig. 2; Supporting Information, Figs S3–S8). *Kallobombus* was consistently placed as the sister-group of the SF + LF clades (Fig. 2; Supporting Information, Figs S3–S8). Within the SF clade, the 'montane grassland' (MG) subgroup (*sensu* Williams *et al.* 2022), which includes *Alpigenobombus*, *Melanobombus*, *Sibiricobombus*, and *Cullumanobombus*, was identified as monophyletic. Likewise, the 'lowland grassland' (LG) subgroup (*sensu* Williams *et al.* 2022), comprising species from subgenus *Thoracobombus*, was also recovered as monophyletic (Fig. 2). Shallow-level relationships (i.e. among congeneric species) were consistently well-supported in all phylogenetic trees, regardless of the data matrix and reconstruction methodology used (Fig. 2; Supporting Information, Figs S3–S8).

The main discrepancies observed in the phylogenetic trees were restricted to poorly supported nodes (Fig. 3). Most trees exhibited an unresolved deeper node, placing *Mendacibombus* and *Bombias* as a polytomy sister to all bumblebee subgenera (Supporting Information, Figs S3–S8), except for the PCG123 + RNA BI tree, which supported *Mendacibombus* as the sister-group to all bumblebee subgenera (Fig. 2). In addition, the recovered trees displayed conflicting and unresolved relationships among LF subgenera, except for the placement of *Psithyrus* and *Thoracobombus* as sister-groups (Supporting Information, Figs S3–S8). Concerning the SF clade, ML trees showed unresolved subgeneric relationships (Supporting Information, Figs S3–S6), while the two BI trees recovered conflicting topologies. The PCG123 + RNA BI tree placed *Melanobombus* external to a trichotomy formed by *Sibiricobombus*, *Cullumanobombus*, and *Alpigenobombus* (Fig. 2; Supporting Information, Figs S3, S7).

