

# Karyotype evolution and speciation in Orthoptera

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## Abstract

Karyotype evolution might fuel speciation and can thereby contribute to species diversity. To test the hypothesis that speciation and karyotype change are linked, we estimated anagenetic and cladogenetic rates of karyotype evolution as well as speciation rates in Orthoptera. We compiled the male diploid chromosome number and the number of visible chromosome arms (the fundamental number) from published sources for 1,541 species. Chromosome-associated speciation rates were estimated by jointly modelling cladogenetic and anagenetic character evolution and the phylogenetic birth–death process in a Bayesian statistical framework using a subset of 516 species from 14 families. Our findings unveiled heterogeneity among orthopteran families in the pace of karyotype evolution and whether it was linked to speciation. In 6/14 clades, we found evidence supporting speciation-associated (cladogenetic) karyotype changes, while in 6/14 clades karyotype evolution was primarily anagenetic. The remaining clades (2/14) showed uncertainty in favour of either model. We further analyzed whether flightless phenotype, and thus less mobile species, showed higher rates of karyotype evolution. We showed that the flightless phenotype is associated with the rate of chromosome loss. The finding indicates contrasting patterns of karyotype evolution within specific orthopteran lineages, thus emphasizing substantial diversity in the pace of this evolutionary process. It also implies that substantial changes in chromosome number, arising from instances of chromosomal gains and losses, are recurring events in orthopterans that are associated with reproductive isolation and speciation, at least in some groups.

**Keywords:** karyotype evolution, chromosomal rearrangements, speciation rates, Orthoptera

## Introduction

The karyotype represents the characteristics of an individual's complete set of chromosomes, including size and number of chromosomes and the distinct number of chromosome arms (the fundamental number, FN), and thus captures important aspects of genome organization. Karyotypes change by chromosomal rearrangements and such changes provide insights into the dynamic nature of the genome. However, these changes tend to unfold gradually due to the potential complications they introduce during meiosis, disadvantaging new variants at low population frequencies (Coyne & Orr, 1998; Hoffmann & Rieseberg, 2008; Sites & Moritz, 1987; White, 1978).

The link between karyotype evolution and speciation has sparked among evolutionary biologists (Coyne & Orr, 1998; Hoffmann & Rieseberg, 2008; Sites & Moritz, 1987; White, 1978). Early contributions highlighted the significance of small populations in facilitating rapid speciation (Coyne & Orr, 1998; Mayr, 1970; Wright, 1931, 1940), which might simultaneously lead to accelerated karyotype change (Coyne & Orr, 1998; Hoffmann & Rieseberg, 2008; White, 1978). Under this view, situations favouring speciation, such as population subdivision caused by founder effects, bottlenecks or populations fragmentation, also facilitate karyotype change (Coyne & Orr, 1998; Hoffmann & Rieseberg, 2008). As both

speciation and karyotype change find impetus in population subdivision, they tend to be linked. However, karyotype change may possess a more active role in speciation by rendering divergent karyotypes incompatible, potentially boosting higher speciation rates.

The two main types of karyotype change are centric fusions and centric fissions. Centric fusions entail the fusion of centromeres from acrocentric chromosomes (chromosomes with a single arm and centromeres located towards the periphery) creating a metacentric chromosome. A metacentric chromosome has two arms with the centromere located midway between the ends of the chromosome (King, 1995; White, 1973). Centric fusions reduce the number of chromosomes but do not change the FN. Conversely, centric fission divides a bi-armed chromosome into two acrocentric ones. This process increases the number of chromosomes while keeping the FN unchanged (King, 1995; White, 1973). Furthermore, pericentric inversions, that involve segmental inversions encompassing the centromere (King, 1995; White, 1973), can alter FN while preserving the diploid number.

A prevailing model of karyotype evolution emphasizes the effect of individual chromosomal rearrangements on the fertility of heterozygotes, either disrupting meiotic segregation or reducing the recombination rate in rearranged areas of the genome (King, 1995; White, 1978). Such polymorphisms

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in chromosomes may contribute to the long-term speciation process (King, 1995; White, 1978). However, the hypothesis of chromosomal speciation caused by impairment of meiotic segregation in hybrids (that is, underdominance of chromosomal polymorphisms) has remained controversial, since it is largely supported by indirect evidence. Uncertainty often arises in identifying whether karyotype divergence is a consequence of speciation-related processes rather than the cause (Coyne, 1984).

The interest in the association of karyotype change and speciation has therefore remained, leading some authors to propose models of speciation based on gene flow and recombination effects rather than the classic underdominance in hybrids (Rieseberg, 2001). While previous work has explored the role of karyotype change in speciation, the causes influencing karyotype change across larger clades have garnered less attention (de Vos et al., 2020; Husemann et al., 2022; Ross et al., 2015; Sylvester et al., 2020). Enhanced phylogenetic analysis tools now enable more precise comparative studies than in the past. Our study investigates karyotype evolution and speciation rates in Orthoptera, offering a unique and fresh contribution to this complex relationship.

The insect order Orthoptera (grasshoppers, katydids, crickets, and allies) contains more than 30,022 valid species (Cigliano et al., 2022). Orthoptera show substantial variation in the diploid number of chromosomes ( $2n$ ) ranging from  $2n = 7, X0$  in *Eunemobius* species (Portugal & Mesa, 2007) and *Gonatoxia helleri* (Warchałowska-Śliwa et al., 2020) to  $2n = 57, X0$  in *Diestramma tachycines asynamorus* (Mesa, 1965). Orthopteran sex chromosome systems are also variable. The most common system is the  $X0$ , in which males have one X chromosome ( $X0$ ) and females have two X chromosomes ( $XX$ ). Other systems such as neo- $XY/XX$ , neo- $X_1X_2Y/X_1X_1X_2X_2$  and even  $X_1X_20/X_1X_1X_2X_2$  can also be found (Castillo et al., 2010; Palacios-Gimenez & Cabral-de-Mello, 2015). Variation in chromosome number and sex chromosomes is mostly attributed to inversions, centric fusions and fissions involving autosomes and sex chromosomes that lead to changes in  $2n$  and/or FN (Castillo et al., 2010; Hewitt, 1979; White, 1973). Gain and losses in either  $2n$  or FN can, therefore, be used as proxies of chromosomal rearrangements that may drive reproductive isolation and lineage diversification (Castillo et al., 2023; Hewitt, 1979; King, 1995; White, 1973). In an ancient group like Orthoptera that had their most recent common ancestor about 355 Mya (Song et al., 2020), karyotype change has accumulated in variable numbers of  $2n$  and FN. Furthermore, karyotype characterization has a long tradition in Orthoptera (due to their unusually large chromosomes), so sufficient data can be compiled. Orthoptera, therefore, provide a strong opportunity and sufficient statistical power to examine the implication of karyotype evolution in speciation and diversification.

We used a Bayesian evolutionary modelling framework to examine associations between karyotype change and speciation. Unlike anagenetic changes that happen within lineages, cladogenetic changes occur at branching points of the evolutionary tree (Freyman & Höhna, 2018; Lucek et al., 2022; Mayrose & Lysak, 2021). We compiled information on  $2n$  and FN for 1,541 species of Orthoptera to test whether speciation and karyotype change are linked, recognizing that both cladogenetic changes during lineage splits and anagenetic changes within lineages can contribute to this process (Lucek et al., 2022). We fitted evolutionary models that

allowed the estimation of rates of karyotype change, both linked and unlinked to speciation, along with speciation rates in a subset of 516 species. The existence of non-zero rates of cladogenetic karyotype change implies a potential causal relationship (direct or indirect) of karyotype change in the speciation processes. Thus, in about half of the clades, karyotype change seems to play a role in the speciation processes while in others it does not (at least not directly). We further demonstrate that the rate of chromosome losses is higher in flightless orthopteran lineages, suggesting that reduced dispersal capabilities promote chromosome changes.

