Molecular Phylogenetics and Evolution 170 (2022) 107439

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Short Communication

Transcriptomic data reveals nuclear-mitochondrial discordance in Gomphocerinae grasshoppers (Insecta: Orthoptera: Acrididae)

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ARTICLE INFO

Keywords:
Caelifera
Chorthippus
Coalescent
Molecular clock
Pseudochorthippus
Taxonomy

ABSTRACT

The phylogeny of many groups of Orthoptera remains poorly understood. Previous phylogenetic studies largely restricted to few mitochondrial markers found many species in the grasshopper subfamily Gomphocerinae to be para- or polyphyletic, presumably because of incomplete lineage sorting and ongoing hybridization between putatively young lineages. Resolving the phylogeny of the *Chorthippus biguttulus* species complex is important because many morphologically cryptic species occupy overlapping ranges across Eurasia and serve important ecological functions. We investigated whether multispecies coalescent analysis of 540 genes generated by transcriptome sequencing could resolve the phylogeny of the *C. biguttulus* complex and related Gomphocerinae species. Our divergence time estimates confirm that Gomphocerinae is a very young radiation, with an age estimated at 1.38 (2.35–0.77) mya for the *C. biguttulus* complex. Our estimated topology based on complete mitogenomes recovered some species as para- or polyphyletic. In contrast, the multispecies coalescent based on nuclear genes retrieved all species as monophyletic clusters, corroborating most taxonomic hypotheses. Our results underline the importance of using nuclear multispecies coalescent methods for studying young radiations and highlight the need of further taxonomic revision in Gomphocerinae grasshoppers.

1. Introduction

Due to their enormous diversity, the phylogenetic relationships among insects are not as well studied as those of many other animal groups. Particularly, relationships at the intra-order and lower taxonomic levels are often insufficiently understood (e.g., Vedenina and Mugue, 2011). This limitation is partially due to taxonomic sampling gaps in such a diverse group of species, but also likely caused both by incomplete lineage sorting and frequent hybridization in rapid radiations of insect species (Nolen et al., 2020).

While advancements have been made in some highly diverse groups, Orthoptera remain poorly understood. The diverse grasshopper family Acrididae is notorious for its large genome size (up to 18 Gb; Husemann et al., 2020), the abundance of mitochondrial pseudogenes (Pereira et al., 2021), and mitochondrial haplotype sharing (Hawlitschek et al., 2017; Vedenina and Mugue, 2011). These factors limited many standard phylogenetic reconstruction attemps at using few (<10) concatenated genes. By using transcriptome data and multispecies coalescent methods, it is now possible to overcome these limitations and use hundreds of functional genes to estimate species trees for rapid radiations of insects (Nolen et al., 2020).

Some of the more diverse subfamilies of Acrididae constitute a significant portion of the biomass of many biomes, are important parts of foodwebs, and sometimes represent important agricultural pests (Hawlitschek et al., 2017). Yet, species identification is difficult if based on morphology alone (Vedenina and Mugue, 2011). Thus, a better knowledge of the phylogenetic relationships and the ability to identify species within these groups of Orthoptera using molecular tools would be highly desirable. One of the most prominent examples of such a morphologically cryptic complex is the bow-winged grasshopper

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https://doi.org/10.1016/j.ympev.2022.107439
Received 8 March 2021; Received in revised form 2 February 2022; Accepted 3 February 2022
Available online 18 February 2022

Chorthippus biguttulus and its closest relatives. The studies of Hawlitschek et al. (2017, and citations therein) found that *C. biguttulus* shares haplotypes of the standard DNA barcoding gene cytochrome *C* oxidase I with *C. brunneus*, *C. mollis*, *C. apricarius*, and *Gomphocerippus rufus*. Here, we use the term "*C. biguttulus* complex" for the assembly of these five species and its close relatives *C. eisentrauti* (Ingrisch, 1995) and *C. rubratibialis* (Nolen et al., 2020). Hybridization has been hypothesized to play a role in this radiation (Gottsberger and Mayer, 2019), which may further increase gene tree discordance.

