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## Phylogenetic insights into Central European *Chorthippus* and *Pseudochorthippus* (Orthoptera: Acrididae) species using ddRADseq data

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### ABSTRACT

The evolution of several orthopteran groups, especially within the grasshopper family Acrididae, remains poorly understood. This is particularly true for the subfamily Gomphocerinae, which comprises cryptic sympatric and syntopic species. Previous mitochondrial studies have highlighted major discrepancies between taxonomic and phylogenetic hypotheses, thereby emphasizing the necessity of genome-wide approaches.

In this study, we employ double-digest restriction site-associated DNA sequencing (ddRADseq) to reconstruct the evolution of Central European *Chorthippus* and *Pseudochorthippus* species, especially *C. smardai*, *P. tatrae* and the *C. biguttulus* group.

Our phylogenomic analyses recovered deep discordance with mitochondrial DNA barcoding, emphasizing its unreliability in Gomphocerinae grasshoppers. Specifically, our data robustly distinguished the *C. biguttulus* group and confirmed the distinctiveness of *C. eisentrauti*, also shedding light on its presence in the Berchtesgaden Alps. Moreover, our results support the reclassification of *C. smardai* to the genus *Pseudochorthippus* and of *P. tatrae* to the genus *Chorthippus*.

Our study demonstrates the efficiency of high-throughput genomic methods such as RADseq without prior optimization to elucidate the complex evolution of grasshopper radiations with direct taxonomic implications. While RADseq has predominantly been utilized for population genomics and within-genus phylogenomics, its application extends to resolve relationships between deeply-diverged clades representative of distinct genera.

### 1. Introduction

The insect order Orthoptera exhibits a remarkable species diversity, with 29,846 species currently recognized worldwide (Cigliano et al., 2023). Despite extensive research, the phylogeny of various orthopteran groups, particularly within the cosmopolitan grasshopper family Acrididae, remains poorly understood (Vedenina & Mugue, 2011; Hawlitschek et al., 2022). The intricate evolutionary history of Acrididae is characterized by multiple dispersal events across biogeographic realms (Song et al., 2018), Quaternary dynamics of expansions and

diversifications (Hewitt, 1999), as well as radiation by sexual selection (Nolen et al., 2020) and has given rise to a vast diversity of 6,898 species assigned to 1,425 genera as of today (Cigliano et al., 2023).

Within the family Acrididae, the subfamily Gomphocerinae is of substantial interest in evolutionary biology, ecology, and biogeography (Song et al., 2018; Gottsberger & Mayer, 2007; Hewitt, 1999). It so far encompasses 1,299 species across 194 genera (Cigliano et al., 2023), including cryptic grasshopper species with similar morphologies, and ecological niches, often occurring in sympatry. Earlier genetic studies have shown wide – though often inconclusive – discrepancies between

**Abbreviations:** ddRADseq, Double-digest restriction site-associated DNA sequencing; M.A.S.I, Meters above sea level; ML, Maximum-Likelihood; NUMT, nuclear mitochondrial pseudogene; OG, Outgroup; OSF, Orthoptera Species File.

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the currently accepted taxonomy and phylogenetic hypotheses (Vedenina & Mugue, 2011; Hawlitschek et al., 2022). This is symptomatic of the genera *Pseudochorthippus* and *Chorthippus*, especially in the subgenus *Chorthippus* (*Glyptobothrus*). According to current taxonomy, *Chorthippus* encompasses 230 species and the recently recognized genus *Pseudochorthippus* (Defaut, 2012) only four species (Cigliano et al., 2023). However, since there are still ambiguities in the classification of the species of both these genera (Chládek, 2014; Kristín et al., 2020), it is necessary to clarify their molecular affiliation and relationships.

Historically, species identification and description in Gomphocerinae have relied predominantly on morphological traits, but also on bioacoustics. The specific songs produced by males are particularly useful for identification and have recently also been used for cladistic reconstructions (Song et al., 2020; Sevastianov et al., 2023). However, this approach proves insufficient for the identification and classification of female individuals, as in many species they either lack the ability to produce sounds or do so to a limited extent only (Sevastianov et al., 2023). In addition, the occurrence of hybrids has the potential to confuse stridulation-based species identification since especially the wing morphology and therefore stridulation-patterns can differ significantly (Gottsberger & Mayer, 2007). Therefore, molecular phylogenetics are necessary to accurately identify and classify the species of Gomphocerinae grasshoppers.

