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# DNA barcoding and species delimitation of crickets, katydids, and grasshoppers (Orthoptera) from Central and Southern Europe, with focus on the Mediterranean Basin

Mattia Ragazzini[1,2,7], Roy M. J. C. Kleukers[3], Luc Willemse[3,4], Baudewijn Ode[4,5], Lara-Sophie Dey[2,6,*], Oliver Hawlitschek[2,7,*]

1 *Dipartimento di Scienze Biologiche, Geologiche ed Ambientali – BiGeA, Università di Bologna, Via Selmi, 3, Bologna 40126, Italy*
2 *Leibniz Institute for the Analysis of Biodiversity Change (LIB), Museum of Nature, 20146 Hamburg, Germany*
3 *EIS Kenniscentrum Insecten / Naturalis Biodiversity Center, Leiden, Netherlands*
4 *IUCN SSC Grasshopper Specialist Group, Trier, Germany*
5 *FLORON Plant Conservation Netherlands, Nijmegen, Netherlands*
6 *Senckenberg German Entomological Institute, 15374 Müncheberg, Germany*
7 *Department of Evolutionary Biology and Environmental Studies, Universität Zürich, 8057 Zürich, Switzerland*
* *Co-senior authors*

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Corresponding author: Mattia Ragazzini (mattia.ragazzini@uzh.ch)

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## Abstract

The Mediterranean Basin, recognized as a global biodiversity hotspot, harbors a remarkable diversity of grasshoppers, katydids, and crickets, many of which are endemic and potentially contain cryptic lineages. In this study, we generated a comprehensive dataset comprising 1,441 barcodes from 270 identified species within the Ensifera and Caelifera suborders. These were combined with existing data to form a dataset of 2,606 barcodes representing 351 species. We employed Maximum Likelihood (ML) topology reconstruction and applied five species delimitation methods (BIN, ABGD, ASAP, GMYC, and PTP) to detect potential incongruences between Operational Taxonomic Units (OTUs) and existing taxonomic classifications. Our analysis revealed that OTUs delimited by these methods corresponded to 71.39% of the evaluated species, with a notably higher congruence in Ensifera (88.53%) compared to Caelifera (52.15%). Across the dataset, we identified 54 lineages comprising cryptic species, indicating significant unrecognized diversity within these groups. Additionally, 21 instances of species being merged into consensus OTUs were observed, suggesting either the need for taxonomic revision or highlighting the limitations of current genetic markers. Among the methods tested, ABGD, particularly with the Kimura two-parameter model, was the most consistent with traditional taxonomy, yielding the highest consensus rates. In contrast, the PTP method exhibited the lowest consensus, often leading to an oversplitting of lineages. These findings underscore the complexity of species delimitation in recently radiated taxa and emphasize the importance of using multiple methodologies to accurately capture biodiversity, especially in regions characterized by a high prevalence of cryptic species.

## Keywords

BOLD (Barcoding Of Life Data), COI (Cytochrome C Oxidase subunit 1), endemism, HGT (Horizontal Gene Transfer), HTS (High-Throughput Sequencing), ILS (Incomplete Lineage Sorting), Mediterranean area, MOTU (Molecularly defined Operational Taxonomic Unit)

# 1. Introduction

Over the last two decades, DNA barcoding has developed into an invaluable tool for taxonomists, enhancing the ability to diagnose species across all life history stages, particularly when traditional morphology-based taxonomy is challenging or when taxa are morphologically ambiguous (Kress and Erickson 2012; Fišer Pečnikar and Buzan 2013; Shadrin 2021). This method also flags potential new species, including undescribed and cryptic species, with applications extending into conservation biology, ecological studies, medicine, pharmaceuticals, and systems biology (Fišer Pečnikar and Buzan et al. 2013; Shadrin 2021). Barcoding employs small, standardized DNA regions, in metazoans particularly the mitochondrial cytochrome C Oxidase I (COI) locus, for rapid and reliable species identification due to their minimal variation within species (Hebert et al. 2003; Ratnasingham et al. 2007; Kress and Erickson 2012). The Barcode of Life Data System (BOLD) provides a platform for the management, quality assurance, and analysis of barcode data while facilitating collaboration among global research communities (BOLD – http://www.boldsystems.org; Ratnasingham and Hebert 2007). As of early-2025, it includes barcodes for 20,768,000 specimens, representing 361,000 species.