## Methods

### Data compilation

We compiled information on diploid chromosome number ( $2n$ ) and number of chromosome arms (fundamental numbers, FN) of male Orthoptera from published sources (Supplementary Table S1).  $2n$  values were later converted to haploid  $n$  values for the analysis. The compilation was done via searches on Google Scholar for keywords “karyotype” and “Orthoptera” as well as names of different suborders, families, subfamilies, and genera. Any resultant paper was screened for additional references that were included if they provided additional karyotype information. Only diploid species were considered (polyploidization is a rare exception in Orthoptera, see below) and B chromosomes were ignored. In total, we assembled data on 1,541 orthopteran species belonging to 27 families (out of 36 families of extant Orthoptera) from 173 publications (Supplementary Table S1). The compiled data concerned families of long-horned grasshoppers (suborder Ensifera) and short-horned grasshoppers (suborder Caelifera), and therefore covered the full range of orthopteran diversity.

### Barcode compilation for phylogenetic analyses

The most reliable sources on phylogenetic relationships among major lineages within Orthoptera (Song et al., 2015, 2020) include less than 16% of the species for which we have karyotype information. We, therefore, reconstructed a more comprehensive phylogeny. We used sequence data of the 5' region of the mitochondrial cytochrome c oxidase subunit I (COI-5P), since this is best represented across Orthoptera in the Barcode of Life Data System (BOLD, <https://www.boldsystems.org>) and NCBI public databases (<https://www.ncbi.nlm.nih.gov>). In total, 41,488 COI-5P barcode sequences of Orthoptera were downloaded from BOLD and NCBI. Barcode sequences were retained if the following criteria were met: (a) sequences were > 500 bp in length to ensure sequence alignment with high fidelity, (b) sequences were unique to a specific species that had karyotype information, and (c) corresponding amino acid sequences did not contain stop codons.

Preprocessing was done using the R package *coil* (Nugent et al., 2020) and primarily involved filtering and evaluation of frameshift errors (<https://github.com/CNuge/coil>). The *coil* package contains functions for placing COI-5P barcodes into a common reading frame, translation of DNA sequences to amino acid sequences, and denoising insertion/deletion errors. We placed 516 COI-5P barcode sequences (a single COI-5P record per species with karyotype data) into the correct reading frame. We added the denoised COI-5P barcode sequences of *Grylloblatta bifratrilecta* (Grylloblattodea, downloaded from BOLD) as an outgroup. The final dataset thus contained

517 denoized COI-5P barcode sequences that were aligned with the regressive mode (Garriga et al., 2019) of T-Coffee (Di Tommaso et al., 2011): “t\_coffee -reg -seq in.fasta -nseq 100 -tree mbed -method mafft\_ginsi\_msa.” This algorithm is considered the most accurate, since it combines the ginsi mode of MAFFT (Katoh & Toh, 2008) with the mbed trees of Clustal Omega (Sievers et al., 2011).

### Reconstruction of a dated phylogeny

We analyzed the aligned COI-5P barcode sequences in a maximum likelihood (ML) framework with IQ-TREE (Nguyen et al., 2015). We used ModelFinder (Kalyaanamoorthy et al., 2017) to fit multiple substitution models and chose the best model that minimizes the BIC score. The best substitution model, by BIC, was a general time reversible model with unequal rates and unequal base frequencies (GTR + F + R10). We estimated node support values via ultrafast bootstrap approximation (UFBoot) (Hoang et al., 2018) with 1,000 bootstrap replicates. We further used the flag --bnni to reduce the risk of overestimating branch support. We then ran IQ-TREE with the following settings: iqtree -s align.fasta -m GTR + F + R10 -B 1000 --bnni -T 6. As in previous studies (Song et al., 2015, 2020), *Grylloblatta bifratrilecta* (Grylloblattodea) was assigned as an outgroup.

We estimated divergence times with treePL (Smith & O’Meara, 2012). treePL uses penalized likelihood, which involves adding a penalty term to the likelihood function to account for variation in the rate of molecular evolution among lineages in a phylogenetic tree (Sanderson, 2002). This approach is especially useful when working with large phylogenetic trees (Smith & O’Meara, 2012). The smoothing parameter determines the degree of penalization applied to the likelihood function. treePL automatically optimizes this parameter using cross-validation or user-defined optimization methods (Smith & O’Meara, 2012). We used the ML tree from IQ-TREE as input, and four fossils and five secondary calibration points as time constraints. Time constraints were given as ranges and directly followed by Song et al. (2015, 2020) (Table 1). To identify the optimal smoothing value in the phylogram, we conducted a data-driven cross-validation analysis with treePL. The optimal smoothing value was found to be  $1 \times 10^{-4}$ .

### Modelling karyotype evolution and speciation

To test for an association between karyotype change and species diversification rates, we fitted Bayesian phylogenetic

models of karyotype evolution as implemented in the *ChromoSSE* model (Freyman & Höhna, 2018) of the statistical software *RevBayes* (Höhna et al., 2016). Fitting the *ChromoSSE* model to the Orthoptera order proved computationally unfeasible because of the extensive species count. The vast number rendered the exponentiation of the instantaneous rate matrix computationally impractical. Since we did suspect heterogeneity among clades, the analysis was thus done separately for different orthopteran families. Subsetting involved pruning the reconstructed phylogenetic tree to subtrees for each family using the list of karyotyped species. Pruning was done using the R packages *phytools* (Revell, 2012) and *ape* (Paradis & Schliep, 2019). In total, we analyzed 14 orthopteran families with a total of 516 species (33% of the karyotyped species). The remaining families had too few species with karyotype information (< 3 species) to infer evolutionary rates.

*ChromoSSE* (Chromosomal State Speciation and Extinction) integrates both discrete and continuous traits to explore the relationship between trait evolution and speciation/extinction rates within a phylogenetic context. By estimating the impact of traits on speciation and extinction rates, *ChromoSSE* enables us to examine hypotheses regarding the influence of traits on the patterns of diversification. We used *ChromoSSE* to infer the dynamics of haploid chromosome number ( $n$ ) evolution through fusion and fission events (that is, changes in chromosome numbers by  $-1$  or  $+1$ , respectively), and the origin and extinction of phylogenetic lineages. The model allows karyotypes to evolve along branches of the phylogeny (anagenetic change) or associated with speciation events (cladogenetic change) and estimates parameters for each of these processes. Specifically, we estimated three speciation rate parameters: fission-associated speciation rate, fusion-associated speciation rate, and speciation rate without karyotype change. Furthermore, we estimated two parameters for anagenetic karyotype change (one for fission and one for fusion), relative extinction rates and total speciation rates per family. We excluded polyploidization from the model by fixing the polyploidization rate to 0. The decision was based on the lack of well-documented cases of polyploidization in Orthoptera, with the only known exception being the pentaploid, parthenogenetic species *Saga pedo* (Tettigoniidae), with  $5n = 70$  (Dutrillaux et al., 2009).

A major limitation for all phylogenetic models of cladogenetic character change lies in addressing unobserved speciation events, which can result from incomplete taxon sampling and

**Table 1.** Fossils and secondary calibration points used as time constraints.

Clade	Fossil	Median age [Myr] (minimum-maximum)	Notes	References
crown-Orthoptera		320-393.8	Secondary calibration point	(Song et al., 2020)
crown-Ensifera		267.4-348	Secondary calibration point	(Song et al., 2020)
crown-Gryllidea		154.1-247.5	Secondary calibration point	(Song et al., 2020)
crown-Tettigoniidea		227.7-308.1	Secondary calibration point	(Song et al., 2020)
crown-Caelifera		282-359.5	Secondary calibration point	(Song et al., 2020)
crown-Tridactyloidea	<i>Monodactylus curtippennis</i>	129.4-132.9	Oldest definitive Tridactyloidea	(Song et al., 2015)
crown-Tetrigidae	<i>Prototetrix reductus</i>	129.4-132.9	Oldest definitive Tetrigidae	(Song et al., 2015)
crow-Eumastacoidea	<i>Archaeomastax jurassicus</i>	145-163.5	Oldest definitive Eumastacoidea and Eumastacidae	(Song et al., 2015)
crown-Acrididae	<i>Tyrbula russelli</i>	33.9-38	Oldest definitive Acridoidea and Acrididae	(Song et al., 2015)