DNA barcoding using a single mitochondrial marker has been the common practice for the genetic classification of organisms for many years, although it has proven unreliable in some groups, such as Acrididae (Hawlitschek et al., 2017). Factors like large genome sizes of up to 22 Gb (Hawlitschek et al., 2023), the abundance of mitochondrial pseudogenes (Pereira et al., 2021), and mitochondrial haplotype sharing (Vedenina & Mugue, 2011; Hawlitschek et al., 2017) due to hybridization or incomplete lineage sorting (Nolen et al., 2020) have limited the effectiveness of mitochondrial DNA barcoding and traditional multi-gene studies in this family. These factors underscore the importance of high-throughput methods to analyze many genetic markers simultaneously (Hawlitschek et al., 2017 and 2022).

In this study, we utilize double-digest restriction site-associated DNA sequencing (ddRADseq) to provide a genomic high-throughput method for the simultaneous analysis of more than 6,700 nuclear markers of Central European *Chorthippus* and *Pseudochorthippus* species, aiming to advance our understanding of their phylogenetic relationships and taxonomy. We specifically aimed at addressing two key questions: a) The systematics of *C. smardai* and *P. tatrae*, utilizing genetic data of these species for the first time, and b) unraveling the *C. biguttulus* group (i.e., *C. biguttulus*, *C. brunneus*, *C. mollis*, and *C. eisentrauti*; Cigliano et al., 2023), with a particular emphasis on determining the position of *C. eisentrauti* within the group. Through the generation of large-scale genomic data, ddRADseq enables the resolution of phylogenetic relationships and provides insights into population genetics, contributing to our broader knowledge of Orthoptera evolution and diversity. To address the nuclear-mitochondrial discordance and to generate barcodes for *C. smardai* and *P. tatrae* we also conducted mitochondrial barcoding.

## 2. Materials and methods

### 2.1. Sampling and DNA extraction

A total of 65 specimens were sampled, comprising ten species of *Chorthippus*, three of *Pseudochorthippus*, and *Stenobothrus crassipes* as outgroup species (Table S1). We followed the taxonomic placement of all species as currently listed on the Orthoptera Species File (Cigliano et al., 2023). The species encompassed all *Chorthippus* and *Pseudochorthippus* species occurring in Germany, augmented by selected species from Slovakia, Austria, and Poland based on sample availability. The specimens, collected and identified by the authors or collaborators, were preserved either in ethanol or by drying. Given the difficulties in identifying female specimens, particularly within the *C. biguttulus* group,

mostly males were sampled and identified based on both morphological and bioacoustic characteristics. A few specimens were temporarily categorized as *C. cf. eisentrauti* due to bioacoustic characters typical for *C. eisentrauti*, i.e., number of verses of male song up to six. However, these specimens otherwise resembled *C. biguttulus* and originated from localities where *C. eisentrauti* had not been detected before. Only the specimen OH22\_11, found in a confirmed *C. eisentrauti* habitat, was identified as a 'true' *C. eisentrauti* based on full song recordings.

Total DNA was extracted from hind femora using a standard DNA salt extraction protocol (Bruford et al., 1992) or the DNeasy Blood & Tissue Kit (QIAGEN). The manufacturer's recommended procedures were followed, and elution was performed in 100 µl elution buffer. To confirm the success of the DNA extraction, we conducted a 1 % agarose gel electrophoresis.

### 2.2. DNA barcoding

For mitochondrial barcoding, the universal primer pair dgLCO1490 and dgHCO2198 (Meyer et al., 2005) was used to amplify a fragment of the cytochrome *c* oxidase subunit I (CO1) gene. In cases where these primers did not work, the Orthoptera-specific primer pair COBU and COBL was utilized (Huang et al., 2013). Amplification was conducted using a standard Taq-Polymerase and primer-specific thermal cycling conditions (Tables S2-S4). PCR products underwent purification using Exo-SAP (Table S5 and S6). Subsequently, the purified PCR products were dispatched to the external company, LGC Genomics (Berlin, Germany), for Sanger sequencing. The obtained raw CO1 sequences underwent quality control and editing using CodonCode Aligner (CodonCode Corporation). Sequence data and metadata were uploaded to Genbank (accession numbers: OR780489 – OR780553).