The problem of barcode sharing and limited phylogenetic resolution in mitochondrial genes does not only concern the *C. biguttulus* complex but also appears to be widespread in other related genera. For example, Hawlitschek et al. (2017) found *Gomphocerus* and *Stauroderus* to be nested within *Chorthippus*, making the genus paraphyletic. This and many other studies (e.g., Bugrov et al., 2005; Sukhikh et al., 2019; Vedenina and Mugue, 2011) suggest that the current assignment of species to genera is in need of a taxonomic revision, which is challenging considering the total of more than 350 described species across the Holarctic (mostly Palearctic) in these genera (Cigliano et al., 2019). It is thus necessary to use a comprehensive subsampling of this large radiation to test if new methodologies using hundreds of nuclear markers can resolve gene tree discordance in the radiation of these grasshoppers and aid future taxonomic revisions. Here, we attempt to answer the following questions: 1) Do nuclear genes provide a tree topology similar to mitochondrial genes? And: 2) Do the *C. biguttulus* complex and other Gomphocerinae form monophyletic clusters, which would be a prerequisite for molecular species identification?

2. Material and methods

2.1. Sample preparation

We collected 24 samples of 18 species of gomphocerine grasshoppers of the following tribes and genera: Gomphocerini (*Chorthippus*, *Gomphocerus*, *Gomphocerippus*, *Stauroderus*, *Pseudochorthippus*), Stenobothrini (*Stenobothrus*, *Omocestus*), and Chrysochraontini (*Euchorthippus*, *Euthystira*) between July and August 2018 (Supplementary File S1; NCBI Sequencing Read Archive project PRJNA801336, numbers SAMN25339079 to SAMN25339102). All specimens were males identified by bioacoustic characters recorded in the field. We stored all specimens in RNAlater, extracted total RNA using the Qiagen RNeasy Mini Kit, and sent the samples to a commercial supplier (BGI, Hong Kong) for mRNA enrichment, library preparation, and sequencing on the DNBSEQ platform. We supplemented our dataset with *Chorthippus* transcriptome data from GenBank originally published in Berdan et al. (2015) and Nolen et al. (2020) (N = 9). We used data of *Locusta migratoria* (Wang et al., 2014) as an outgroup.

2.2. Data processing

Raw sequencing reads were adaptor-trimmed and quality-filtered using 'bbduk' from BBTools v. 38.73 (https://jgi.doe.gov/data-and-tools/bbtools/). Adaptor reference sets were platform-specific to accommodate the samples sequenced either on Illumina's HiSeq 2000 or BGI's DNBSEQ platforms. Two consecutive rounds of adaptor trimming were performed, during which poly-A tails were also removed. Leading and trailing bases with a PHRED quality score lower than 10 were trimmed, and whole reads with an average PHRED quality score lower than 12 were excluded from downstream analyses.

Clean reads were *de novo* assembled with MEGAHIT v. 1.2.9 (https://doi.org/10.1093/bioinformatics/btv033) using the options '–no-mercy, –k-min 27, –k-max 147, and –k-step 24'. Mitochondrial contigs were identified using the BLAST feature within the genomic visualization tool Bandage v. 0.8.1 (Wick et al., 2015). These contigs were annotated using Geneious v. 11.1.5 (Kearse et al., 2012), followed by the extraction of protein-coding genes for building alignments. The remaining (non-mitochondrial) assembled contigs were clustered across samples at 75% identity using MMseqs2 v. 11-e1a1c (Steinegger and Söding, 2018). Then, clusters were aligned using MAFFT v. 7.455 (Katoh et al., 2009, 2002) with options '–max-iterate 1000 –adjust-direction'. Clusters were selected using custom Python scripts so that selected alignments had to 1) contain at least half the samples (N = 19), and 2) contain a single copy of the marker (i.e. single sequence per sample) to exclude potential paralogs. Such filtering resulted in 540 alignments with a concatenated length of 791,978 sites, of which 92,112 were informative, with a global 14.4% of missing data. The size of the alignments ranged from 496 to 8,399 sites (average 1,466.6) containing 23 to 39 sequences (average 32.9).