lineages going extinct without leaving any extant descendants (Bokma, 2002). This has the potential to skew the relative rates of anagenetic and cladogenetic changes. Nonetheless, the *ChromoSSE* model employs the Cladogenetic State change Speciation and Extinction (*ClaSSE*) model (Goldberg & Igić, 2012) to mitigate this bias by explicitly incorporating unobserved speciation events. The *ClaSSE* framework not only allows for modelling unobserved speciation but also offers a flexible framework for examining state-dependent speciation and extinction rates. To control for sampling biases, we estimated the probability of sampling species at the present ( $\rho_{bd}$ ) by dividing the number of sampled species in each family by the total number of extant species in that family (obtained from Orthoptera Species File (Cigliano et al., 2022) and Google Scholar searches). The  $\rho_{bd}$  values ranged from 0.0053 to 0.13 depending on the family. Exponential distributions centred on 10 were used as priors for the two anagenetic parameters as recommended in the *ChromoSSE* protocol. Also following the established *ChromoSSE* protocol (Freyman & Höhna, 2018), priors for the three cladogenetic parameters were exponentially distributed, centred at the inverse of the log taxonomic count divided by the root age of the phylogeny. Two independent runs of 25,000 MCMC generations (sampling every 10th generation) were used to infer model parameters. For the particularly species-rich families Acrididae and Tettigoniidae, however, 10 independent runs of 100,000 MCMC generations each (sampling every 10th generation) were performed. The first 25% of samples of each run were discarded as burn-in, and the traces were combined once the simulations were completed. Convergence of chains was checked with Tracer (Rambaut et al., 2018), ensuring an effective sample size (ESS) > 200 for all parameters.

### Inference based on rate estimates

We summarize posterior distributions of rate parameters by their medians, which is preferable over posterior means if parameters are near a boundary (Pick et al. 2023). Furthermore, we determined the 95% highest posterior densities (95% HPD) for each parameter. Since rate parameters are strictly positive, it can be difficult to interpret the strength evidence for positive rates from posterior distributions. We therefore assessed whether (a) 95% HPD touched zero (or close to zero), (b) if posterior distributions were shifted away from zero with a clear peak in the middle (rather than near the boundary), and (c) we used Bayes factors for assessing the evidence for non-zero cladogenetic karyotype change rates as described below.

To test for differences among rates, we subtracted rate estimates from one another and evaluated whether the posterior distribution of differences overlapped with zero. Two-tailed  $p$  values were calculated as twice the fraction of posterior samples of differences that were greater/smaller (whatever the smaller fraction) than zero. Differences among rates for different families were tested in the same way, except that vectors of posterior samples were first randomized. This was done for all pair-wise comparisons of families and an overall  $p$  value for heterogeneity among families was calculated using Fisher's method of combined probabilities.

To further test the hypothesis that the total rate of speciation is related to the total rate of karyotype change across families, we fitted a linear-mixed model controlling for phylogenetic relatedness among families using the MCMCglmm R package (Hadfield, 2010; Hadfield & Nakagawa, 2010).

The response variable, speciation rate, was defined as the sum of all estimated speciation rate parameters, while the predictor, karyotype change rate, was calculated as the sum of all cladogenetic and anagenetic rate parameters. We fitted 100 separate models each using a random sample from the posterior distributions (from the *ChromoSSE* model). Each model was run for 13,000 iterations with a burn-in of 3,000, and thinning every 10 iterations. We summarize each model by the posterior mean, leaving us with 100 slope estimates, each one based on an independent sample from the posterior distribution of the rate parameters.

### Evaluating rates of changes against a null hypothesis

We were particularly interested in whether there is statistical evidence for non-zero rates of cladogenetic karyotype change and therefore employed Bayes factors (BF) to evaluate the statistical evidence favouring positive rates of cladogenetic karyotype change (referred to as model 1, M1) in comparison to a null model where rates of cladogenetic chromosomal fusions and fissions were fixed to zero (referred to as M2). We estimated BF within the order Orthoptera and within orthopteran families because we suspected heterogeneity in the mode of karyotype evolution among different clades. To do this, we first estimated the marginal likelihood of M1 and M2 employing the stepping-stone algorithm as implemented in *RevBayes*. This algorithm involves iteratively sampling from distributions representing a series of steps between posterior and prior probabilities. We used 10 steps between 1 and 0 as power for the likelihood term in the model, producing a diminishing influence of the data as the power exponent approaches zero. The first step draws samples from the untransformed posterior, while the last step samples only from the prior. The process started with a burn-in for 10,000 generations to sample from the posterior (tuning interval of 1,000). Then the powers were raised stepwise for the likelihood with 1,000 generations sampled at each step (3,000 for Orthoptera, Acrididae, and Tettigoniidae).

After obtaining the marginal likelihood of M1 and M2 for Orthoptera and for each orthopteran family, we computed BF by subtracting the estimates from one another:  $K = \ln[B-F(M1, M2)] = \ln[P(X | M1)] - \ln[P(X | M2)]$ , where  $\ln[P(X | M1)]$  and  $\ln[P(X | M2)]$  are the marginal likelihoods of M1 and M2, respectively, and summarized the 3,000 posterior samples by their means. We interpreted the strength of evidence in favour of one model over the other by evaluating average  $K$  values (Jeffreys, 1922). If  $K > 1$ , model M1 is preferred. If  $K < -1$ , M2 is preferred. Absolute values of  $|K| > 1$  are generally interpreted as statistical support for a particular model (with larger values indicating stronger support). Values between 0 and 1 indicated ambiguity in support for either model.

### Modelling karyotype evolution with phenotype

We used the *BiChroM* model (Zenil-Ferguson et al., 2017) implemented in *RevBayes* (Höhna et al., 2016) to determine whether the rates of karyotype evolution are linked to the capability of incapability of flight in Orthoptera. The *BiChroM* model describes the joint evolution of both the phenotypic traits and chromosome evolution. For this purpose, we defined a binary code to represent flight (state 0) and flightless (state 1). The motivation for focusing on flightless phenotype in Orthoptera stems from its potential evolutionary

significance. Flightless phenotype is a common trait in many insects (McCulloch et al., 2021), including Orthopteran species (Voje et al., 2009; Yagui et al., 2024), and has been linked to ecological and evolutionary processes such as reduced dispersal abilities, which can lead to population isolation and increased genetic divergence. The phenotype is easy to quantify based on the presence/absence of wings.

We retrieved phenotype data for orthopterans from Orthoptera Species File (Cigliano et al., 2022). The data matrix represents both the observed haploid  $n$  chromosome counts and the observed phenotype. In this file, state 0 represents the haploid  $n$  chromosomes for lineages with the ability to flight, and state 1 represents the haploid number  $n + 40$  chromosomes for flightless lineages. Forty represents the maximum haploid  $n$  chromosome count (39) plus 1. The *stateLabels* argument was set to twice the maximum number of chromosomes. This represents the way of coding required for *BiChroM*.

In contrast to the *ChromoSSE* model, we fitted the *BiChroM* model to all lineages with karyotype information that aligns with the phylogeny, since speciation rates were not estimated in this analysis. Like before, we used exponential distributions centred on 10 as priors to model the rates of chromosome gains ( $\gamma$ ) and losses ( $\delta$ ) along the branches of the phylogeny. We set up two rate parameters for each type of karyotype change: one for phenotype state 0 and one for phenotype state 1. We thus used a simple *ChromEvol* model (without speciation rates) that includes only the rate of chromosome gains and losses. Twenty independent runs of 200,000 MCMC generations (sampling every 10th generation) were used to infer model parameters. The first 25% of samples of each run were discarded as burn-in, and the traces were combined once the simulations were completed. The convergence of chains was checked with Tracer (Rambaut et al., 2018), ensuring an ESS > 200 for all parameters.

## Results

We assembled records of  $2n$  for 1,541 orthopterans belonging to 27 families, including Ensifera and Caelifera, and therefore covering the full range of orthopterans diversity. The median and mode of chromosome numbers was  $2n = 23$  (range 7–57,  $N = 1,541$ ; Figure 1A and B, Supplementary Table S1). The median and mode of FN was FN = 25 (range 11–64; Figure 1C). Broadly, orthopteran chromosomes were recognized as metacentric, submetacentric and/or acrocentric in nature.