In the construction of the mitochondrial reference tree, a 622 bp fragment of the Cytochrome *c* oxidase subunit I (CO1) was used, except for the *C. smardai* samples where only 298 bp were of sufficient quality. Subsequently, maximum likelihood phylogenies were reconstructed using IQTree 2.2.0 (Minh et al., 2020). The best substitution model was determined using ModelFinder (Kalyaanamoorthy et al., 2017), and branch support was assessed by performing 1,000 replicates of ultrafast bootstraps (UFBoot) (Hoang et al., 2018). The resulting trees were visualized using FigTree (Rambaut & Dummer, 2012) and CorelDraw X7 (Corel Corporation).

### 2.3. ddRAD sequencing

ddRADseq was employed, following an adapted version of the protocol by Brelsford et al. (2016) [<https://dx.doi.org/10.17504/protocols.io.kxygx3nzwg8j/v1>]. Enzymatic digestion was performed using SbfI and MseI restriction enzymes, and unique 4–8 bp barcodes on the SbfI end were utilized for adaptor ligation. Fragments ranging between 400 and 500 bp were selected by performing a gel extraction using the Monarch DNA Gel Extraction Kit (New England Biolabs) and purified for further analysis. The purified libraries underwent paired-end sequencing on an Illumina NextSeq platform using a High Output 150 Cycles kit (2 x 75 bp).

Raw reads were processed with STACKS 2.6.1 (Catchen et al., 2013), involving the following steps: demultiplexing, data filtering, and trimming to 65 bp (*process\_radtags*), de novo assembling and cataloging (*denovo\_map.pl*), as well as SNP calling (*populations*). The Stacks catalog contained 1,755,588 loci with an average coverage of 12.1x per locus. Default stacking parameters (-m, -n and -M) were applied, following the suggestions by Paris et al. (2017). For each sub-dataset, the filtering options (-p and -r) were optimized to achieve a balance between the number of loci and missing data across all species.

### 2.4. Phylogenetic and population genomic analyses of ddRAD data

For the RAD phylogeny, a total of 6,763 loci (135,221 variant sites)

were obtained using the *populations* module (n = 65, -p 32 and -r 1) of STACKS by using p as the number of individuals. The alignment was exported with the *-phylip-var-all* option. For phylogenetic analyses, we utilized identical software and analytical procedures employed in the DNA barcoding analysis.

Genotype matrices for the main dataset (including all species) and two sub-datasets (*C. biguttulus* group and genus *Pseudochorthippus*) were generated in STACKS by considering only unlinked markers, utilizing the filter option *-write-random-snp*. For the main dataset, a total of 872 SNPs (n = 64, -p 50 and -r 1) were identified, while the *C. biguttulus* group dataset yielded 970 SNPs (n = 35, -p 33 and -r 1), and the *Pseudochorthippus* dataset 3,808 SNPs (n = 11, -p 11 and -r 1).

Individual ancestries for each of these datasets were estimated using Structure V.2.3.4 (Pritchard et al., 2000). The admixture model was applied without prior assignment of localities (LOCPRIOR). Twenty replicates were performed for each K value (K = 1 to K = n<sub>species</sub> + 1), with 100,000 iterations executed after a burn-in period of 10,000. To mitigate the impact of outlier runs (i.e., that did not converge), the ten best replicates of each K value were selected based on the estimated Ln probability of data. Summation and graphical representation of the structure results were achieved using CLUMPAK (Kopelman et al., 2015). The rate of likelihood increases and the ΔK index were determined using STRUCTURE HARVESTER (Earl & vonHoldt, 2012).