Orthoptera are prime candidates for DNA barcoding due to their significant diversity and ecological importance (Hawlitschek et al. 2016). With over 30,000 species described (Grimaldi and Engel 2005), they make up a substantial part of the biomass of grassland ecosystems (Belovsky and Slade 2018), serve as key indicators of environmental changes (Bazelet and Samways 2011), and are linked to human agriculture as both pests and species threatened by intensive land use (Aragón et al. 2012; Huang et al. 2013; Hawlitschek et al. 2016; Dey et al. 2021). Europe is home to over 1,033 orthopteran species, with the highest diversity in the Mediterranean region (Cigliano et al. 2024). The Mediterranean has experienced various geomorphological and climatic changes over geological history, which have influenced species distribution. These changes facilitated orthopteran radiation through the evolution of new genetic variants and allopatric speciation, resulting in high endemism in areas such as the southern Balkan Peninsula, western Asia Minor, and the Iberian Peninsula (Kenyeres et al. 2009; Keppel et al. 2011; Hochkirch et al. 2023). At the same time, more than a quarter of European orthopteran species are under threat due to limited distribution areas and anthropogenic impacts (Samways and Lockwood 1998; Lemonnier-Darcemont et al. 2018; Iorio et al. 2019).

So far, comparatively few dedicated barcoding studies worldwide have targeted Orthoptera (Huang et al. 2013), and barcodes have long been available only for a small number of European species (Vedenina and Mugue et al. 2011). Recent studies using DNA barcoding for species delimitation in orthopterans involved species from Central Europe (Hawlitschek et al. 2016; De Jesús-Bonilla et al. 2017), Portugal (Pina et al. 2024), the Canary Islands (López et al. 2006; López et al. 2013), Mexico (Pedraza-Lara et al. 2015), and the Andean area (Pocco et al. 2015). The Mediterranean basin, stretching across southern Europe, northern Africa, and parts of western Asia, covers diverse habitats such as temperate forests, grasslands, wetlands, and arid zones. Mediterranean climate with hot, dry summers and mild, wet winters fosters remarkable biodiversity (Blondel et al. 2010). Renowned for its high endemism, supporting a wide range of flora and fauna, the Mediterranean is recognized as one of the most ecologically significant regions globally (Myers et al. 2000). The current study thus aims at enriching the publicly available dataset of COI sequences for orthopterans of the Mediterranean and other parts of Europe.

Shortcomings still affect DNA barcode efficiency, especially in caeliferans (Hawlitschek et al. 2016). Hybridization and mtDNA introgression are common in orthopterans, leading to potential identification errors (Ballard 2000; Babik et al. 2005; Gottsberger and Mayer 2019; Hawlitschek et al. 2022). Symbiont exchange, especially with *Wolbachia* bacteria, further complicates barcode accuracy (Mandel et al. 2001; Zabal-Aguirre et al. 2010; Boto 2014; Bugrov et al. 2016; Ilinsky et al. 2022; Zhou and Luo et al. 2022). Additionally, the prevalence of nuclear mitochondrial pseudogenes (numts) in grasshopper genomes can result in erroneous phylogenetic patterns (Moulton et al. 2010; Hanrahan and Johnston 2011; Leite 2012; Wang et al. 2014; Pereira et al. 2020; Hawlitschek et al. 2023).

Nabholz et al. (2023), analysing data from Hawlitschek et al. (2016, 2022), highlighted incomplete lineage sorting (ILS) as a likely reason for DNA barcoding failures in Orthoptera. This finding aligns with Nolen et al. (2020), who observed extensive ILS across independent gene trees in *Chorthippus* Fieber 1852 (Caelifera: Acrididae). Such genomic studies on ecological radiations, driven by divergent natural selection, suggest that rapid speciation can lead to extensive ILS, resulting in non-monophyletic nominal species (Lamichhaney et al. 2015). In this context, Acrididae represents one of the comparatively most recently diverged lineages within Orthoptera. Their cosmopolitan distribution was likely achieved through dispersal followed by rapid radiation (Chintauan-Marquier et al. 2014; Song et al. 2018). ILS may thus obscure species boundaries in DNA barcoding efforts (Vedenina and Mugue et al. 2011; Nabholz et al. 2023).

Species delimitation requires integrating multiple independent lines of evidence, in order to obtain accurate results (Dayrat 2005; Tang et al. 2014; Blair and Bryson 2017). COI barcoding represents one of these and is widely used in combination with species delimitation algorithms such as Barcode Index Numbers (BINs) from the BOLD system (Ratnasingham and Hebert 2013). Given the challenges posed by DNA barcoding of orthopterans, a multifaceted approach based on different theoretical frameworks has been used for species delimitation and identification of Molecularly defined Operational Taxonomic Units (MOTUs).