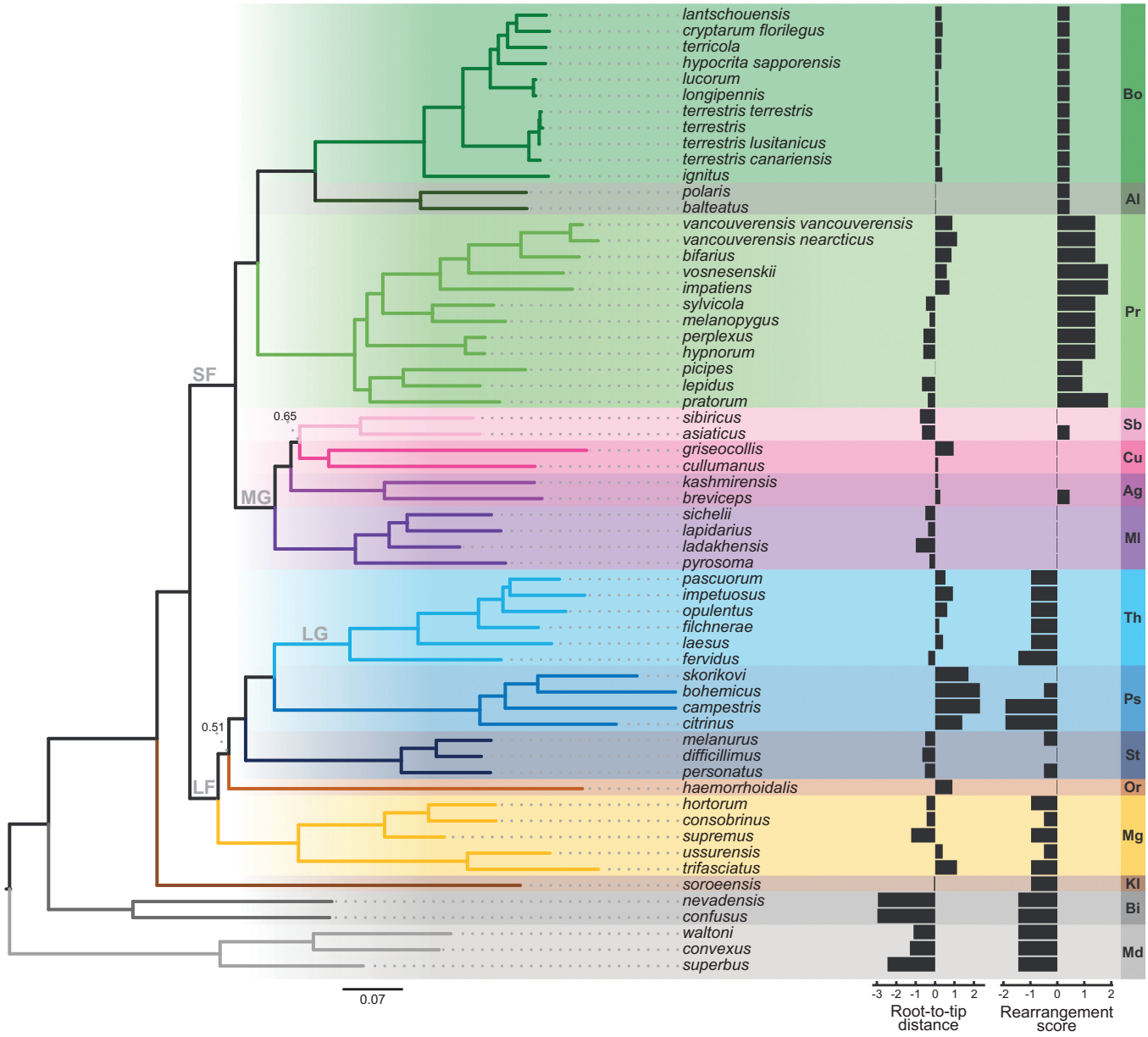

Figure 2. Phylogenetic tree of *Bombus* built based on the PCG123 + RNA dataset using Bayesian analysis. Only low node support values (PP < 0.90) are shown. The scale bar indicates estimated substitutions per site. Groups of bumblebee subgenera are labelled: LF, the 'long-faced' group; SF, the 'short-faced' group; MG, the 'montane grassland' group; LG, the 'lowland grassland' group (*sensu* Williams *et al.* 2022). Outgroups were pruned from the phylogenetic tree to improve visualization. The standardized root-to-tip distance and rearrangement score for each species are shown on the right, and the X axis represents the number of standard deviations from the mean. The subgenus that each bumblebee species belongs to is colour-coded: Bo, *Bombus*; Al, *Alpinobombus*; Pr, *Pyrobombus*; Sb, *Sibiricobombus*; Cu, *Cullumanobombus*; Ag, *Alpigenobombus*; Ml, *Melanobombus*; Th, *Thoracobombus*; Ps, *Psithyrus*; St, *Subterraneobombus*; Or, *Orientalibombus*; Mg, *Megabombus*; Kl, *Kallobombus*; Bi, *Bombias*; Md, *Mendacibombus*.

However, the PCG12 + RNA BI tree fully resolved subgeneric relationships, positioning *Melanobombus* as external to the other three subgenera and revealing a paraphyletic *Cullumanobombus* (Supporting Information, Figs S3, S8).

Gene order and rearrangements

When comparing the structure of mitogenomes, we found that PCG and rRNA gene order and orientation are conserved among bumblebee species, matching the proposed ancestral pancrustacean gene order (Boore 1999). However, we identified several tRNA gene rearrangements, including translocations, inversions, adjacent shuffling, and tandem duplications. Interestingly, the extent of rearrangement was not correlated with branch lengths (Fig. 2), indicating that species with rapidly evolving mitogenomes do not necessarily exhibit higher rearrangement rates (Supporting Information, Table S3). Species from the clade comprising subgenera *Pyrobombus*, *Alpinobombus*, and *Bombus s.s.* displayed the highest rearrangement scores, while *Psithyrus* exhibited the longest branch lengths (Fig. 2).