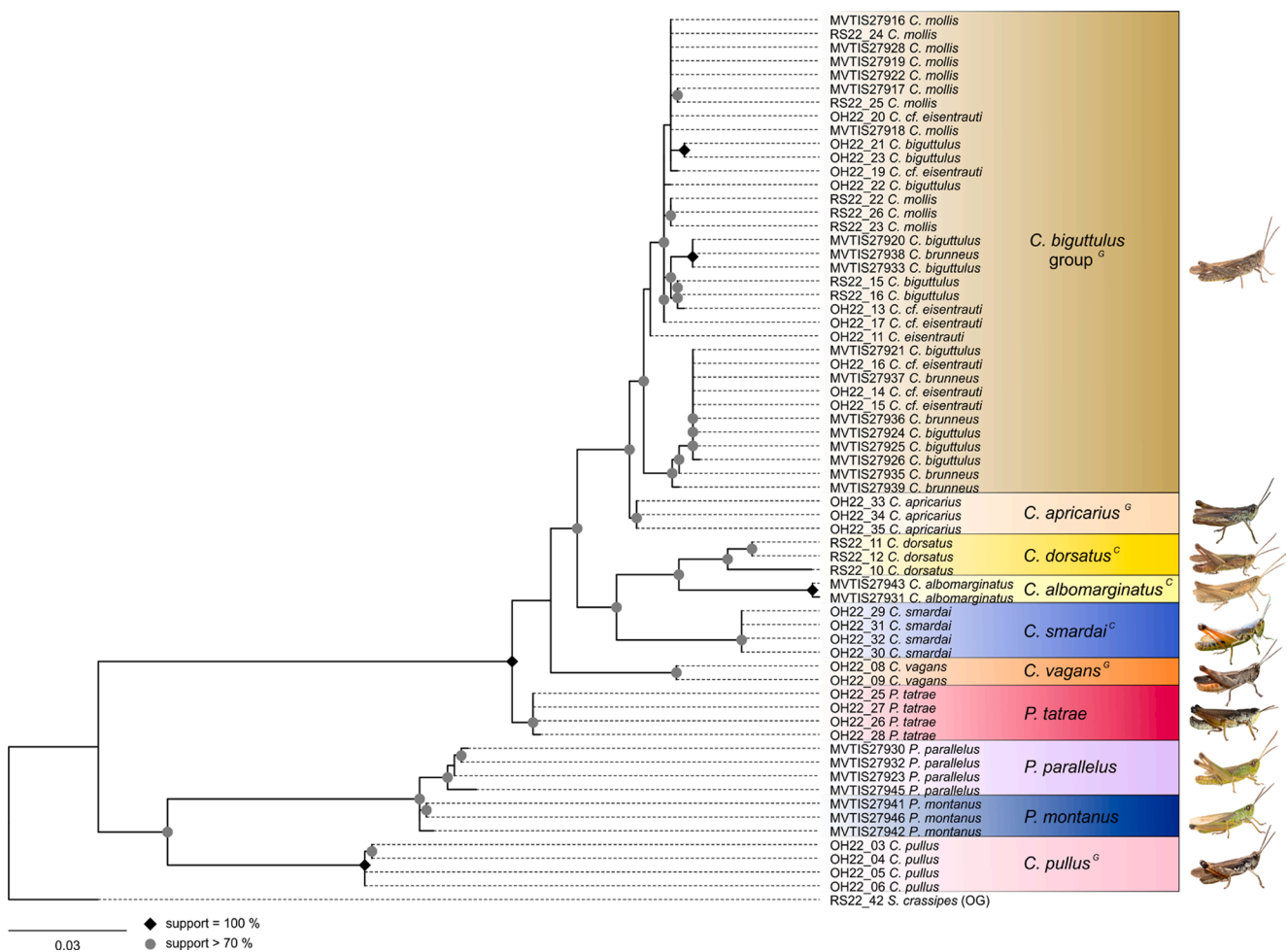
All aligned datasets, resulting trees, structure plots, and other

relevant data were deposited in Zenodo [<https://doi.org/10.5281/zenodo.10288896>].

### 3. Results

#### 3.1. DNA barcoding reference tree

The Maximum-Likelihood (ML) tree based on CO1 sequences (Fig. 1) had weak bootstrap support for most nodes, particularly within the *C. biguttulus* group. Notably, the topology in the *C. biguttulus* group itself is unresolved, as none of the four species are retrieved as monophyletic. Conversely, all other species were retrieved as monophyletic. *C. apricarius* was recovered as sister species to the *C. biguttulus* group, although previous studies (e.g., Hawlitschek et al., 2017) placed it within the group. *Chorthippus dorsatus* and *C. albomarginatus* were placed as sister species, and together formed the sister clade to *C. smardai*, recovering the subgenus *Chorthippus* as monophyletic. In contrast, the subgenus *Glyptobothrus* (comprising *C. apricarius*, *C. vagans*, *C. pullus*, and the *C. biguttulus* group) is found to be polyphyletic in this analysis. Additionally, *P. tatrae* does not cluster with *P. parallelus* and *P. montanus*, indicating its possible affiliation with the genus *Chorthippus* (subgenus *Glyptobothrus*). Among the *Chorthippus* species, *C. vagans* is sister to the clade of all other species of the genus excluding *C. pullus*, which instead was sister to the clade containing *P. parallelus* and *P. montanus*.