### Phylogenetic reconstruction and time calibration

We reconstructed the Orthoptera phylogeny using COI-5P barcode sequences of 516 species along with *Grylloblatta bifratrilecta* as outgroup (Figure 2A). As in previous phylogenetic studies (Song et al., 2015, 2020), our calibrated tree confirmed the monophyly of the two suborders Ensifera and Caelifera. Within Ensifera, our analysis confirmed two monophyletic infraorders, Gryllidea and Tettigoniidea, with the former containing Gryllidae, Trigonidae, and Gryllotalpidae, and the latter consisting of Tettigoniidae, Rhaphidophoridae, Stenopelmatidae, and Anostostomatidae. Moreover, the estimated tree also confirms the monophyly of five superfamilies within Caelifera: Tridactylidea (Tridactylidae), Tetragoidea (Tetrigidae), Eumastacoidea (Morabidae), Pyrgomorphaidea (Pyrgomorphidae), and Acridoidea (Romaleidae, Pamphagiidae, and Acrididae). The estimated divergence

times of the phylogenetic tree spanning 300 million years of orthopterans evolution (Figure 2A) are broadly consistent with previous phylogenetic studies (Song et al., 2015, 2020).

### Rates of karyotype evolution

We used *ChromoSSE* to estimate rates of karyotype evolution and speciation in 14 families. Posterior medians for anagenetic fusions varied between  $3.05 \times 10^{-3}$  (Acrididae) and 0.105 (Gryllotalpidae) events/sp/Myr (Table 2). These rates differed significantly among families (Fisher's combined probability,  $p = 0.0019$ ). In two families (Rhaphidophoridae and Gryllotalpidae), posterior distributions appeared significantly positive, while for other families, posterior distributions piled up near the boundary (Figure 2B). Posterior medians of anagenetic fissions ranged from  $2.61 \times 10^{-3}$  (Romaleidae) to 0.032 (Gryllidae) events/sp/Myr (Table 2). These rates also differed significantly among families (Fisher's combined probability,  $p = 0.0227$ ). As for anagenetic fusions, Rhaphidophoridae and Gryllotalpidae showed posterior distributions that appeared significantly positive (Figure 2C).

Rates of cladogenetic (speciation-related) karyotype changes were generally lower than rates of anagenetic karyotype change. Posterior medians for cladogenetic chromosomal fusion varied between  $2.92 \times 10^{-3}$  (Tetrigidae) and 0.025 (Morabidae) events/sp/Myr (Table 2). These rates did not differ significantly among families (Fisher's combined probability,  $p = 0.99$ ). In two families (Morabidae and Acrididae), posterior distributions appeared significantly positive (Figure 2F). Posterior medians for cladogenetic chromosomal fission varied between  $2.61 \times 10^{-3}$  (Romaleidae) and 0.032 (Gryllidae) events/sp/Myr (Table 2). These rates also did not significantly differ among families (Fisher's combined probability,  $p = 1$ ). In one family (Acrididae), posterior distributions appeared significantly positive (Figure 2G).

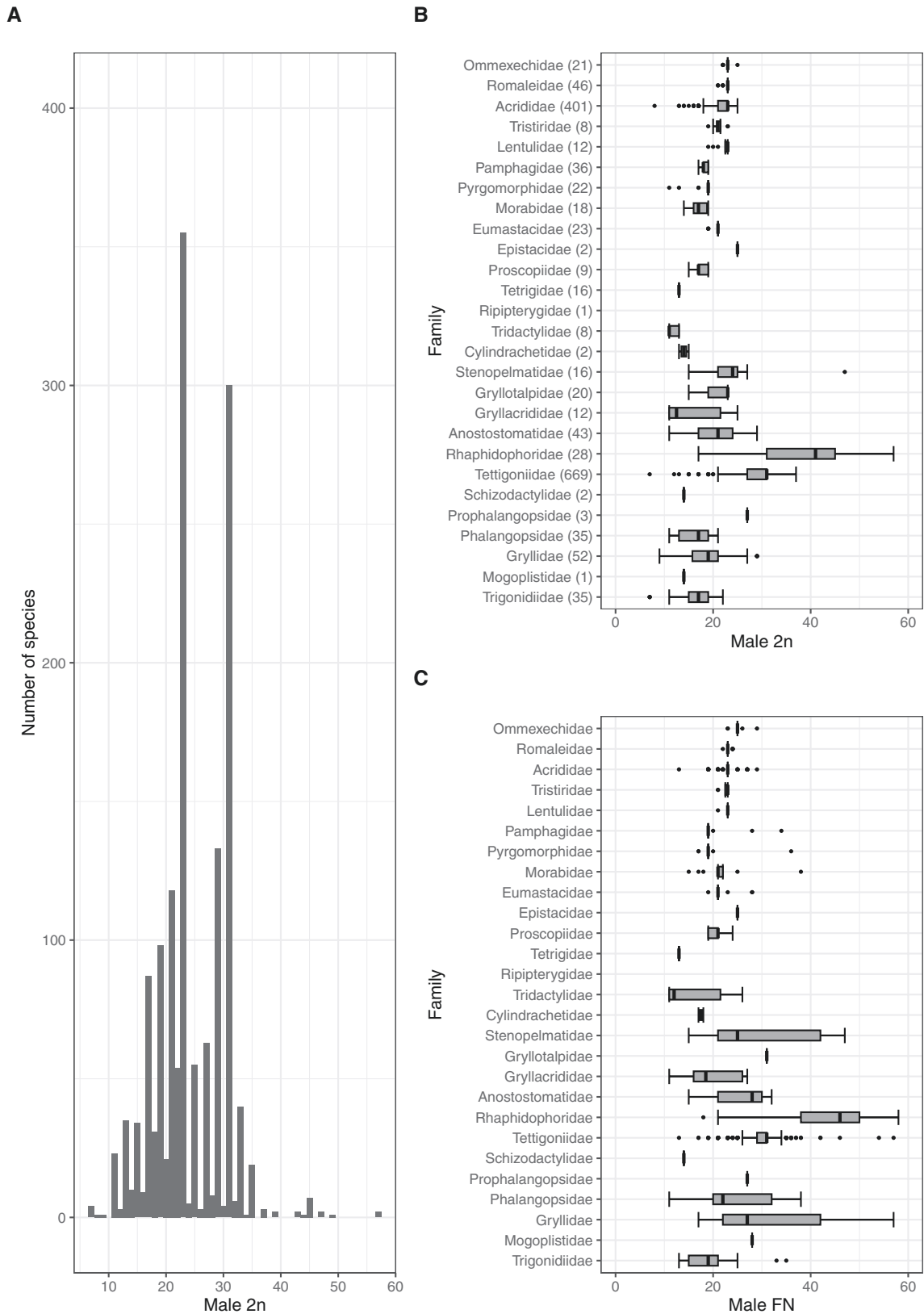
We tested for positive rates of cladogenetic chromosomal changes (M1) relative to a null model that assumes there is no cladogenetic fusion and fission and all karyotype change is anagenetic (M2). The order Orthoptera had  $K = 0.83$  indicating ambiguity in support for either model.  $K > 1$  (supporting M1) was found in 6/14 analyzed families: Gryllidae, Gryllotalpidae, Morabidae, Pamphagiidae, Rhaphidophoridae, and Stenopelmatidae.  $K < -1$  (supporting M2) was found in 6/14 families: Tettigoniidae, Trigonidae, Pyrgomorphidae, Acrididae, Romaleidae, and Tetrigidae.  $K$  near 0 was found in 2/14 analyzed families (Anostostomatidae and Trydactylidae) indicating no preference for either model (Table 3).

Total rates of fusion (anagenetic fusion + cladogenetic fusion) were similar to total rates of fission (anagenetic fission + cladogenetic fission) across clades, but in Tettigoniidae, estimated fusion rates exceeded fission rates (Figure 3A–O).