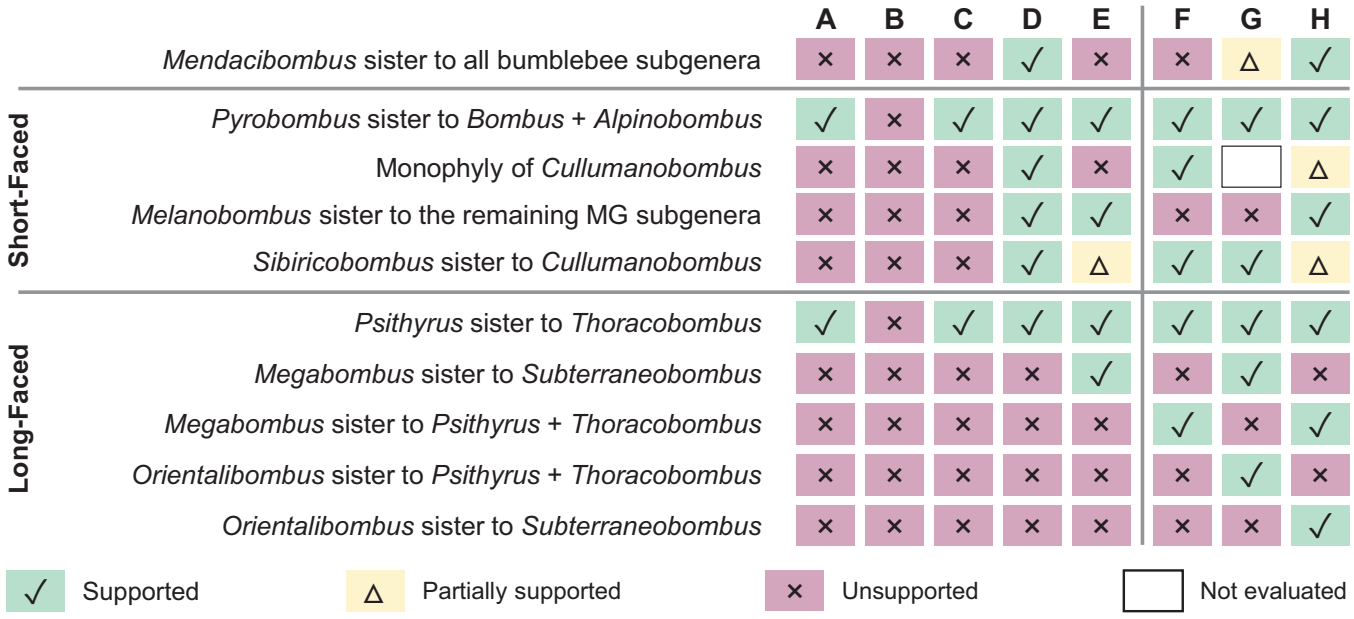

Figure 3. Major points of tree conflict in phylogenetic relationships of *Bombus* subgenera. Rows correspond to phylogenetic hypotheses, and columns correspond to the results from different datasets and methods (A–E, this study; F–H, other studies). A, maximum likelihood, PCG123 + RNA dataset; B, maximum likelihood, PCG12 + RNA dataset; C, maximum likelihood, AA dataset; D, Bayesian inference, PCG123 + RNA dataset; E, Bayesian inference, PCG12 + RNA dataset; F, topology from Cameron *et al.* (2007) using one mitochondrial (mt) and four nuclear (nu) genes; G, topology from Sun *et al.* (2021) using 2918 nu genes; H, topology from Santos Júnior *et al.* (2022) adding two mt genes to the matrix of Cameron *et al.* (2007). Nodes with PP ≥ 0.90 or BS ≥ 80 were considered supported, and partially supported nodes mainly refer to the paraphyly of *Cullumanobombus* in some of the recovered trees.

We classified the rearrangements into major and minor events based on the magnitude of changes and the number of tRNA genes affected. Major rearrangements involved significant changes in the order of tRNA genes (as in tRNA clusters 1 and 6; Fig. 1), whereas minor rearrangements entailed relatively small or punctual changes (as in tRNA clusters 2, 8, 11, and 12; Fig. 1).

In cluster 1, we found four distinct tRNA gene orders: (i) $tRNA^{Ala}$-$tRNA^{Ile}$-$tRNA^{Met}$, (ii) $tRNA^{Ala}$-$tRNA^{Met}$-$tRNA^{Ile}$, (iii) $tRNA^{Met}$-$tRNA^{Ile}$-$tRNA^{Ala}$, and (iv) $tRNA^{Met}$-$tRNA^{Ala}$-$tRNA^{Ile}$. These tRNA genes were typically located on the light strand, but we also detected inversion events for all three tRNAs (Supporting Information, Table S4). Rearrangement events within cluster 1 were homoplasic, occurring independently multiple times during the evolutionary history of bumblebees. Additionally, the copy number of $tRNA^{Met}$ varied among species, ranging from one (most species) to four (*Bombus haemorrhoidalis* and *Bombus skorikovi*) (Supporting Information, Table S4). Notably, the clade comprising *Alpinobombus* and *Bombus s.s.* shared a $tRNA^{Met}$ duplication event (Fig. 4), with the additional copy being lost in *Bombus hypocrita sapporensis* and *Bombus ignitus* (Supporting Information, Table S4). Furthermore, the available mitogenome sequences of *Bombus s.s.* in GenBank lack the annotation of the duplicated $tRNA^{Met}$.