2.3. Phylogenetic analyses and divergence time dating

We conducted two separate analyses for the datasets containing 1) all linked 13 mitochondrial protein-coding genes, the two rRNA genes, and the control region (D-loop), and 2) all independent 540 nuclear transcripts. We used the mitochondrial dataset for estimating divergence times, assuming a molecular clock. We applied Partitionfinder v. 2.1.1 (Lanfear et al., 2016) to find the best partitioning scheme (Supplementary File S2). After initial runs with these models resulted in low effective sample size also at 100 m + generations, we selected the less complex HKY + G model for all partitions to achieve sufficient effective sample sizes. We then reconstructed a phylogeny with a log-normal relaxed clock in BEAST v. 2.6.1 (Bouckaert et al., 2019). We calibrated the molecular clock using splitting events for which time estimates were available from a previous study by Song et al. (2015): 1) the split of the lineages comprising *Locusta* and *Stauroderus* at 37.9 ± 3.1 mya, and 2) the split between *Euchorthippus* and *Stauroderus* at 12.2 ± 5.6 mya. We ran BEAST twice for 10 million generations, sampling every 1,000 generations. We checked for convergence and sufficient effective sample size in Tracer v. 1.7.1 (https://beast.community/tracer) and removed 10% burn-in.

We reconstructed individual Maximum Likelihood trees of all 540 genes separately in IQ-TREE v. 2.0-rc1 (Minh et al., 2020b). A phylogenomic Concordance Factor analysis (Minh et al., 2020a) was run on the 540 nuclear alignments with IQ-TREE following the guidelines detailed in http://www.iqtree.org/doc/Concordance-Factor. This analysis estimated a phylogeny based on the concatenation of all the nuclear markers (i.e., concatenated species tree) with support from 1,000 ultrafast bootstraps (UFB) (Hoang et al., 2018), followed by the estimation of a phylogeny for each locus (i.e., gene trees). The percentage of decisive gene trees (gCF or gene concordance factor) supporting every node in the species tree was added as node support. Furthermore, in order to mitigate the potential support bias on the gCF caused by including gene trees with poor resolution (i.e., increased phylogenetic estimation uncertainty), the percentage of decisive alignment sites supporting a node was also added to the species tree (sCF, site concordance factor).

After that, we conducted a multispecies coalescent analysis using ASTRAL-II v. 5.7.5 (Mirarab and Warnow, 2015) to reconstruct a species tree from the 540 independent gene trees, using the default settings and assessing node support via local posterior probability. We visualized the trees using FigTree v. 1.4.4. and Inkscape v. 0.92.

2.4. Testing phylogenetic incongruence

In order to determine the statistical significance of the phylogenetic incongruence between the mitochondrial and nuclear phylogenies, we used the discordance tests included in IQ-TREE v. 2.0-rc1 (see references in Supplementary File S3). A set of 98 topologies was considered for the tests: the coalescent nuclear species tree, the mitochondrial tree, and the 96 gene trees (out of 540) containing the full set of 38 taxa, all with branch lengths removed. We then tested these 98 topologies against 1) the mitochondrial alignment and 2) the concatenated alignment of the 540 nuclear genes, calculating the likelihoods of each tree to either accept or reject topologies according to their fit to the data. The tests were run with the command: 'iqtree2 -s alignment.fasta -n 0 -z

trees_to_test.trees -zb 10,000 -au -zw -nt 8 -pre test_alignment'. In order to visually assess and highlight the discordance, we used the function 'cophyloplot' from the R package *phytools* v. 0.7–70 (Revell, 2012), which plots both topologies side by side after rotating branches and matching taxa names whenever possible.

Furthermore, we tested mito-nuclear discordance at contentious nodes one-by-one, similar to the methods described in Shen et al., (2017). We tested three alternative topologies of the nuclear phylogeny: the unaltered topology resulting from our coalescent analysis; *C. apricarius* as sister to the *C. biguttulus* complex; and *O. viridulus* as sister to the other *Omocestus* species. We also tested eight alternative topologies of the mitochondrial phylogeny: the unaltered topology resulting from our BEAST analysis; *Pseudochorthippus* as sister to the rest of Gomphocerinae; *O. haemorrhoidalis* as sister to the other *Omocestus* species; *C. alticola* as sister to *S. scalaris* + *G. sibiricus*; and monophyly of each *C. biguttulus*, *C. brunneus*, *C. eisentrauti*, and *C. mollis*. We then used IQ-TREE v. 2.0-rc1 to calculate site-wise (-wsl) and partition-wise (-wpl) likelihoods for each alternative topology and produced plots showing the realtive support of each site or partition for a particular node as shown in Shen et al. (2017).