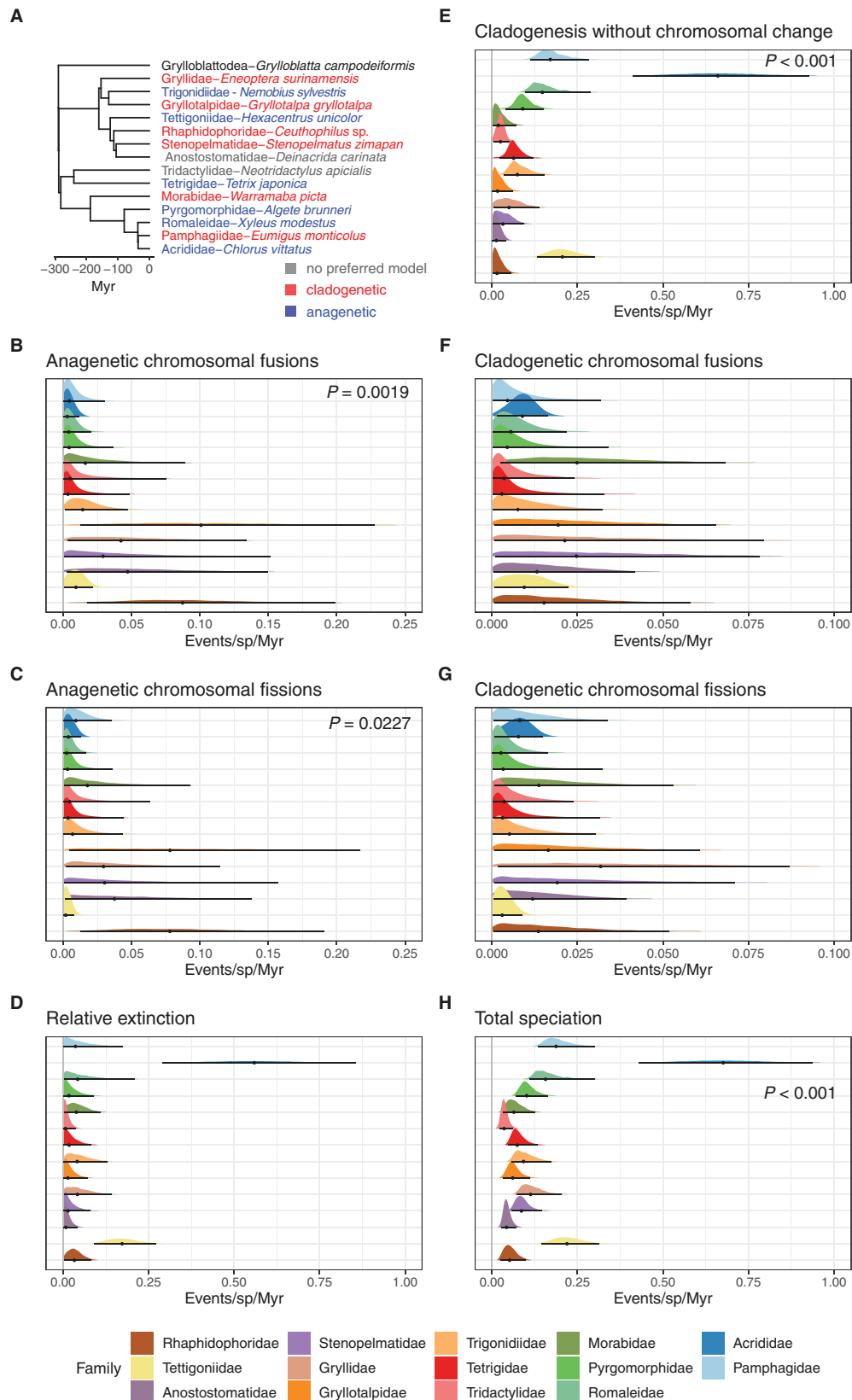
We tested for an association between total speciation rate and total rate of karyotype change across families (using the posterior distributions of the rate parameters). Results showed on average a negative association ( $b = -0.69 \pm 0.27$ ), indicating that families with low rates of karyotype change do not necessarily show a low rate of speciation. This is exemplified by Acrididae which show a relatively stable karyotype, but one of the highest speciation rates in our dataset.

### Speciation and extinction rates

Posterior medians for speciation without karyotype change varied between 0.013 (Anostostomatidae) and 0.669 (Acrididae) events/sp/Myr (Table 2). These rates differed



**Figure 1.** (A) Distribution of chromosome numbers in males (male 2n) of Orthoptera based on 1,541 karyotyped species. (B) Boxplots summarizing chromosome numbers (male 2n) for the 27 families of Orthoptera (1,541 species). The number in parentheses next to the family name represents the count of species within that family with documented chromosome numbers. (C) Boxplots summarizing the number of chromosome arms (fundamental numbers, FN) for 27 families of Orthoptera (1,541 species).



**Figure 2.** Summary of anagenetic and cladogenetic chromosomal change estimates for 14 families of Orthoptera. (A) Time calibrated phylogeny (in Myr) based on DNA barcoding 5' region of the mitochondrial cytochrome c oxidase subunit I (COI-5P) gene, pruned to simply represent the phylogenetic relationships among the 14 Orthoptera families and the outgroup (Grylloblattodea). (B–H) Posterior distributions for (B) anagenetic fusions, (C) anagenetic fissions, (D) relative extinction rates, (E) cladogenetic changes without chromosomal change, (F) cladogenetic chromosomal fusions, (G) cladogenetic chromosomal fissions, and (H) total speciation rates. All rate parameter are expressed in events per species per million years (events/species/Myr). Bars and dots indicate the confidence interval of 95% of the posterior probability space. Two-tailed  $p$  values testing for heterogeneity in rates among families are shown for each panel where the results were significant. Families highlighted in bold lend support for cladogenetic chromosomal change.

**Table 2.** Rate parameter estimates from the *chromoSSE* analyses for 14 Orthoptera families. Indicated are posterior medians and the 95% highest posterior density interval (in brackets).

Family	Model parameters				Compound (constructed) parameters			
	Speciation without chromosomal change (events/sp/Myr)	Cladogenetic chromosomal fusion (events/sp/Myr)	Cladogenetic chromosomal fission (events/sp/Myr)	Anagenetic chromosomal fusion (events/sp/Myr)	Anagenetic chromosomal fission (events/sp/Myr)	Total speciation (events/sp/Myr)	Extinction (events/sp/Myr)	
Rhaphidophoridae	0.015 [1.18E-6, 0.045]	0.015 [5.91E-6, 0.045]	0.013 [5.91E-6, 0.045]	0.087 [7.68E-3, 0.189]	0.078 [3.63E-3, 0.1771]	0.051 [0.022, 0.092]	0.033 [3.32E-5, 0.073]	
Tettigoniidae	0.206 [0.132, 0.293]	9.45E-3 [5.718E-9, 0.020]	2.99E-3 [1.75E-7, 7.68E-3]	9.32E-3 [5.61E-7, 0.019]	1.89E-3 [3.64E-8, 6.53E-3]	0.219 [0.144, 0.306]	0.173 [0.091, 0.264]	
Anostostomatidae	0.013 [3.09E-5, 0.037]	0.012 [6.41E-6, 0.034]	0.012 [6.41E-6, 0.034]	0.048 [3.13E-4, 0.132]	0.038 [1.21E-6, 0.120]	0.042 [0.025, 0.066]	7.86E-3 [2.11E-6, 0.034]	
Stenopelmatidae	0.032 [1.79E-5, 0.081]	0.025 [6.32E-6, 0.072]	0.019 [6.35E-6, 0.064]	0.029 [2.14E-5, 0.126]	0.031 [2.43E-5, 0.130]	0.086 [0.052, 0.135]	0.014 [7.51E-7, 0.067]	
Gryllidae	0.050 [4.41E-5, 0.121]	0.021 [9.52E-6, 0.073]	0.032 [3.50E-5, 0.085]	0.042 [4.03E-4, 0.117]	0.030 [9.13E-6, 0.097]	0.113 [0.069, 0.189]	0.042 [1.37E-5, 0.121]	
Gryllotalpidae	0.016 [2.12E-5, 0.053]	0.019 [4.91E-5, 0.057]	0.016 [1.52E-5, 0.053]	0.105 [3.05E-3, 0.238]	0.080 [2.13E-4, 0.217]	0.061 [0.030, 0.105]	0.014 [3.66E6, 0.057]	
Trigonidiidae	0.074 [0.029, 0.144]	7.44E-3 [6.37E-6, 0.026]	5.04E-3 [4.83E-7, 0.025]	0.014 [2.58E-4, 0.041]	6.94E-3 [1.95E-6, 0.034]	0.092 [0.050, 0.157]	0.041 [3.17E-6, 0.112]	
Tettigidae	0.063 [0.020, 0.118]	2.92E-3 [1.25E-6, 0.023]	3.06E-3 [4.24E-6, 0.023]	3.37E-3 [2.06E-6, 0.035]	3.67E-3 [1.38E-7, 0.034]	0.074 [0.043, 0.120]	0.018 [1.51E-5, 0.07]	
Tridactylidae	0.025 [1.71E-3, 0.046]	3.57E-3 [8.06E-7, 0.020]	3.59E-3 [6.69E-7, 0.019]	5.23E-3 [2.85E-6, 0.054]	4.80E-3 [2.25E-6, 0.046]	0.036 [0.019, 0.058]	6.87E-3 [1.52E-6, 0.030]	
Morabidae	0.018 [3.38E-6, 0.059]	0.025 [3.29E-5, 0.061]	0.014 [8.25E-6, 0.045]	0.016 [7.32E-6, 0.076]	0.017 [5.08E-6, 0.076]	0.064 [0.028, 0.116]	0.038 [1.31E-5, 0.096]	
Pyrgomorphidae	0.090 [0.041, 0.152]	4.40E-3 [1.32E-6, 0.024]	3.24E-3 [5.22E-7, 0.023]	4.37E-3 [5.77E-6, 0.025]	3.26E-3 [6.64E-6, 0.025]	0.102 [0.065, 0.154]	0.017 [1.91E-5, 0.071]	
Romaleidae	0.148 [0.088, 0.260]	5.46E-3 [1.38E-7, 0.018]	2.61E-3 [7.05E-7, 0.013]	4.17E-3 [2.16E-5, 0.016]	2.58E-3 [6.50E-6, 0.013]	0.157 [0.104, 0.279]	0.043 [6.69E-6, 0.174]	
Pamphagidae	0.170 [0.107, 0.269]	4.60E-3 [8.12E-7, 0.024]	8.166E-3 [5.31E-6, 0.028]	4.533 [8.14E-6, 0.023]	9.34E-3 [2.71E-5, 0.003]	0.187 [0.129, 0.284]	0.036 [1.57E-5, 0.140]	
Acrididae	0.669 [0.429, 0.931]	8.92E-3 [1.041E-3, 0.016]	7.71E-3 [3.99E-5, 0.014]	3.05E-3 [3.53E-7, 0.010]	3.93E-3 [9.98E-7, 0.011]	0.686 [0.452, 0.953]	0.567 [0.326, 0.854]	