Within cluster 6, we found three different tRNA orders: (i) $tRNA^{Arg}$-$tRNA^{Asn}$-$tRNA^{Glu}$-$tRNA^{Ser1}$-$tRNA^{Phe}$ in *Mendacibombus*, *Bombias*, and the SF clade; (ii) $tRNA^{Asn}$-$tRNA^{Arg}$-$tRNA^{Glu}$-$tRNA^{Ser1}$-$tRNA^{Phe}$ in *Kallobombus* and the LF clade (excluding *Psithyrus*); and (iii) $tRNA^{Glu}$-$tRNA^{Arg}$-$tRNA^{Asn}$-$tRNA^{Ser1}$-$tRNA^{Phe}$ exclusive to *Psithyrus* (Fig. 4). We also detected unique arrangements in *Bombus bohemicus* ($tRNA^{Glu}$-$tRNA^{Asn}$-$tRNA^{Arg}$-$tRNA^{Ser1}$-$tRNA^{Phe}$) and *Bombus fervidus* ($tRNA^{Arg}$-$tRNA^{Ser1}$-$tRNA^{Asn}$-$tRNA^{Glu}$-$tRNA^{Phe}$). In *Bombus consobrinus*, $tRNA^{Arg}$ translocated from cluster 6 to cluster 12 (Supporting Information, Table S4).

In Cluster 2, tRNA order was largely conserved across species: $tRNA^{Cys}$-$tRNA^{Tyr}$-$tRNA^{Trp}$. However, we observed a shuffling event between $tRNA^{Tyr}$ and $tRNA^{Cys}$ in *Bombus consobrinus* and *B. skorikovi*, and duplication of $tRNA^{Tyr}$ in *Bombus picipes*. Within cluster 8, the ancestral condition of bumblebee mitogenomes was $tRNA^{Thr}$-$tRNA^{Pro}$, and the shuffling between these tRNA genes was synapomorphic for the SF clade (Fig. 4). Furthermore, both subspecies of *Bombus vancouverensis* exhibited a duplication of $tRNA^{Pro}$. Lastly, $tRNA^{Val}$ translocated from cluster 11 to cluster 12 in all *Pyrobombus* species (Fig. 4).

Selection tests in *Psithyrus* and *Thoracobombus*

We employed PAML and HyPhy to investigate signs of positive or relaxed purifying selection in mitochondrial PCGs of cuckoo bumblebees. By conducting a branch-site test in PAML, we identified a significant signature of positive selection in *NAD2* and *NAD6* of *Psithyrus* (q = 0.004), with several individual sites being detected under positive selection (Supporting Information, Table S5). Utilizing RELAX, we detected evidence of selection relaxation in *COI*, *COII*, *Cytb*, *NAD5*, and *NAD6* of *Psithyrus* (Supporting Information, Table S5).

When examining signs of selection in *Thoracobombus*, the non-parasitic sister-subgenus of *Psithyrus*, we observed