3. Results

The phylogenetic tree reconstructed from mitochondrial data (Fig. 1) showed overall very high posterior probability on all nodes (all nodes listed below are supported with pp = 1). Chrysochraontini (*Euchorthippus* + *Euthystira*) were retrieved as monophyletic and sister to all other ingroup taxa. *Pseudochorthippus* + (*Stenobothrus* + *Omocestus*) were all found reciprocally monophyletic and formed a clade that was estimated to split from other ingroup taxa at 13.77 (95% HPD: 21.30–9.53) mya, leaving Gomphocerini polyphyletic due to the placement of *Pseudochortippus* and *Chorthippus* as unrelated groups. Stenobothrini (*Stenobothrus* + *Omocestus*) was retrieved as monophyletic, and *O. rufipes* was paraphyletic with regard to *O. haemorrhoidalis*. *Gomphocerippus*, *Gomphocerus*, and *Stauroderus* were nested within *Chorthippus*. The *C. biguttulus* complex was retrieved as a clade in which *C. apricarius* was sister to all other members, splitting from *Gomphocerus* + *Stauroderus* at 5.27 (6.61–4.08) mya. *Chorthippus biguttulus*, *C. brunneus*, *C. eisentrauti*, *C. mollis*, and *Gomphocerippus rufus* formed a clade estimated to 1.38 (2.35–0.77) mya, but none of these species were retrieved as monophyletic (only one sample of *G. rufus* was included). The oldest split within this clade was dated to 0.94 (1.75–0.58) mya.

The coalescent species tree reconstructed from nuclear data (Fig. 2) also showed overall high support of 0.99 or 1 for all nodes outside the