significantly among families (Fisher's combined probability,  $p < 0.001$ ; **Figure 2F** and **G**). Speciation rates without karyotype change were significantly larger than speciation rates with karyotype change in 5 of 14 families (Acrididae, Pamphagidae, Pyrgomorphidae, Romaleidae, Tettigoniidae; Fisher's combined probability,  $p < 0.05$ ). Posterior medians for total speciation rates (with and without karyotype change) varied between 0.036 events/sp/Myr (Tridactylidae) and 0.686 events/sp/Myr (Acrididae) and differed significantly among families (**Figure 2H**; **Table 2**; Fisher's combined probability,  $p < 0.001$ ). Posterior medians for relative extinction rates varied between 0.007 (Tridactylidae) and 0.567 (Acrididae) (**Figure 2D**; **Table 2**) and did not differ among families (Fisher's combined probability,  $p = 1$ ). Across families, the correlation between relative extinction and total speciation was 0.98 (among posterior medians,  $t = 15.372$ ,  $df = 12$ ,  $p = 2.935 \times 10^{-9}$ ).

### Karyotype evolution and phenotype

We annotated 60% (308/516) of the species in the phylogenetic tree as potentially capable of flight in that they have fully developed wings (state 0), while 40% (208/516) were classified as flightless with undeveloped wings (state 1) (**Supplementary Table S2**). Trigonidiidae and Stenopelmatidae are mostly flightless, while Rhabdophoridae, Tridactylidae, and Morabidae are entirely flightless. Tettigidae included only flight-capable species, and Gryllidae, Gryllotalpidae, Romaleidae, and Acrididae are predominantly flight-capable. The other families show a mix of flight and flightless species (**Figure 4A**). We then used the *BiChrom* model to test the association between karyotype evolution and phenotype in orthopterans (**Figure 4B**). The posterior medians for the rate of chromosome gains were  $8.99 \times 10^{-3}$  events/sp/Myr for the flight phenotype (gamma 0) and  $1.66 \times 10^{-3}$  events/sp/Myr for the flightless phenotype (gamma 1). These rates differed significantly between the two states ( $p < 0.01$ ). The posterior medians for the rate of chromosome losses were 0.018 events/sp/Myr for the flight phenotype (delta 0) and 0.037 events/sp/Myr for the flightless phenotype (delta 1). These rates differed significantly between the two states ( $p < 0.001$ ).

### Discussion

We conducted a comprehensive analysis of karyotype evolution in Orthoptera. Our study involved a total of 1,541 orthopteran species with karyotype information, making it the most extensive study on this topic to date. The karyotype survey included 27 out of the 36 extant families of Orthoptera, covering both the suborders Ensifera and Caelifera, resulting in a nearly complete representation of the diverse range of karyotypes found within the Orthoptera. While there was one previous study that compiled karyotype diversity across the suborder Caelifera (**Husemann et al., 2022**), our study extends to karyotype diversity in the complete order Orthoptera and explicitly estimates evolutionary change parameters. Increasing the number of species examined would contribute to a more robust assessment of the phylogenetic signals. While utilizing a single mitochondrial gene for phylogenetic reconstruction may not capture the entire evolutionary history, this approach is a practical choice given the available data.

The data illustrated the large diversity of karyotypes in Orthoptera as summarized by  $2n$  and FN. We found two

**Table 3.** Summary statistics of marginal likelihood estimated through the stepping-stone algorithm for M1 and M2, and the Bayes factor calculations within Orthoptera and across 14 Orthopteran families.

Order Family	Marginal likelihood M1	Marginal likelihood M2	$K = \ln[\text{BF}(M1, M2)] = \ln[\text{P}(X M1)] - \ln[\text{P}(X M2)]$	Evidence based on $K$ (Jeffreys, 1922)
Orthoptera	-5,563.751	-5,564.578	0.83	No preference
Acrididae	-1,547.58	-1,545.71	-1.93	Supports M2
Anostomatidae	-63.46	-64.11	0.66	No preference
Gryllidae	-139.37	-141.79	2.42	Supports M1
Gryllotalpidae	-36.89	-39.05	2.16	Supports M1
Morabidae	-91.40	-95.89	4.48	Supports M1
Pamphagidae	101.03	-96.84	197.87	Supports M1
Pyrgomorphidae	-69.59	-65.87	-3.72	Supports M2
Rhaphidophoridae	-109.68	-112.47	2.78	Supports M1
Romaleidae	-114.32	-109.74	-4.58	Supports M2
Stenopelmatidae	-48.09	-49.29	1.19	Supports M1
Tetrigidae	-71.94	-68.20	-3.73	Supports M2
Tettigoniidae	-1,432.34	-1,429.02	-3.01	Supports M2
Tridactylidae	-48.39	-48.66	0.27	No preference
Trigonidiidae	-167.85	-165.87	-1.97	Supports M2

M1 = rates of cladogenetic karyotype change; M2 = rates of cladogenetic karyotype change with chromosomal fissions and fusion rates both fixed to zero;  $\ln[\text{P}(X|M1)]$  and  $\ln[\text{P}(X|M2)]$  are the marginal likelihood estimate for M1 and M2, respectively. If  $K > 1$ , M1 is preferred. If  $K < -1$ , M2 is preferred. Values of  $K$  around 0 indicate no preference for either model.