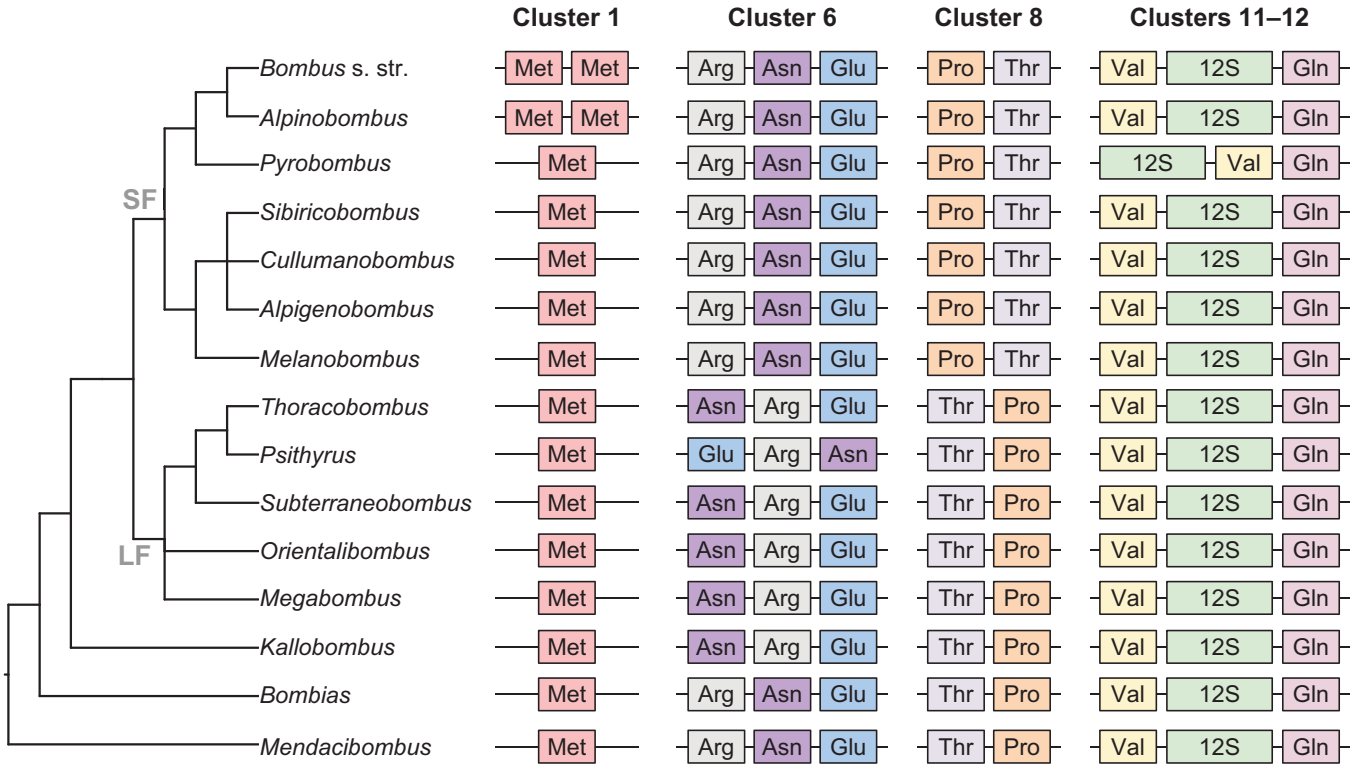

Figure 4. Putative structural synapomorphies of bumblebee mitogenomes plotted against the PCG123 + RNA BI tree. Groups of bumblebee subgenera are labelled: LF, the 'long-faced' group; SF, the 'short-faced' group. Transfer RNA (tRNA) genes are represented by the three-letter IUPAC-IUB abbreviation for their corresponding amino acid. In cluster 1, we emphasize the marked duplication event of $tRNA^{Met}$ shared by *Bombus s.s.* and *Alpinobombus*; however, this cluster comprises other tRNA genes that rearranged multiple times (see Results). In clusters 11–12, the 12S ribosomal RNA gene is also depicted.

positive selection in *NAD2* (q = 0.004) using PAML (Supporting Information, Table S5). In contrast, RELAX revealed no significant changes in the selection constraints acting upon *Thoracobombus* mitogenomes, except for an intensified purifying selection in *COIII* (P = .001) (Supporting Information, Table S5). These findings demonstrate that positive selection in *NAD2* is shared between the two subgenera, while the widespread relaxation of selection in mitochondrial genes is a characteristic feature of cuckoo bumblebees.

DISCUSSION

Phylogenetic relationships

We utilized mitochondrial phylogenomics to infer the evolutionary relationships of bumblebees, a diverse and ecologically important group of bees. Our analyses yielded well-supported phylogenetic trees that confirmed the monophyly of *Bombus* and its subgenera, supporting the currently accepted taxonomic classification (Williams *et al.* 2008). The resulting trees corroborated the presence of two major clades, the LF and SF clades, as documented in previous studies (Cameron *et al.* 2007), and the monophyly of the MG clade (Williams *et al.* 2022). Despite the limited taxon sampling for evaluating shallow nodes, our dataset provided robust support for these relationships. However, our findings revealed some discrepancies with previous studies using nuclear genes, highlighting instances of mitonuclear discordance in the phylogenetic placement of certain subgenera.