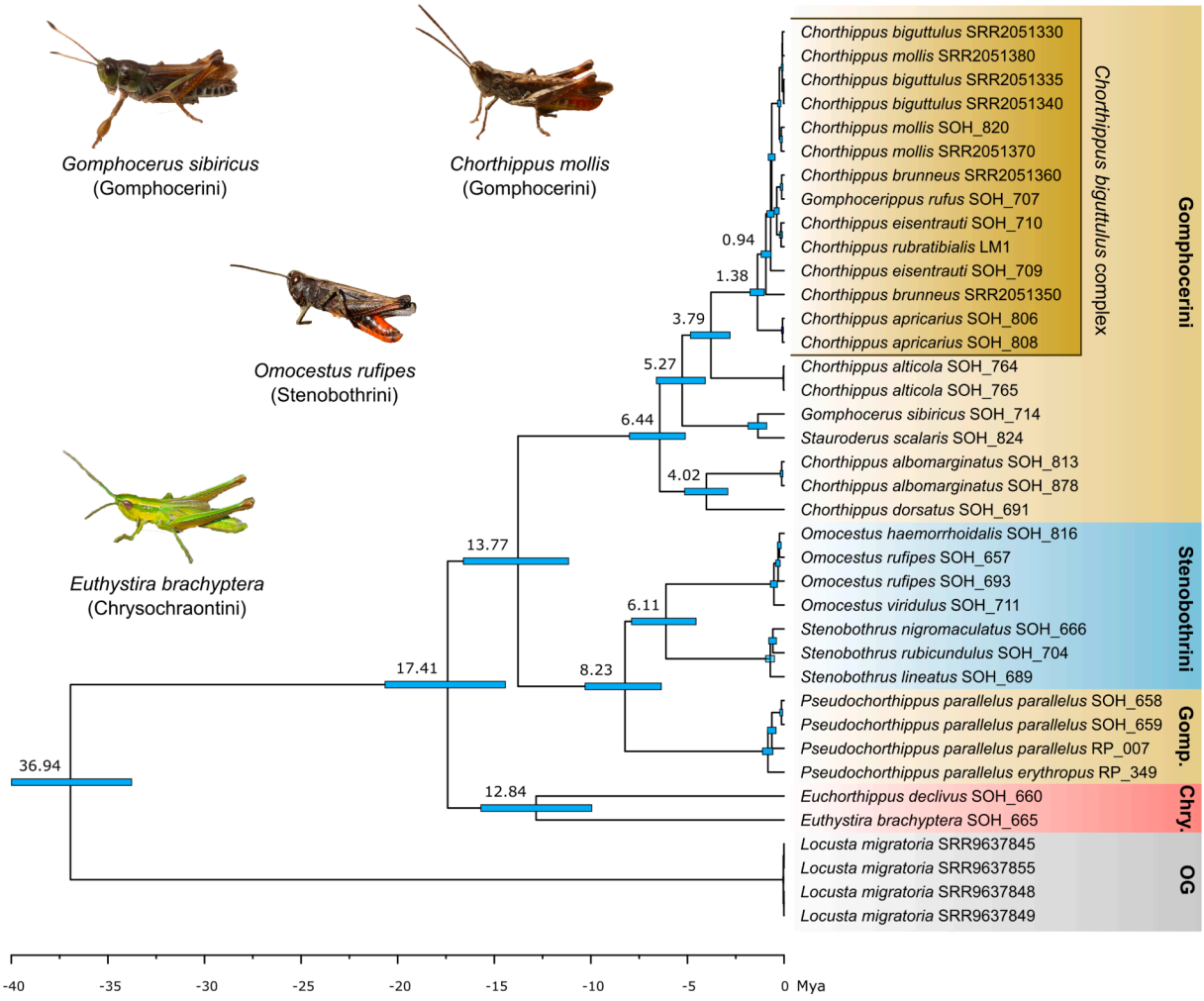

Fig. 1. Chronogram of the study species of *Chorthippus* and related genera, reconstructed in BEAST v. 2.6.1 based on mitochondrial genes. Node age estimates are given above nodes, blue bars represent 95% HPD. All nodes for which age estimates are given are supported with pp = 1. Colors highlight the *Chorthippus biguttulus* complex and the tribes of the Gomphocerinae subfamily represented here: Gomp. = Gomphocerini (retrieved as paraphyletic in the mitochondrial tree), Stenobothrini, and Chry. = Chrysochraontini. OG = Outgroup, belonging to the subfamily Oedipodinae. Photographs by OH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