Several European orthopteran species were barcoded for the first time in this study, contributing to the expand-

ing dataset of COI sequences available for Orthoptera. Given the challenges posed by incomplete lineage sorting (ILS), mtDNA introgression, and nuclear mitochondrial pseudogenes, this study highlights the importance of integrating multiple complementary species delimitation methods to overcome these limitations. By employing a multifaceted approach, we aim to enhance species identification accuracy, uncover cryptic diversity, and define more reliable Molecular Operational Taxonomic Units (MOTUs). Through the integration of molecular and morphological evidence, this study reveals hidden biodiversity and establishes a foundation for future taxonomic revisions and conservation efforts. Our findings will help refine species delimitation in Orthoptera, supporting biodiversity assessments and guiding targeted conservation strategies, particularly in the Mediterranean, a region renowned for its high endemism and ecological significance.

# 2. Materials and Methods

## 2.1. Sampling and species identification

Since 1999, the entomologists and orthopterologists Baudewijn Odé, Rob Felix, Luc Willemse, and Roy Kleukers have been organizing Orthoptera collection expeditions on the field, in southern Europe, focusing primarily on Spain, Portugal, Italy, Greece, and Romania. Voucher specimens were killed using ethyl acetate, dried, mounted, and identified utilizing literature and identification keys specifically designed for European orthopterans (e.g., Willemse et al. 2018; Iorio et al. 2019). Some individuals were included in the dataset even if they could not be identified to species level. Based on the barcode data results, more precise information will be possible in future projects. The right middle legs were taken from freshly killed specimens and preserved in 97% ethanol (Rentz 2010; Willemse et al. 2018). A total of 1,698 voucher specimens are stored at the collections at the Naturalis Biodiversity Center, the national research institute for biodiversity located in Leiden, Netherlands.

## 2.2. Laboratory procedures

The DNA extraction, amplification and sequencing were performed in two parts. 630 samples were processed at the Leibniz Institute for the Analysis of Biodiversity Change (LIB) in Hamburg (Germany), and 1,068 samples at the Naturalis Biodiversity Center in Leiden (Netherlands).

At LIB, DNA was extracted according to the methods of Chelex® 100 resin-based protocol (de Lamballerie et al. 1992). For PCR amplification of the DNA barcode region, the COBL (forward) and COBU (reverse) primers were used, tailored for Orthoptera to improve amplification and sequencing results (Huang et al. 2013). The reaction master mix composition and cycling protocol are outlined as supplementary material (File S1 [protocol a]). The PCR products were checked for successful amplification on an agarose gel and recorded using a gel imaging system. Samples were sequenced by Macrogen Europe (Amsterdam).

The Sanger sequencing results (ab1 files) underwent a quality check. Sequences chromatograms were analysed using Geneious Prime 2023.1.1, trimming poor-quality edges and manually correcting bases with low-quality peak maps. Sequences with unreliable peaks were subjected to PCR and sequencing repetition, adjusting the annealing temperature to 49°C, which improved outcomes in several instances. Sanger sequencing data were filtered, selecting sequences longer than 500 bp (base pairs), except for some species where only few longer sequences were available.

At Naturalis, DNA extraction was performed adhering to the ARISE (Authoritative and Rapid Identification System for Essential biodiversity information) protocol (van Ommen Kloeke 2022; protocols.io; bomb.bio). Following DNA extraction and cleanup using KingFisher, PCR was conducted on all samples. This step employed the same primers (COBL and COBU).

PCR products were verified on a 2% agarose E-Gel, with nearly all samples showing positive results, prompting continuation with all samples (detailed PCR protocol in File S1 [protocol b]). Subsequently, the Nanopore dual barcoding protocol with kit 14 was applied for the sequencing. In this protocol, samples with no bands in the elektrophoresis were also used for library preparation.

## 2.3. Sequences quality check and upload to BOLD

All sequences were aligned using MAFFT V 7.505 (Katoh and Standley 2013) on the CIPRES Science Gateway (Miller et al. 2010). Sequences with excessively wide gaps and those containing internal stop codons were removed. Next, all sequences underwent contamination or misidentification checks using BLASTn (Camacho et al. 2009).