Overall, our results were consistent with the most recent phylogeny of *Bombus* subgenera inferred from 2918 nuclear loci (Sun *et al.* 2021). However, we observed two major topological differences. First, in the nuclear data phylogeny recovered by Sun *et al.* (2021), *Megabombus* and *Subterraneobombus* were positioned as sister-subgenera within the LF clade (Supporting Information, Fig. S3). In our mtDNA-based analyses, we only recovered this relationship in the PCG12 + RNA BI tree (Supporting Information, Fig. S8), while the remaining trees exhibited unresolved topologies or placed *Subterraneobombus* as sister to *Thoracobombus* + *Psithyrus* (Fig. 3; Supporting Information, Figs S4–S7). Second, Sun *et al.* (2021) identified discordant relationships within the MG clade, particularly concerning the position of *Melanobombus*. Their ASTRAL tree mirrored our findings, placing *Melanobombus* as an external group to the remaining MG subgenera (Supporting Information, Fig. S2). However, when a concatenated matrix was employed for phylogenetic reconstruction, Sun *et al.* (2021) recovered a different relationship, with (*Cullumanobombus*, *Sibiricobombus*) as sister to (*Melanobombus*, *Alpigenobombus*). None of our phylogenetic trees supported this relationship (Fig. 3; Supporting Information, Fig. S3).

Furthermore, our phylogenetic trees can be compared to the topologies obtained by Cameron *et al.* (2007), utilizing one mitochondrial and four nuclear genes, and Santos Júnior *et al.* (2022), which expanded upon the matrix of Cameron *et al.* (2007) by including two additional mitochondrial genes

(Fig. 3; Supporting Information, Fig. S3). Our phylogenetic trees exhibited slight differences compared to theirs, primarily regarding the position of *Subterraneobombus*, *Megabombus*, and *Orientalibombus*. While Cameron *et al.* (2007) and Santos Júnior *et al.* (2022) recovered *Megabombus* as the external group to *Psithyrus* and *Thoracobombus*, we found *Subterraneobombus* occupying that position in the PCG 123 + RNA BI tree (Fig. 3; Supporting Information, Fig. S7). Moreover, the placement of *Orientalibombus* and *Megabombus* within the LF clade was mostly inconclusive in our phylogenies (Fig. 3), except for the PCG12 + RNA BI tree, which showed *Subterraneobombus* and *Megabombus* as sister-subgenera (Fig. 3; Supporting Information, Fig. S8). In contrast, Santos Júnior *et al.* (2022) reported *Subterraneobombus* and *Orientalibombus* as sister-subgenera (Fig. 3; Supporting Information, Fig. S3). Interestingly, these topologies conflict with the findings of Sun *et al.* (2021) based on nuclear loci (Fig. 3; Supporting Information, Fig. S3).

In our dataset, we included two species belonging to the subgenus *Cullumanobombus*, which were recovered in all trees as part of the MG clade. However, the precise phylogenetic placement of *Cullumanobombus* within this clade varied. We identified three distinct scenarios for *Cullumanobombus*, influenced by the data matrix and methodology employed: well-supported monophyly (Supporting Information, Fig. S7), paraphyly due to low statistical support (Supporting Information, Figs S4–S6), or paraphyly with high support (Supporting Information, Fig. S8). In the latter, *B. griseocollis* was recovered external to *Sibiricobombus*, and *B. cullumanus* was placed external to *Alpigenobombus* (Supporting Information, Fig. S8). Notably, Santos Júnior *et al.* (2022) also encountered instances of paraphyletic *Cullumanobombus* in their analyses due to low statistical support, while Sun *et al.* (2021) did not assess the monophyly of this subgenus. Considering our restricted sampling and the relatively weak signal provided by mtDNA for understanding the phylogenetic relationships of the MG clade, a comprehensive investigation using an expanded dataset is necessary to test the monophyly of *Cullumanobombus*.

The taxonomy and systematics of *Bombus* have long puzzled biologists (Moure and Sakagami 1962), and our findings highlight the challenges in inferring the phylogenetic relationships of this group. The mitonuclear discordance and the sensibility to the chosen method emphasize the complexity of unravelling evolutionary relationships in *Bombus*, underscoring the importance of considering multiple data sources in bumblebee phylogenetics. Our results demonstrate that mitogenomes contribute to resolving shallower phylogenetic relationships within *Bombus* but have limited power in disentangling the deeper nodes of the bumblebee tree of life.