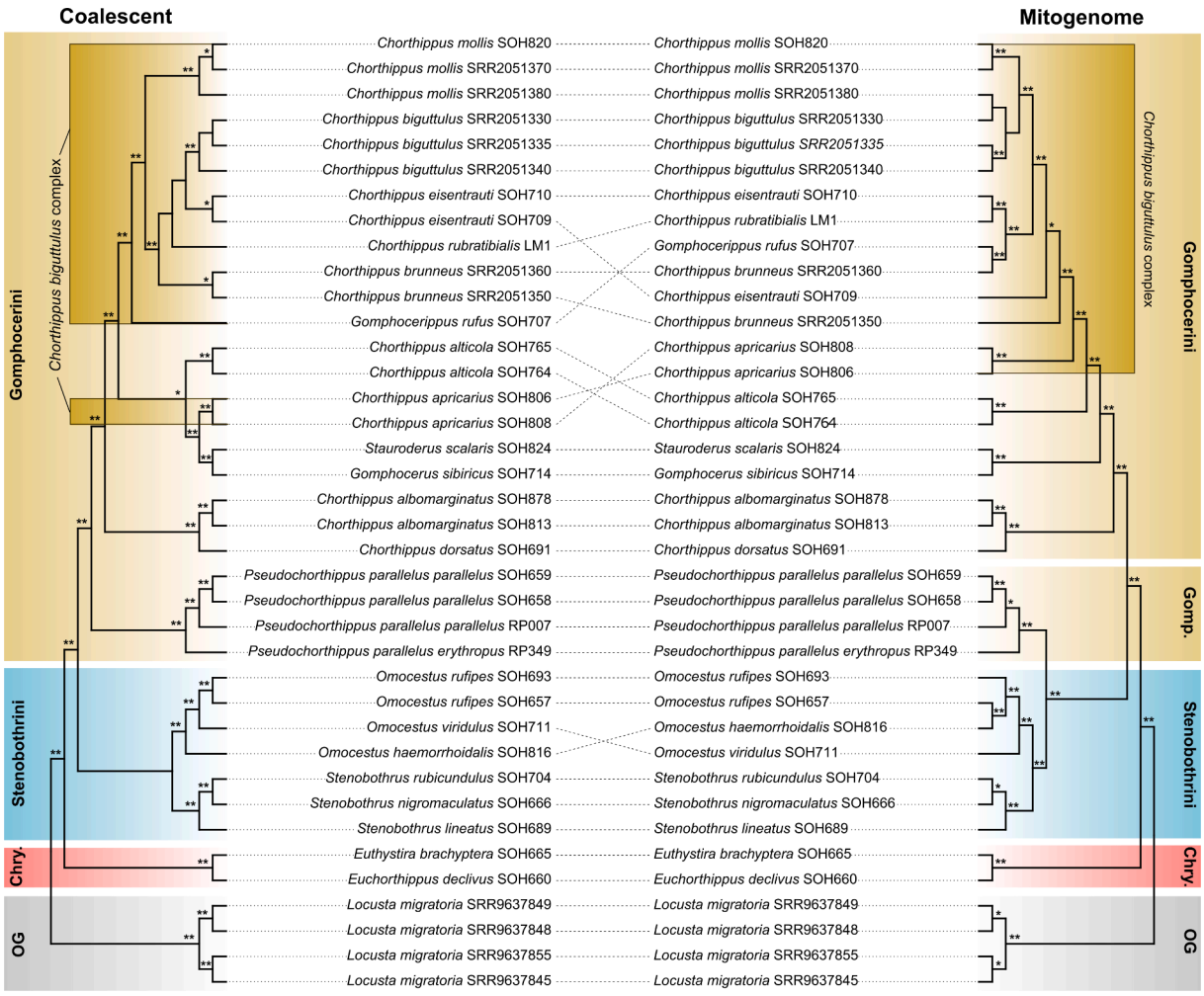

Fig. 2. Coalescent tree of the study species of *Chorthippus* and related genera, based on 540 genes extracted from transcriptomes and reconstructed in ASTRAL-II, plotted against the tree generated from the mitochondrial data. Support values are shown above nodes: ** = 1, * = 0.80–0.99. Gomp. = Gomphocerini; Chry. = Chrysochraontini; OG = Outgroup, belonging to the subfamily Oedipodinae.

C. biguttulus complex and shared an identical topology with the phylogeny obtained by the Concordance Factor analysis in IQ-TREE. The topology disagreed with that of the mitochondrial tree in several points. Gomphocerini was retrieved as monophyletic in the nuclear species tree, while it was polyphyletic in the mitochondrial tree. *Pseudochorthippus* was placed as the sister group to *Chorthippus* + all genera nested therein in the nuclear tree, while it was sister to Stenobothrini in the mitochondrial tree. *Chorthippus apricarius* was retrieved as sister to *Gomphocerus* + *Stauroderus*, while sister to the other members of the *C. biguttulus* complex in the mitochondrial tree. Unlike in the mitochondrial tree, all species of the *C. biguttulus* complex, except *C. apricarius*, were found reciprocally monophyletic, with *Gomphocerippus rufus* retrieved as sister to all species of *Chorthippus* in this clade.

We tested for phylogenetic incongruence between the mitochondrial gene tree and the coalescent species tree based on nuclear markers (Supplementary File S3). All topologies except the mitochondrial topology were rejected by the mitochondrial alignment ($p = 0.0467–6.21E-156$). Similarly, the only topology accepted by the nuclear alignment was the nuclear species tree topology, and all other topologies were rejected ($p = 5.80E-5–5.08E-159$). The individual UFB, gCF, and sCF node values are given in Supplementary File S4. gCF and sCF are < 95 for all ingroup nodes except gCF for the split between Chrysochrantini and Gomphocerini + Stenobothrini and gCF/sCF for the split between the two individuals of *C. albomarginatus*. These results indicate that the phylogenetic incongruence between the trees is statistically significant. Not a single nuclear gene tree out of the 96 trees tested showed concordance with either the mitochondrial or the coalescent species tree, reflecting a general conflict among independent gene trees.