**Fig. 1.** Phylogenetic tree analysis of CO1 gene (622 bp) using IQTree for a sample size of n = 65 individuals. The Maximum Likelihood method was employed with 1000 ultrafast Bootstrap replicates. The model used for the analysis was TPM2u + F + G4. *Stenobothrus crassipes* was utilized as the outgroup (OG) for rooting the tree. Subgenus affiliation is denoted by uppercase letters: “G” for *Glyptobothrus* and “C” for *Chorthippus*. Support values were indicated on the tree, with black diamonds representing 100 % support and grey circles indicating support of at least 70 %. The raw tree was processed using FigTree and the final visualization was created using CorelDraw.



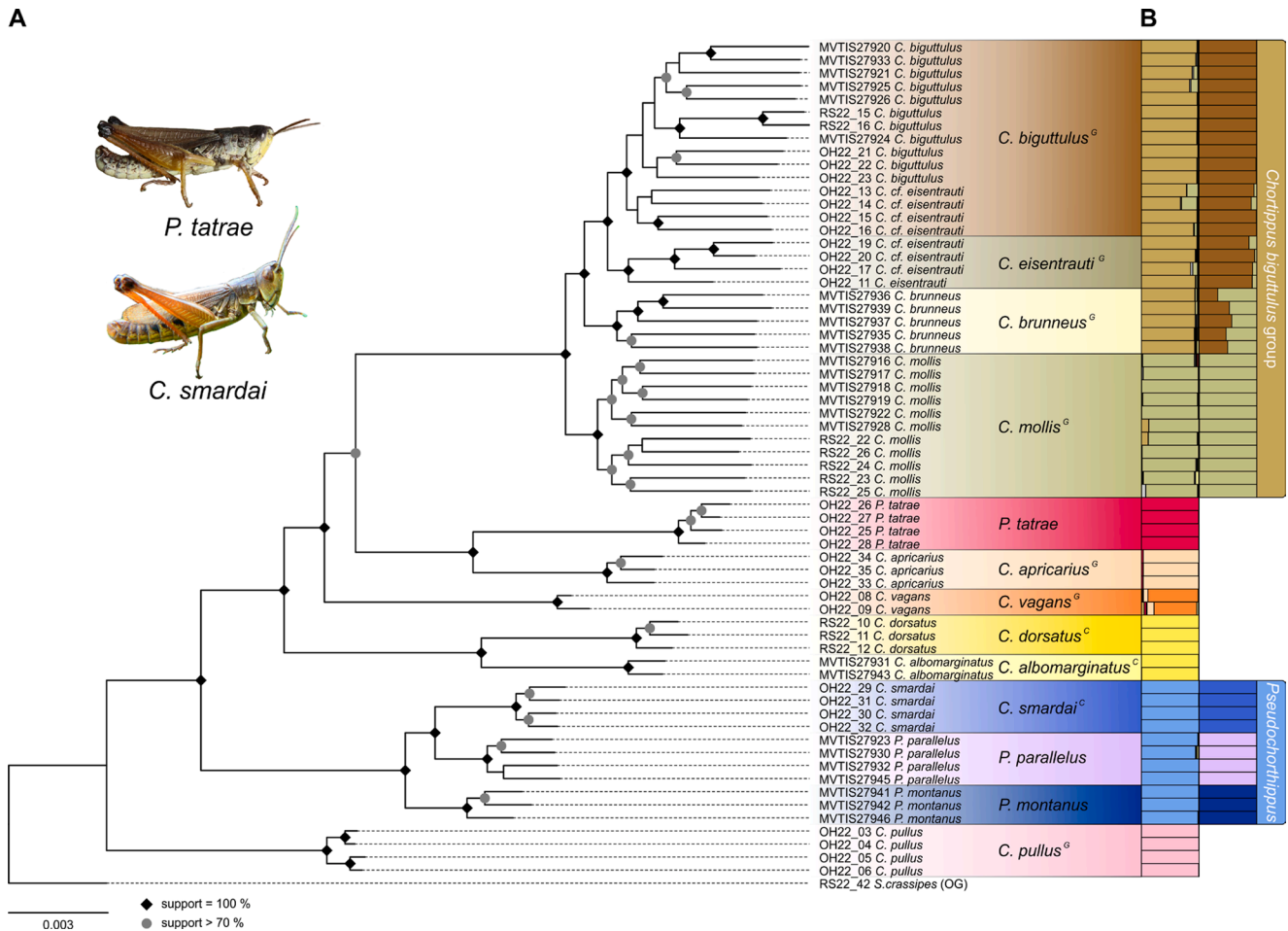
### 3.2. Phylogenetic analyses using RAD