Gene rearrangements

We investigated the gene rearrangements in bumblebee mitogenomes and found that PCG and rRNA gene order remained conserved, while rearrangements in tRNA genes were frequent. Interestingly, we observed recurrent rearrangements in tRNA clusters 1 and 6, which aligns with previous studies conducted on other hymenopteran species (Dowton and Austin 1999, Oliveira *et al.* 2008, Mao *et al.* 2015, Françoso *et al.* 2020). Furthermore, unique rearrangements were observed in certain subgenera or subgeneric groups (Fig. 4), offering valuable insights into the evolutionary history of bumblebees and providing additional characters for subgeneric taxonomy.

Insect mitogenome rearrangements are commonly explained by the tandem duplication and random loss (TDRL) model, involving the duplication of a contiguous gene set followed by the random loss of one copy of each duplicated gene (Boore 2000). Here we identified mitogenomes with tandem duplicated tRNA genes, which could indicate ongoing TDRL events. However, some rearrangements are inconsistent with the TDRL model, such as the long-range translocations or inversion of tRNA genes. It has been proposed that intramitochondrial recombination may drive this type of rearrangement in invertebrate mitogenomes, notably in hymenopterans (Dowton and Austin 1999, Mao *et al.* 2014, 2015). Furthermore, rearrangements by slipped-strand mispairing are prone to occur in the replication origin regions (Levinson and Gutman 1987, Macey *et al.* 1998), which explains the higher frequency of rearrangements in clusters 1 and 6 that coincide with the origin of replication of the heavy and light mtDNA strands, respectively (Brown *et al.* 2005, Duarte *et al.* 2008). These tRNA clusters have been identified as regions of high rearrangement frequency in other bees, such as the tribe Meliponini (Silvestre *et al.* 2002, 2008, Wang *et al.* 2021) and genus *Tetrapedia* (Françoso *et al.* 2020). Since mitogenomic rearrangements within the same genus are generally uncommon in insects (Cameron 2014), the multiple and complex events of tRNA gene rearrangements we detected suggest a certain degree of structural plasticity in bumblebee mitogenomes.

Although tRNA gene rearrangements were common, certain positions remained conserved across all species, particularly in clusters 3–5 and 7–10. One notable exception was a single shuffling event between $tRNA^{Thr}$ and $tRNA^{Pro}$ in cluster 8, which was synapomorphic among SF bumblebees (Fig. 4). The conserved position of $tRNA^{Pro}$ in bee mitogenomes is attributed to the change in transcriptional polarity at these sites and the role these genes may play in mRNA maturation (Dowton *et al.* 2009, Françoso *et al.* 2020). This rare and conserved shuffling event might reflect the functional significance and constraints associated with the specific position of these tRNA genes, aligning with previous hypotheses (Dowton *et al.* 2003).

We did not find a direct association between the degree of rearrangement and evolutionary rates (Fig. 2), contrary to previous studies that have suggested such a relationship (Shao *et al.* 2003, Hassanin 2006, Xu *et al.* 2006, Bernt *et al.* 2013a, Zou *et al.* 2022). Although the high degree of rearrangements in bumblebees can be attributed to the inherent high substitution rates in hymenopteran mitogenomes, it remains uncertain if this relationship holds at lower taxonomic levels. For instance, our phylogeny exhibited accelerated substitution rates in cuckoo bumblebees (*Psithyrus*), as often observed in parasitic taxa (as discussed below). However, the most rearranged mitogenomes belonged to subgenus *Pyrobombus*, specifically *B. impatiens*, *B. pratorum*, and *B. vosnesenskii*. Further investigations are necessary to determine whether the high degree of rearrangement in these species is adaptive.

Besides their phylogenetic utility, mtDNA rearrangements hold crucial implications for speciation. Their capacity to disrupt

gene flow between populations may facilitate the formation of new species (Burton and Barreto 2012, Hill *et al.* 2019). This disruption stems from the coordinated functioning of mitochondrial and nuclear genes, essential for electron transport chain complexes and cellular respiration. Any disturbance to this coadaptation may create barriers to gene flow once coadapted genotypes from one population become incompatible with those of another, further contributing to speciation (Burton and Barreto 2012, Hill 2016). After speciation, these rearrangements may persist within the mtDNA of the resulting lineages, effectively acting as molecular fossils of their evolutionary history (Richardson *et al.* 2013). This explains why many of the rearrangements we observed are shared among related species, tracing back to their common ancestors. Supporting the findings for other taxa (Tan *et al.* 2019), it is likely that the causes of mitogenome rearrangements in bumblebees are multifactorial and lineage-specific, warranting additional research to unravel the underlying mechanisms.