Separate testing of specific alternative topologies at contentious nodes also revealed a pervasive pattern of mito-nuclear discordance (Supplementary File S5). The majority of nuclear genes (greater than 65%) supported the original nuclear topology. In contrast, mitochondrial data showed no support for nuclear topologies, reflecting a discordance between the coalescent and mitochondrial phylogenies. Alternative mitochondrial topologies received only minor support.

4. Discussion

This study assessed the power of transcriptomic data to resolve the phylogenetic relationships of the rapid radiation of Gomphocerinae grasshoppers. We found that the topology of the species tree based on 540 nuclear genes differed from the topology of a mitogenome tree in several points. Unlike this and earlier mitochondrial trees (Hawlitschek et al., 2017; Sukhikh et al., 2019; Vedenina and Mugue, 2011), our species tree retrieved all species, including those of the *Chorthippus biguttulus* complex, as monophlyetic.

All results must be treated with caution due to the necessarily limited sampling of taxa from a radiation with more than 350 species. Nevertheless, our finding of mito-nuclear discordance can only increase with

further sampling of this radiation. Most importantly, this study demonstrates the potential of using transcriptomic data and multispecies coalescent methods to estimate a well-supported species tree despite extensive discordance among independent gene trees. This represents an advancement towards a more comprehensive phylogeny of Acrididae and towards finding a method of molecular identification in taxonomically challenging species, such as the *C. biguttulus* complex (Hawlitschek et al., 2017).

The subfamily Gomphocerinae and specifically the *C. biguttulus* complex have been suggested to be of young age based on the shallow genetic distances found using few mitochondrial markers (Hawlitschek et al., 2017; Sukhikh et al., 2019; Vedenina and Mugue, 2011). We estimated the divergence within the *C. biguttulus* complex (excluding *C. apricarius*) to < 1.75 mya, placing it in the Quaternary. Nolen et al. (2020), using rates of mitochondrial evolution, estimated the split between *C. biguttulus*, *C. brunneus*, and *C. mollis* to 0.51 (0.40–0.62) mya (using the rate of Brower, 1994) or 0.49 (0.39 – 0.59) mya (using the rate of Papadopoulou et al., 2010). This is slightly younger than our estimate of 0.94 (1.75–0.58) mya but is also in the Pleistocene. Nolen et al. (2020) estimated the split between *Pseudochorthippus* and *Chorthippus* to 5.90 (4.67–7.14)/5.64 (4.55–6.80) mya, which is significantly younger than our estimate of 13.77 (21.30–9.53) mya. This may be caused by the differences in taxon sampling and the topologies retrieved: in our mitochondrial tree (but not in the nuclear tree) *Pseudochorthippus* clustered with *Stenobothrus* + *Omocestus*, which were not included in the tree of Nolen et al. (2020). Nevertheless, divergence times estimated here and by previous studies (Bugrov et al., 2005; Hawlitschek et al., 2017; Vedenina and Mugue, 2011; Nolen et al. 2020) suggest that climate oscillations have likely played an important role in the speciation of this complex.

Besides our molecular clock analyses based on mitochondrial data, our observation of strong discordance among independent nuclear gene trees (Fig. 2) is also consistent with a very recent radiation of the *Chorthippus biguttulus* complex, which can explain the incomplete lineage sorting observed in mitochondrial markers by this and previous studies (Hawlitschek et al., 2017; Nolen et al., 2020; Vedenina and Mugue, 2011). We focused our topology tests on the mitochondrial genome, because, while representing a single gene tree, mitochondrial data has been, and will continue to be, instrumental for understanding the distribution and abundance of these species (Bugrov et al., 2005; Contreras and Chapco, 2006; Sukhikh et al., 2019; Vedenina and Mugue, 2011). Our topology testing shows that the phylogenetic discordance observed between the mitochondrial and the nuclear estimates are statistically significant, implying that future taxonomic revisions of this group should not be based on mitochondrial data alone, even if the entire mitochondrial genome is considered. Discordance affected not only very young nodes: the oldest incongruent node is dated to 5.27 mya. We also determined that not a single nuclear gene tree was fully concordant with the species tree, underlining the importance of multigene coalescent methods for reconstructing the phylogenies, especially of young radiations and for future taxonomic revisions. Therefore, we consider our multispecies coalescent tree as the best reflection of the real evolutionary relationships within the study group.