The RAD phylogenetic tree (Fig. 2A) achieved a high resolution with strong bootstrap support for nearly all nodes up to species level. The *C. biguttulus* group is largely resolved, except for the *C. cf. eisentrauti* specimens OH22\_13-16, which cluster together with *C. biguttulus*. The other *C. cf. eisentrauti* specimens constitute a clade with the *C. eisentrauti* sample (OH22\_11), representing the true *C. eisentrauti*. *C. brunneus* and *C. mollis* form monophyletic groups, with *C. brunneus* being the sister species of *C. biguttulus* and *C. eisentrauti*. *P. tatrae* is positioned within the genus *Chorthippus* (subgenus *Glyptobothrus*) as the sister species of *C. apricarius*. This finding is intriguing since *P. tatrae* was initially described as a subspecies of *C. apricarius* by Harz (1971). *C. vagans* emerges as the sister species to all other species of the subgenus *Glyptobothrus*, except of *C. pullus*. *C. dorsatus* and *C. albomarginatus* are determined to be sister species, forming a monophyletic group that represents the subgenus *Chorthippus*, in agreement with the CO1 results. *C. smardai* clusters within the genus *Pseudochorthippus* as the sister species of *P. parallelus*. Notably, *C. pullus* splits basally from all other species of the group (i.e., *Chorthippus* and *Pseudochorthippus*).

### 3.3. Structure analyses

Determining the most likely number of genetic clusters (K) inferred from the SNP dataset, which includes on one hand species that are genetically highly divergent from each other, and on the other hand lineages that are genetically closely related but clearly delimited as species due to divergent morphology and bioacoustics in sympatry (e.g., the *C. biguttulus* group), required a careful evaluation of data and results. As a starting point, a best K analysis using Structure Harvester was conducted (Fig. S1, Table S7). The analysis suggested an unrealistic number of genetic clusters of only K = 2 as most likely, but with likelihood values for different K values being very close to each other.

In a second step, we therefore evaluated all K values from K = 2 to K = 14 (Fig. S2). For K = 2, the analysis resulted in the separation of the *C. biguttulus* group from all other species. Increasing K to 3 led to the division of the *C. biguttulus* group, all other *Chorthippus* species, and *Pseudochorthippus*. K = 4 further split *Chorthippus* into the subgenera *Chorthippus* and *Glyptobothrus*. Progressing to K = 5 isolated *C. pullus*. Within subgenus *Glyptobothrus*, divisions occurred at K = 8 (*P. tatrae* and *C. apricarius*) and K = 14 (*C. mollis* and *C. vagans*). Notably, no distinct separation was observed within other *C. biguttulus* group species, *Pseudochorthippus*, or the subgenus *Chorthippus* (i.e., *C. albomarginatus* and *C. dorsatus*). In general, as the K value increases, more artefactual



**Fig. 2.** A: Phylogenetic tree analysis using IQTree for a sample size of  $n = 65$  individuals and 6,763 RAD loci. The Maximum Likelihood method was employed with 1,000 ultrafast Bootstrap replicates. The model used for the analysis was TVM + F + I + I + R2. *Stenobothrus crassipes* was utilized as the outgroup (OG). Subgenus affiliation is denoted by uppercase letters: "G" for *Glyptobothrus* and "C" for *Chorthippus*. Support values were indicated on the tree, with black diamonds representing 100% support and grey circles indicating support of at least 70%. B: Structure plots depicting the genetic structure for the main dataset and two sub-datasets. Main-dataset a) includes *Chorthippus* and *Pseudochorthippus* with 872 SNPs and K = 14 clusters. Sub-dataset b) consists of *Pseudochorthippus* with 3,808 SNPs and K = 3 clusters. Sub-dataset c) represents the *C. biguttulus* group with 970 SNPs and K = 2 clusters. The figures were processed using FigTree and the final visualization was created using CorelDraw.

patterns became apparent. Based on these findings, the most likely number of genetic clusters should be at least  $K = 8$ . However, since most species were recovered at  $K = 14$ , this specific configuration was used for plotting next to the RAD phylogeny. (Fig. 2B).