The newly generated sequence data and metadata were then uploaded to the Barcode of Life Data Systems (BOLD PROJECT "MEDOR Barcoding of Mediterranean Orthoptera"). Thanks to our preliminary quality assessments, all barcode sequences met the automatic quality criteria of BOLD upon upload.

## 2.4. Merged dataset and phylogenetic analyses

The initial dataset was augmented with two additional datasets of European Orthoptera sequences available on the BOLD system, named GBORT-GBOL (Hawlitschek et al. 2016; 745 records) and IBIOR (Pina et al. 2024; 420 records), resulting in the merged dataset DS-MEDOR1.

The former consists of records primarily from Austria, Germany, and Switzerland, while the latter comprises records from continental Portugal. This integration aimed to achieve a more accurate subdivision of the samples by species within the phylogenetic tree. This was facilitated by the fact that the datasets from other European and Mediterranean basin areas share several species and genera with those in the current study.

DNA barcoding shows variable efficiency between Caelifera and Ensifera due to factors such as numts frequency (Kaya and Çıplak 2018), hybridization rate (Hawlitschek et al. 2016), ILS (Nabholz 2023), and *Wolbachia* infections (Ilinsky et al. 2022). Thus, phylogenetic and species delimitation analyses were conducted separately for each suborder (unless stated otherwise), which are well-supported as monophyletic lineages (Song et al. 2015; Cigliano et al. 2024), dividing the merged dataset into two subsets. This division significantly reduced the initial dataset size, facilitating the management of the extensive number of sequences.

Two preliminary Maximum Likelihood (ML) trees (Felsenstein 1981) were reconstructed of the aligned dataset using IQ-Tree v2.2.2.7 (Nguyen et al. 2015). The GTR (General Time Reversible) + I + G model (Tavaré 1986), identified by ModelFinder (Kalyaanamoorthy et al. 2017) as the best-fit model for nucleotide substitution, was used in both analyses. The merged datasets revealed sequences occupying potentially incorrect or ambiguous positions within the phylogenetic trees. To resolve possible issues of contamination or misidentification, voucher specimens were re-verified with the assistance of experts at the Naturalis Biodiversity Center in Leiden. In accordance with the approaches recommended by Song et al. (2008) and Leite (2012), chromatograms and sequence editing were meticulously re-examinated. Furthermore, bioacoustic, biogeographic, and morphological data were consulted to ensure accurate species verification. Sequences presumed to represent numts or contaminations were excluded from the final datasets and trees.

Specimens not identified at the species level were retained for further analysis, provided their most specifically identified clade was accurately placed within the ML tree, aligning with currently accepted Orthoptera taxonomy (Cigliano et al. 2024).

After quality measures, each definitive subset was realigned and trees reconstructed using the same parameters as for the preliminary analyses described above.

All phylogenetic analyses were executed with XSEDE (eXtreme Science and Engineering Discovery Environment) through the CIPRES Science Gateway (www.phylo.org; Miller et al. 2010).

The effectiveness of DNA barcoding in species identification was assessed by calculating the ratio between the number of species not exhibiting BIN sharing and the total number of species assigned a BIN code, following the methodology described by Hawlitschek et al. (2016). Additionally, a weighted average was computed to account for the varying number of samples per genus, ensuring that genera with a larger number of samples had a proportional influence on the final estimate (Sokal and Rohlf 1995). The weighted average of species per genus was calculated by multiplying the number of species within each genus by the number of samples representing that genus, summing these products, and dividing by the total number of samples across all genera. Both statistics were calculated for the entire dataset and for caeliferans and ensiferans separately.

## 2.5. Species delimitation analyses

Species delimitation analyses were conducted on the definitive datasets using various species delimitation approaches to detect and compare MOTUs, to explore species diversity and deepen the understanding of species boundary delimitation issues. For this purpose, both similarity (BIN, ABGD, ASAP) and clustering-based (GMYC, PTP) approaches designed for a single-locus strategy were utilized. In all methods we employed, the term "partition" refers to the grouping of sequences into distinct clusters or units, typically representing putative species. In methods such as ABGD and ASAP, partitions emerge from the identification of genetic gaps or distance thresholds, while in GMYC and PTP, partitions are determined by tree-based criteria, such as branch length distributions or coalescent processes (Pons et al. 2006; Puillandre et al. 2011, 2020; Ratnasingham and Hebert 2013; Zhang et al. 2013).