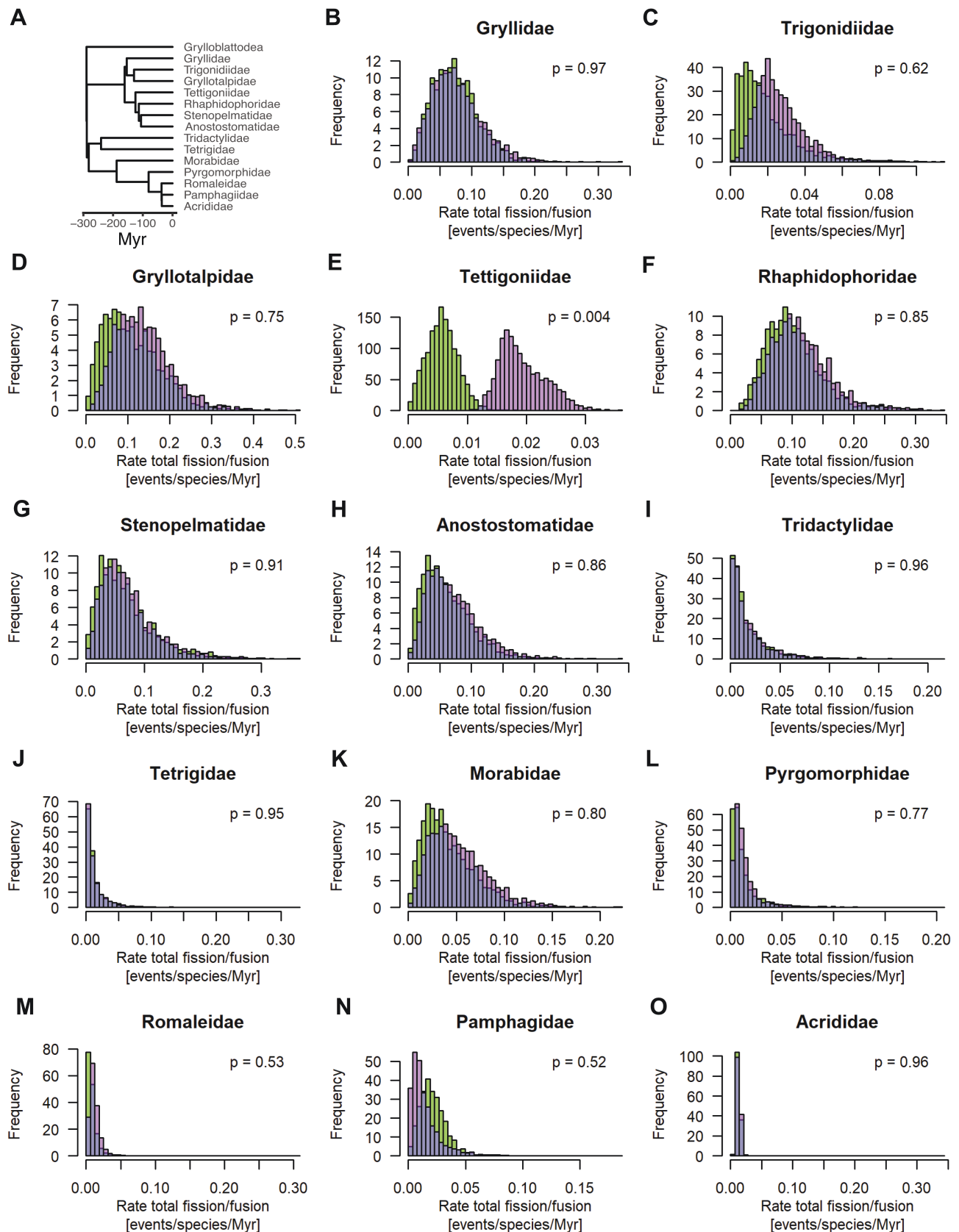
major peaks in the distribution of chromosome number (Figure 1A). The first peak corresponds to Caelifera, which mostly shows a conserved karyotype with  $2n = 23, X0$ , but sometimes extremes such as  $2n = 8, \text{neo-XY}$  in *Dichroplus silveiraguidoi* (Castillo et al., 2010) or  $2n = 25, X0$  in *Dirshacris aridus* (Fossey, 1990). The second peak ( $2n = 31$ ) corresponds to Ensifera, with extremes of  $2n = 7, X0$  in *Eunemobius* species (Portugal & Mesa, 2007) and *Gonatoxia helleri* (Warchałowska-Śliwa et al., 2020) and  $2n = 57, X0$  in *Diastrammema tachycines asynamorus* (Mesa, 1965). Karyotypes of  $2n = 23$  and  $2n = 31$  may represent the ancestral karyotype (or close to these numbers) for Caelifera and Ensifera, respectively. The data demonstrated that karyotype diversification is more prevalent in Ensifera than in Caelifera, as the latter exhibited a more stable karyotype. We chose not to formally estimate the ancestral karyotype because the reconstructed tree includes 516 species (~2% of the 28,000 extant Orthopteran species). While we recognize the value of exploring karyotype variation across taxa, the current data limitations make it challenging to construct a robust ancestral state for reliable karyotype reconstruction.

The results of an empirical analysis of a subset of 516 orthopteran species identified a dichotomy between clades (6/14) with statistical evidence for positive rates of cladogenetic karyotype change, while in other clades (6/14) karyotype change appeared to be largely anagenetic. The prevalence of cladogenetic chromosomal changes in nearly half of all orthopteran families (both Ensifera and Caelifera) imply that major changes in chromosome number contribute to reproductive isolation and speciation in some clades, but that karyotype changes played no immediate role in speciation in 6/14 families (2/14 cases ambiguous). These results support the hypothesis put forth by White (1978) which suggested that instances of karyotype changes resulting from chromosomal gains and losses, may hold substantial importance in speciation events.

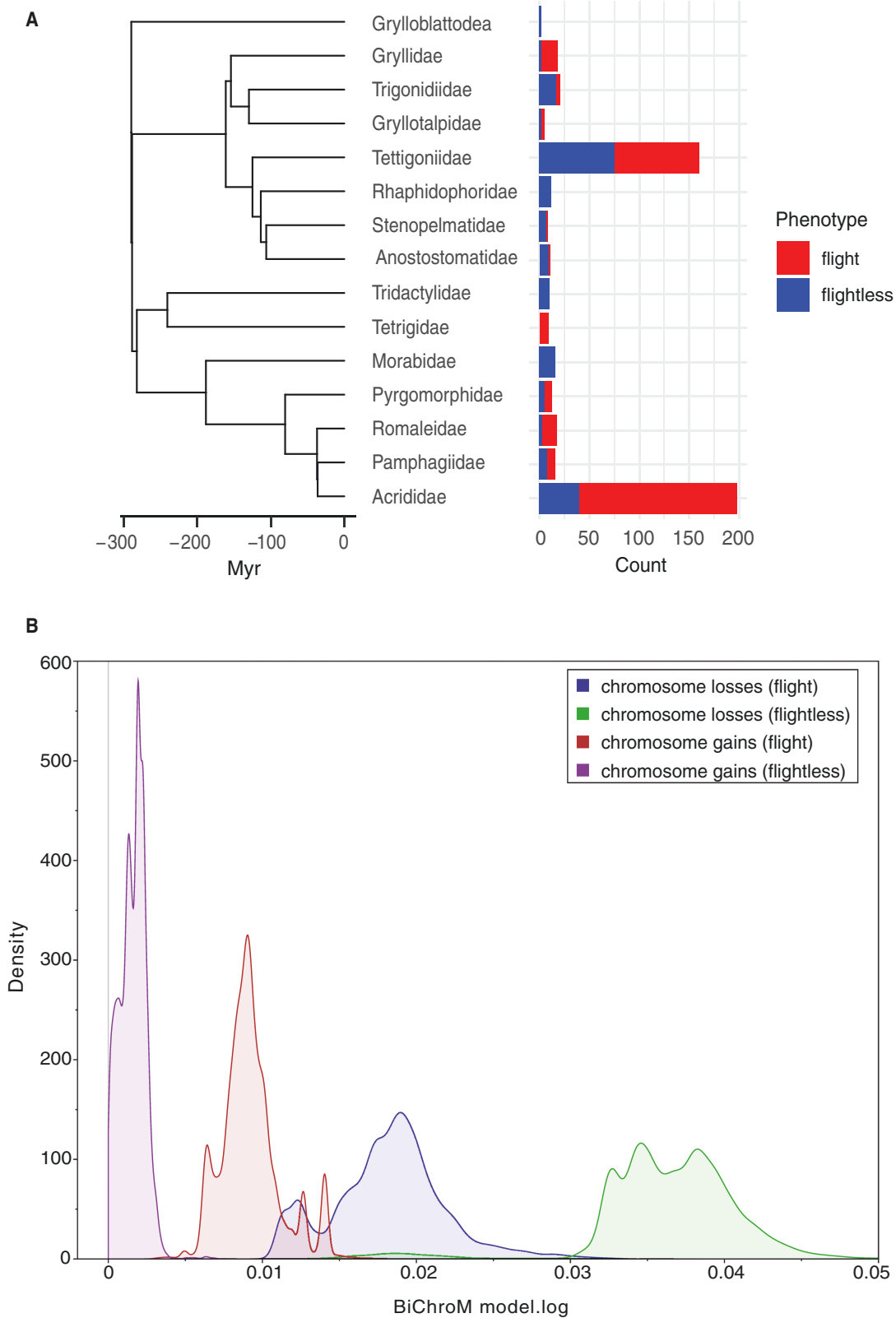
Indeed, chromosomal fusion and fission might have contributed to the emergence of reproductive isolation and speciation in organisms as diverse as apes (Ayala & Coluzzi, 2005), lizards (Leaché et al., 2016), flies (Ayala & Coluzzi, 2005), butterflies (de Vos et al., 2020; Lukhtanov et al., 2005), and plants (De Storme & Mason, 2014).