Analyzing the *C. biguttulus* group dataset separately revealed distinct patterns (Fig. S3). For  $K = 2$ , a clear separation of *C. mollis* was observed, along with pronounced structure within *C. brunneus* and limited introgression within *C. eisentrauti* (see also Fig. 2B). However, no further structure was detected for other  $K$  values (Fig. S3).

Based on the outcome of the phylogenetic analysis, *C. smardai* was included in the structure analysis of the genus *Pseudochorthippus*, whereas *P. tatrae* was excluded. Regarding *Pseudochorthippus*, the structure analysis initially separated *C. smardai* from *P. parallelus* and *P. montanus* at  $K = 2$  (Fig. S4), and subsequently, at  $K = 3$ , *P. parallelus* was distinguished from *P. montanus* (see also Fig. 2B).

#### 4. Discussion

Our analyses highlight substantial discrepancies between nuclear and mitochondrial phylogenies, thus confirming the inaccuracy of mitochondrial markers in Gomphocerinae grasshoppers demonstrated by Hawlitschek et al. (2022). The RAD dataset further supports the close relationship of *C. smardai* with *P. parallelus* and *P. montanus*, confirming an earlier assessment based on morphological similarity (Chládek, 2014). Additionally, it provides evidence supporting the polyphyletic nature of the genus *Chorthippus*. Consequently, the division of the genus into the subgenera *Glyptobothrus* and *Chorthippus* also presents significant challenges, necessitating substantial taxonomic revisions using a larger dataset, as previously emphasized (Hawlitschek et al., 2017, 2022; Nolen et al., 2020).

The examination of the *C. biguttulus* group confirms the nuclear-mitochondrial discordance observed within the Gomphocerinae by Hawlitschek et al. (2022). In contrast, the RAD data largely aligns with the traditional taxonomy, despite the challenges posed by the young evolutionary history and frequent hybridization of these species, making their phylogenetic recovery difficult.

In the past, the species status of *C. eisentrauti* has been challenged by Perdeck (1958) based on weak morphological differences but has been confirmed through observation of stridulation and mating behavior by Ingrisch & Bassangnova (1995) and Koschuh (2012). However, genetic studies using single mitochondrial markers (Hawlitschek et al., 2017) or complete mitochondrial genomes (Hawlitschek et al., 2022) have not provided a clear picture of the *C. biguttulus* group and the position of *C. eisentrauti*. Only the implementation of hundreds of nuclear markers by Hawlitschek et al. (2022) showed a clear distinction of *C. eisentrauti* from *C. biguttulus*, though in a very small set of samples. Our data now confirms these results. Notably, the *C. cf. eisentrauti* specimens (OH22\_13-16) from Poland appear to belong to *C. biguttulus*, which was expected as *C. eisentrauti* is so far only known from the Alps (Koschuh, 2012). Furthermore, the remaining *C. cf. eisentrauti* specimens (OH22\_17-20) discovered on the Watzmann in the Berchtesgaden Alps form a monophyletic group with the confirmed *C. eisentrauti* specimen. Historical findings from Hölzel (1955) and Harz (1957) from the Berchtesgaden Alps support this hypothesis even if the findings were doubted by v. Helversen und Meyer in the past (see Koschuh, 2012). However, it should be noted that our analysis was restricted to a single confirmed *C. eisentrauti* specimen (OH22\_11). To enhance the robustness of these results, future analyses should include *C. eisentrauti* specimens from different populations.