**BIN method**: the Cluster Sequences tool, implemented by BOLD, generates MOTUs through the REfined Single Linkage (RESL) algorithm, grounded on uncorrected pairwise distances (p-distance). The Barcode Index Number (BIN) informatics system provides a unique alphanumeric code for MOTUs (BOLD: 3 letters, 4 numbers (Ratnasingham and Hebert 2013)). BIN-assignment data were retrieved from the dedicated web interface for all chosen specimens. Cases of BIN discordance arise when traditionally recognized species encompass more than one BIN, whereas BIN sharing pertains to single BINs containing members from multiple recognized species (Hebert et al. 2004).

**ABGD method**: the Automatic Barcode Gap Discovery was adopted to divide samples based on genetic distance, detecting the so-called "barcode gap" (Puillandre et al. 2011). The analysis used a relative gap width of 1 and 20 bins, and results were interpreted using a prior intraspecific divergence limit of P = 0.01 (Puillandre et al. 2011; Gonçalves et al. 2021) with four combinations of different substitution models and partitions employed: Jukes-Cantor (Jukes and Cantor 1989) or Kimura two-parameter (Kimura 1980) models and initial or recursive partition.

**ASAP method**: the Assemble Species by Automatic Partitioning creates new partitions by amalgamating sequences at equal pairwise distances into progressively larger groups until the final partition encompasses all records (Puillandre et al. 2020). This analysis used Jukes-Cantor distances and default parameters, with the

best initial and recursive partitions selected based on the lowest "asap-score".

For both ABGD and ASAP, the initial partition refers to the first grouping of sequences based on broader genetic thresholds, while the recursive partition further refines these clusters by progressively splitting them to detect finer genetic distinctions, potentially identifying cryptic species. Both ABGD and ASAP analyses were run on the respective online platforms (available at https://bioinfo.mnhn.fr/abi/public/abgd and https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html, respectively).

**GMYC method**: the Generalized Mixed Yule Coalescent discerns the most likely putative species clusters basing on an ultrametric tree (Pons et al. 2006). Concatenated sequence alignments were analysed using BEAST v.2.6.7 (Drummond and Rambaut 2007; Suchard and Rambaut 2009; Bouckaert et al. 2014), a Bayesian inference tool generating ultrametric trees. Parameters included a range of clock and speciation model priors (strict or optimized relaxed clock model and Yule or Coalescent Constant Population process). According to ModelFinder (implemented by IQ-Tree), GTR+I+G was chosen as the most suitable model of nucleotide substitution. Due to predominantly non-converging runs and lower-than-expected Effective Sample Size (ESS) values, likely from over-parametrization, the partition scheme was adjusted to employ the simpler HKY model (Hasegawa et al. 1985). Each run, totaling eight, was set for 100 million iterations, adjusting the burn-in rate for optimal convergence. Tree and parameter sampling occurred every 1,000 steps, with TRACER v.1.7.2 employed to ensure ESS values exceeded 200, as advised by Drummond et al. (2007). The posterior distribution was summarized into the maximum clade credibility tree using TreeAnnotator v.2.6.6 (Drummond and Rambaut 2007). Newick files, post-burnin, were then subsampled to 10,000 trees each for processing. The resulting ultrametric trees supported only single-threshold GMYC analyses to prevent species number overestimation, a common issue with multiple-threshold approaches (Fujisawa and Barraclough 2013). The GMYC species delimitation analysis was then executed within R Software (Team, R Core 2014), using the "ape" package for phylogenetic analysis (Paradis and Schliep 2019), "paran" for parallel analysis (Dinno 2012), "rncl" for reading and manipulating phylogenetic data (Michonneau et al. 2016), and "splits" for species delimitation and network analysis (FitzJohn et al. 2009).

**PTP method**: this method delineates hypothetical species clusters by analysing branch length distributions in a rooted non-ultrametric gene tree. The PTP model, enhanced by the bPTP version with Bayesian support for delimited species on the input tree (Zhang et al. 2013), underwent analysis via an online server for both PTP and bPTP versions (http://species.h-its.org), targeting the maximum likelihood tree as input. Analysis parameters included 500,000 MCMC generations, a thinning of 500, and a burn-in of 0.1, with convergence checked as Zhang et al. (2013) recommended.