Relaxed selection constraints in *Psithyrus*

Our findings revealed evidence of relaxed purifying selection in several mitochondrial genes of *Psithyrus* bumblebees. Purifying selection typically removes deleterious mutations and is essential for mitochondrial PCGs due to their fundamental role in ATP production (Stewart *et al.* 2008, Palozzi *et al.* 2018). The relaxed purifying selection suggests that these genes in *Psithyrus* bumblebees undergo more changes or variations than expected. This pattern could be attributed to reduced N_e and decreased functional constraints on these genes in social parasites.

Subgenus *Psithyrus* exhibited longer branch lengths in our phylogenetic trees, illustrating the accelerated substitution rates within this clade. Parasitic lineages often show accelerated mtDNA evolution compared to their non-parasitic counterparts, possibly due to reduced N_e (Castro *et al.* 2002, Jakovlić *et al.* 2021 , Oliveira *et al.* 2008). The nearly neutral theory predicts that a small N_e leads to the accumulation of slightly deleterious mutations through genetic drift, relaxing purifying selection (Ohta 1972). Since cuckoo bumblebees occupy a higher level in food webs and are quite rare in nature, their N_e is expected to be lower than their host species (Suhonen *et al.* 2015, Lhomme and Hines 2019). Thus, the reduced N_e may contribute to the relaxation of purifying selection on mitochondrial genes in *Psithyrus* bumblebees.

The relaxed purifying selection observed in these genes may also stem from decreased functional constraints. Social parasitism has independently evolved multiple times in Hymenoptera (Michener 2007) and is often associated with degenerative processes characterized by the loss of behavioural, physiological, and morphological traits (Schrader *et al.* 2021). Cuckoo bumblebees lack the pollen-collecting apparatus on their hind legs, cannot produce a worker caste, and have limited wax production for nest-building (Lhomme and Hines 2019). These losses extend to the molecular level, as *Psithyrus* bumblebees lost 11 odorant receptor genes (Sun *et al.* 2021). As obligate parasites with limited dispersal capabilities and complete reliance on their host workers for thermoregulation and foraging, cuckoo bumblebees probably experience distinct selective pressures on their mitochondrial genes compared to non-parasitic bumblebees, owing to their reduced metabolic needs. Furthermore, the selective constraints on mitochondrial genes directly impact insect mobility (Mitterboeck *et al.* 2017, Chang *et al.* 2020), and a relaxation of purifying selection may explain the slower and less energetic flight observed in cuckoo bumblebees compared to their non-parasitic counterparts (Lhomme and Hines 2019, Fisogni *et al.* 2021). Since obligate social parasitism has also arisen in other non-*Psithyrus* bumblebee species—in subgenus *Alpinobombus* with *Bombus natvigi* and *Bombus hyperboreus*, and in subgenus *Thoracobombus* with *Bombus inexpectatus* (Lhomme and Hines 2019)—an intriguing avenue for future research would be to investigate whether the relaxed purifying selection is a convergent phenomenon in the mitogenomes of these species or if it is unique to subgenus *Psithyrus*.

CONCLUSIONS

Here we provide new insights into the mtDNA evolution of bumblebees. Our findings support the monophyly of *Bombus* and its subgenera, while revealing discrepancies with nuclear DNA topologies at certain deep nodes. Moreover, we demonstrate the prevalence of mitochondrial tRNA rearrangements, which hold potential as informative markers for subgeneric classification. We found no association between rearrangement and evolutionary rates, challenging the prevailing notion that faster-evolving mitogenomes exhibit higher gene rearrangement rates. These results highlight the need for a more nuanced understanding of the factors influencing mitogenome evolution in hymenopterans. Finally, the observed relaxed selection constraints on mitochondrial genes in *Psithyrus* bumblebees provide valuable insights into the mitochondrial biology and evolutionary history of these parasitic species. Our study highlights the potential of comparative mitogenomics in uncovering previously unexplored aspects of bumblebee evolution and paves the way for exciting avenues of future research in this field.

SUPPLEMENTARY DATA

Supplementary data are available at *Zoological Journal of the Linnean Society* online.

ACKNOWLEDGEMENTS

We thank Pedro Henrique Pezzi for his valuable feedback on this manuscript. This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY

Resulting alignments, phylogenies, and scripts are available as Supporting Information. The newly assembled mitogenomes are available on GenBank under the accession numbers listed in Table 1.

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