The position of *C. brunneus* and *C. mollis* within the RAD phylogeny is consistent with previous studies (Nolen et al., 2020; Hawlitschek et al., 2022). Based on Nolen et al. (2020), the divergence of *C. mollis* likely occurred first, followed by *C. biguttulus*, and finally *C. brunneus* and *Chorthippus rubratibialis* (not included in this analysis). In previous studies, additional species like *C. rubratibialis* or *Gomphocerippus rufus* were clustering within the *C. biguttulus* group and their phylogenetic

relationships could not be resolved either through mitochondrial markers (Hawlitschek et al., 2022) or nuclear markers (Nolen et al., 2020). Hence, future RAD studies should include these species to obtain a more comprehensive understanding of the *C. biguttulus* group.

*Chorthippus smardai*, a species endemic to higher elevation regions of the Tatra mountains (1,650 – 2,150 m.a.s.l.), was described by Chládek in 2014 (found already in 1967 – 1971) and has not previously been genetically examined. Our RAD analysis strongly suggests that *C. smardai* is positioned between *P. parallelus* and *P. montanus*, presumably representing their sister species. A notable contradiction arises when comparing the CO1 and RAD phylogenies in terms of the position of *C. smardai*. However, the CO1 results are based on a shorter sequence consisting of only 298 bp for *C. smardai* while all other taxa are represented by 622 bp which could result in an incorrect assignment of *C. smardai* into the genus *Chorthippus*. An alternative analysis of the CO1 gene, excluding *C. smardai*, revealed the same species-level topology for all other species (Fig. S5).

The allocation of *C. smardai* to the genus *Pseudochorthippus* has been previously discussed. Chládek (2014) stated in his species description that *C. smardai* is closely related to *P. parallelus* and *P. montanus* and Kristín et al. (2020) recently highlighted that *C. smardai* shares morphological resemblances with these species. Certainly, the genus *Pseudochorthippus* was established by Defaut (2012) only two years before the species description of *C. smardai*. At this time, the majority of orthopterologists had not yet recognized the genus.

Another species that has not previously been genetically analyzed is *Pseudochorthippus tatrae* (Harz, 1971), another endemic of the Northern Carpathian Mountain region but with much larger extent of occurrence and altitudinal distribution than *C. smardai* (Chobanov et al., 2016). Both the RAD and CO1 data consistently place *P. tatrae* within the genus *Chorthippus*. Moreover, the RAD data specifically indicates a sister group relationship between *P. tatrae* and *C. apricarius*. Historically *P. tatrae* has been described as a subspecies of *C. apricarius* by Harz in 1971 and was only later associated with *P. montanus* (formerly *Chorthippus montanus*). Over the following decades, especially with the introduction of the genus *Pseudochorthippus*, the species was then assigned to the genus *Pseudochorthippus*. However, this has already been questioned by Kristín et al. (2020), due to the allocation of the species to the subgenus *Glyptobothrus*.

Both the CO1 and RAD datasets indicate that *C. pullus* forms a distinct cluster between the genus *Pseudochorthippus* and the outgroup *S. crassipes*. Due to the lack of other outgroups, the current dataset makes it difficult to determine the status of *C. pullus*. Nonetheless, it further underlines the need to revise *Chorthippus* and *Pseudochorthippus* or to re-synonymize them again. However, to obtain more robust conclusions, it is crucial to expand the sampling effort to include all relevant Holarctic Gomphocerinae species, particularly type species. Extending the RAD analysis to the entire subfamily or tribus may encounter limitations when genetic lineages are deeply divergent and the differences between groups become substantial (Rubin et al., 2012). Nevertheless, our dataset serves as an empirical demonstration for the use of RAD-based phylogenetic analysis to deliver reliable results on the relationships of relatively deep clades (i.e., genera). That the data set simultaneously allows addressing population genetic and alpha-taxonomic research questions corroborates its versatility and its value among the various genomic approaches available to molecular systematists.

#### CRedit authorship contribution statement

**Robin Schmidt:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. **Christophe Dufresnes:** Funding acquisition, Methodology, Supervision, Validation, Writing – review & editing. **Anton Kristín:** Resources, Writing – review & editing. **Sven Künzel:** Investigation, Writing – review & editing. **Miguel Vences:** Conceptualization, Funding acquisition, Resources, Supervision,

Writing – review & editing. **Oliver Hawlitschek**: Conceptualization, Methodology, Resources, Validation, Writing – original draft, 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.

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### Statement of Ethics

None of the species studied in this project is protected by EU or national legislation.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2024.108012>.

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