Our study contributes to the significance of chromosomal fusion and fission as a potential catalyst in the processes of evolutionary diversification in orthopterans. The clades where cladogenetic chromosome changes were prominent (that is, Gryllidae, Gryllotalpidae, Morabidae, Pamphagidae, Rhaphidophoridae, and Stenopelmatidae) have long been recognized for their intricate history of chromosomal fusion and fission, affecting either autosomes only or combinations of autosomes and sex chromosomes (Buleu et al., 2020; Ferreira & Mesa, 2010; Hewitt, 1979; Palacios-Gimenez & Cabral-de-Mello, 2015; Palacios-Gimenez et al., 2015; Vandergast et al. 2017; White, 1978). However, our study represents a statistical demonstration of the prevalence of chromosome-associated speciation within some groups of Orthoptera.

The dichotomy in models supporting or not-supporting non-zero rates of cladogenetic change implies that the evolutionary mechanisms underlying the role of karyotype change in speciation vary across clades. When families undergo pronounced cladogenetic modifications, the selective fixation of karyotype changes and the occurrence of negative interactions among loci in closely related species can establish robust reproductive barriers, acting as Dobzhansky–Muller incompatibilities (Coyne & Orr, 2004). This phenomenon may be further reinforced during secondary contact, where heterozygotes experience diminished fertility due to impaired meiotic segregation, thereby contributing to the long-term speciation processes (King, 1995; White, 1978). Conversely, if karyotype changes primarily occur through anagenesis, the presence of novel rearrangements can disrupt recombination



**Figure 3.** Summary statistics of the rates of fusion and fission in 14 families of Orthoptera. (A) Time calibrated phylogeny (in Myr) based on DNA barcoding 5' region of the mitochondrial cytochrome c oxidase subunit I (COI-5P) gene, pruned to simply represent the phylogenetic relationships among the 14 Orthoptera families and the outgroup (Grylloblattodea). Annotation as in Figure 2A. (B–O) Posterior distributions for total rate of fissions (anagenetic fission + cladogenetic fission, in green) and total rate of fusions (anagenetic fusion + cladogenetic fusion, in purple). All rate parameter are expressed in events per species per million years (events/species/Myr). The two-tailed  $p$  values are shown for each panel.



**Figure 4.** Results of the BiChroM analysis performed in RevBayes. The plot shows the posterior distributions for the rate of chromosome losses ( $\delta$ ) in flightless lineages ( $\delta_1$ ) and winged lineages ( $\delta_0$ ) as well as for the rates of chromosome gains ( $\gamma$ ) in flightless ( $\gamma_1$ ) and winged lineages ( $\gamma_2$ ). All rate parameters are expressed in events per species per million years (events/species/Myr).

and promote the emergence of linked haplotypes that can later act as Dobzhansky–Muller incompatibilities. The influence of anagenetic chromosomal changes on the speciation process is therefore more indirect. Karyotype changes may

play a significant role when they function as intrinsic barriers to gene flow, causing hybrid dysfunction (King, 1995; White, 1978), physically bringing together selected sites (Jay et al., 2021), or involving sex chromosomes (Fraïsse & Sachdeva,

2020). Consequently, chromosomal fusions and fissions may accompany rapid lineage diversification and contribute to the emergence of species radiations, as observed in the case of orthopteran organisms.

Another crucial aspect of karyotype evolution that has never been tested in Orthoptera is whether the rates of karyotype change are associated with flightless phenotype in Orthoptera. We investigated this by fitting the *BiChroM* model. We found that chromosome losses are higher in flightless lineages. Families with a high proportion of flightless lineages (such as Pamphagidae, Trigonidiidae, Rhabdophoridae, and Stenopelmatidae) or those composed solely of flightless lineages with restricted/fragmented distributions (like Rhabdophoridae and Morabidae) are also prominent for chromosome-associated cladogenetic and anagenetic changes (Table 3). This suggests that evolutionary reduction in chromosome number primarily occurred within these lineages. We believe that local chromosomal characteristics can change due to adaptation or genetic drift. This can promote the establishment of reproductive isolation, leading to the differentiation of emerging species (Faria & Navarro, 2010). Different environments impose varying selective pressures, allowing advantageous chromosomal rearrangements to spread and become fixed (Hooper & Price, 2015; Kirkpatrick & Barton, 2006). Species with fragmented distributions and small populations are more prone to the fixation of chromosomal changes by drift (Hooper & Price, 2015; Martinez et al., 2015). High gene flow between populations generally reduces the likelihood of fixation, but strong selective pressure can overcome the homogenizing effect of gene flow (Coyne & Orr, 2004; Martinez et al., 2015; Olson-Manning et al., 2012). Some chromosomal rearrangements, like centric fusions and inversions, can protect beneficial genes and become fixed despite high gene flow (Bidau et al., 2001; Lowry & Willis, 2010). Broad geographical distributions expose species to diverse selective pressures, facilitating the fixation of different rearrangements in different environments.

Overall, we provide a novel and comprehensive perspective on the extent of karyotype diversity within Orthoptera. Notably, our study incorporates significantly enlarged taxonomic sampling, allowing for a comprehensive examination of the evolutionary processes involved. Moreover, we employed modern phylogenomic models within a subset of orthopterans characterized by a notable diversity in terms of karyotypes. Our analysis yielded evidence supporting the macroevolutionary significance of karyotype change, but this impact seems to be variable among clades. The evidence supporting chromosome-associated speciation appears to hold broader significance, extending beyond Orthoptera to encompass diverse animal taxa (Bush et al., 1977; de Vos et al., 2020; Leaché et al., 2016; Potter et al., 2022; Wellband et al., 2019). Lastly, the mode of karyotype evolution of Orthoptera signifies its potential as a valuable resource for investigating the implications of chromosomal rearrangements in evolutionary processes and speciation events. Consequently, it merits heightened attention and further exploration within this context. Future research on phylogenetic chromosome evolution in Orthoptera presents several intriguing avenues for exploration. By integrating our knowledge of chromosome evolution with models of geographic range expansion, we can statistically investigate whether the incidence of cladogenetic chromosome change is more prevalent in sympatric speciation scenarios relative to allopatric speciation instances. This approach would allow us

to assess the interplay between these two distinct processes of reproductive isolation and evolutionary divergence.

## Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

## Data availability

The software and codes used in this study are publicly available, with corresponding versions indicated in Materials and Methods. The datasets and scripts generated as part of the study are available as supplementary information and on Zenodo: <https://doi.org/10.5281/zenodo.14894106>.

## Author contributions

Octavio Manuel Palacios-Gimenez (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration [equal]), Elio Castillo (Data curation, Formal analysis, Investigation [equal]), and Holger Schielzeth (Data curation, Formal analysis, Investigation, Methodology [equal])

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## Conflicts of interest

The authors declare no conflict of interest.

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