In contrast to our mitochondrial analysis and earlier studies (Bugrov et al., 2005; Contreras and Chapco, 2006; Sukhikh et al., 2019; Vedenina and Mugue, 2011), our nuclear species tree retrieves all species for which more than one sample was included as monophyletic. The exceptions are *C. brunneus*, *C. biguttulus* + *C. eisentrauti*, and *C. rubratibialis*, which form a polytomy. Nolen et al. (2020) also could not resolve the relationship between *C. rubratibialis* and its close relatives using nearly 4,000 nuclear gene trees, suggesting a biological rather than methodological explanation for this lack of phylogenetic resolution. An acoustic study (Ragge et al., 1990) hypothesized that *C. rubratibialis* may have a hybrid origin of *C. biguttulus* and *C. mollis*. Likewise, the species status of *C. eisentrauti* has been challenged (Perdeck, 1957, but see Ingrisch, 1995). In agreement, we find extensive mito-nuclear discordance in this species, suggesting that hybridization may be an additional source of gene tree discordance that should be considered in future studies.

All other members of the *C. biguttulus* complex are distinct with well-supported clusters in the tree. This was already established for *C. biguttulus*, *C. brunneus*, and *C. mollis* by Nolen et al. (2020), but we find this result to be robust to the inclusion of other closely related taxa such as *C. eisentrauti* that share mitochondrial lineages. This indicates that these morphologically cryptic species can be distinguished using multiple nuclear loci and corroborates the species status of all taxa, which has been disputed despite ecological and bioacoustical differences (Ramme, 1921).

In line with previous studies (Hawlitschek et al., 2017; Vedenina and Mugue, 2011), our analysis places *Pseudochorthippus parallelus* outside of *Chorthippus* – in our case as sister to all *Chorthippus* species included. Our study is the first to use genomic data from species of the subgenus *Chorthippus,* specifically *C. albomarginatus* and *C. dorsatus*. This supports the erection of the genus *Pseudochorthippus* as proposed by Defaut (2012) based on two mitochondrial genes alone, and we advocate the use of this name in all fields of biology. We note that the name has already been implemented by the Orthoptera Species File (Cigliano et al., 2019) and has been used in evolutionary studies and faunistic literature for several years.

The placement of *Gomphocerus*, *Stauroderus,* and *Gomphocerippus* within *Chorthippus* in our tree supports the view that *Chorthippus* is in need of revision. Either *Chorthippus* will have to be split, as already suggested by Storozhenko (2002), or the other species will have to be assigned to *Chorthippus*. Our tree includes the type species of *Chorthippus,* subgenus *Chorthippus* (Fieber, 1852), *C. albomarginatus* (De Geer, 1773) but not the type species of *Chorthippus* subgenus *Glyptobothrus* (Chopard, 1951), *C. binotatus* (Charpentier, 1825). Due to this, and due to the still limited taxon sampling of our study, we advocate for postponing any taxonomic changes until more data becomes available. Nevertheless, we note that the methodology used here can easily be scaled up to include more taxa, or alternatively, probes could be designed based on the genes we used to estimate the species tree in order to extend sampling even to museum specimens by using sequence capture methods. These approaches will likely help to establish sound phylogenetic relationships in grasshopper radiations, despite extensive gene discordance.

CRediT authorship contribution statement

Oliver Hawlitschek: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. **Edgardo M. Ortiz:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft, Writing – review & editing. **Sajad Noori:** Formal analysis, Visualization. **Kathleen C. Webster:** Formal analysis, Methodology. **Martin Husemann:** Writing – review & editing. **Ricardo J. Pereira:** Conceptualization, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Carmen Argudo Peña, Amelie Höcherl (Munich), Michael Gebhardt (Freising), Inge Illich (Salzburg), and Günther Wöss (Vienna) for field sampling support. This work was supported by the German Research Foundation (DFG) grants HA7255/2-1 to OH and Stefan Schmidt, Munich, and SCHA1875/4-1 to Hanno Schäfer, Freising, and benefitted from the sharing of expertise within the DFG priority program

SPP 1991 Taxon-Omics.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2022.107439.

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