### 2.6. Species delimitation results comparison

The Python-based script SPdel v.2.0 (Ramirez et al. 2023) was utilized to visualize a summary representation of all delimitation methods, including the ML tree, specimen labels, and putative species clusters. This was computed according to each of the five species delimitation methods implemented and their different combinations of parameters, along with a manually designed consensus, which compares features summarizing all results based on a majority criterion (MOTUs found in more than 50% of methods). Records lacking a BIN code were excluded from the species delimitation summary, as SPdel can only provide graphical output for records assessed by all involved methods. All species were still represented except for *Glyphanus obtusus* Fieber, 1853, *Tettigonia caudata* (Charpentier, 1845), and *Isophya lemnotica* Werner, 1932. For these species, the delimitation analysis was also conducted, but results were only described and not displayed in the summary representation.

A comprehensive description of the command, input files, and options used for the SPdel.py analysis can be found in File S8.

## 3. Results

### 3.1. Dataset description and barcoding effectiveness

The final MEDOR (Barcoding of Mediterranean Orthoptera) alignment comprises 1,441 newly generated, quality-checked barcodes, each 726 bp in length (accession numbers for newly generated sequences on BOLD (Ratnasingham and Hebert 2013) are listed in File S5). These barcodes correspond to 270 identified species, including 10 subspecies. Additionally, 209 barcodes lack species-level identification, with most assigned to the genus level (49 genera). Among these, three records are identified as Pamphagidae sp., two as Ephippigerini sp., and one as Tettigoniinae sp. Notably, the dataset includes barcodes for 26 species (and two subspecies) of ensiferans and four species (and one subspecies) of caeliferans that had no publicly available barcodes before this study.

Incorporating data from Hawlitschek et al. (2016) and Pina et al. (2024), the complete dataset totals 2,606 specimens across 351 identified species, with a total of 14 subspecies and an equivalent number of taxa not identified at the species level (209). A significant portion of specimens were collected in Spain and the Canary Islands (851), Germany (544), Portugal (511), Italy (173) and Greece (145), followed by 15 other countries in the Mediterranean Area, plus two specimens from Sri Lanka and two from the United Arab Emirates (Fig. 1, with the exclusion of non-European specimens). Relative to the known Orthoptera species richness described in each country (Cigliano et al. 2024), the coverage of Orthoptera

biodiversity is 100.00% in Germany, 93.44% in Portugal, 63.47% in Spain, 43.89% in Italy, and 31.12% in Greece (further details in File S5).

The analysis of the merged dataset revealed significant variation in barcoding effectiveness between ensiferans and caeliferans. Overall, 76.72% of all studied Orthoptera species were accurately identified through DNA barcoding. However, the success rate differs markedly between the suborders: 63.64% for Caelifera, primarily due to BIN sharing among certain species, compared to 88.53% for Ensifera. The datasets for ensiferans and caeliferans also highlight differences in the species richness of the respective genera, with a weighted mean of 3.78 and 8.29 species per genus, respectively.

The ML trees of Caelifera and Ensifera are shown in Figs 2 and 3, and more in detail in File S2 [graphs a, b] respectively. Species delimitation analyses were then performed separately on two distinct datasets: Caelifera, with 1,478 records, and Ensifera, with 1,128 records.

## 3.2. Species delimitation results

The various species delimitation approaches employed yielded hypothetical OTU counts ranging from 355 to 505, compared to 351 previously identified species (Table 1). BOLD identified 445 BINs (162 for caeliferans and 283 for ensiferans). 278 species demonstrated BIN concordance, where all barcodes from specimens of a given species grouped into a single BIN, exclusively containing barcodes from that species alone. 95 species displayed singleton BINs, characterized by containing only a single record without additional sequences. BIN discordance was observed in several species, indicating a mismatch between the BIN system and traditional taxonomic classifications. 21 and 10 BINs, in caeliferans and ensiferans respectively, were identified as shared across multiple species, impacting 60 and 21 species in total. Additionally, 35 and 17 records were not assigned any BIN due to the absence of sequences longer than 500 bp, the minimum length required for BIN assignment on BOLD. These records were omitted from the BIN discordance analyses. However, for each of these, there was at least one co-specific record that did possess a BIN code, with the sole exceptions being *Glyphanus obtusus*, *Isophya lemnotica*, and *Tettigonia caudata*. Detailed associations between BIN codes and the associated species are provided in File S4 [tables a, b] and in File S6.

The ABGD method, especially its initial partition using the Kimura two-parameter model, emerged as the most conservative, contrasting with the PTP method, which identified the highest number of hypothetical species. Parameter variations within ABGD resulted in partitions of varying sizes, with the Jukes-Cantor model generally less conservative than the Kimura two-parameter, leading to a higher MOTUs count across both suborders,

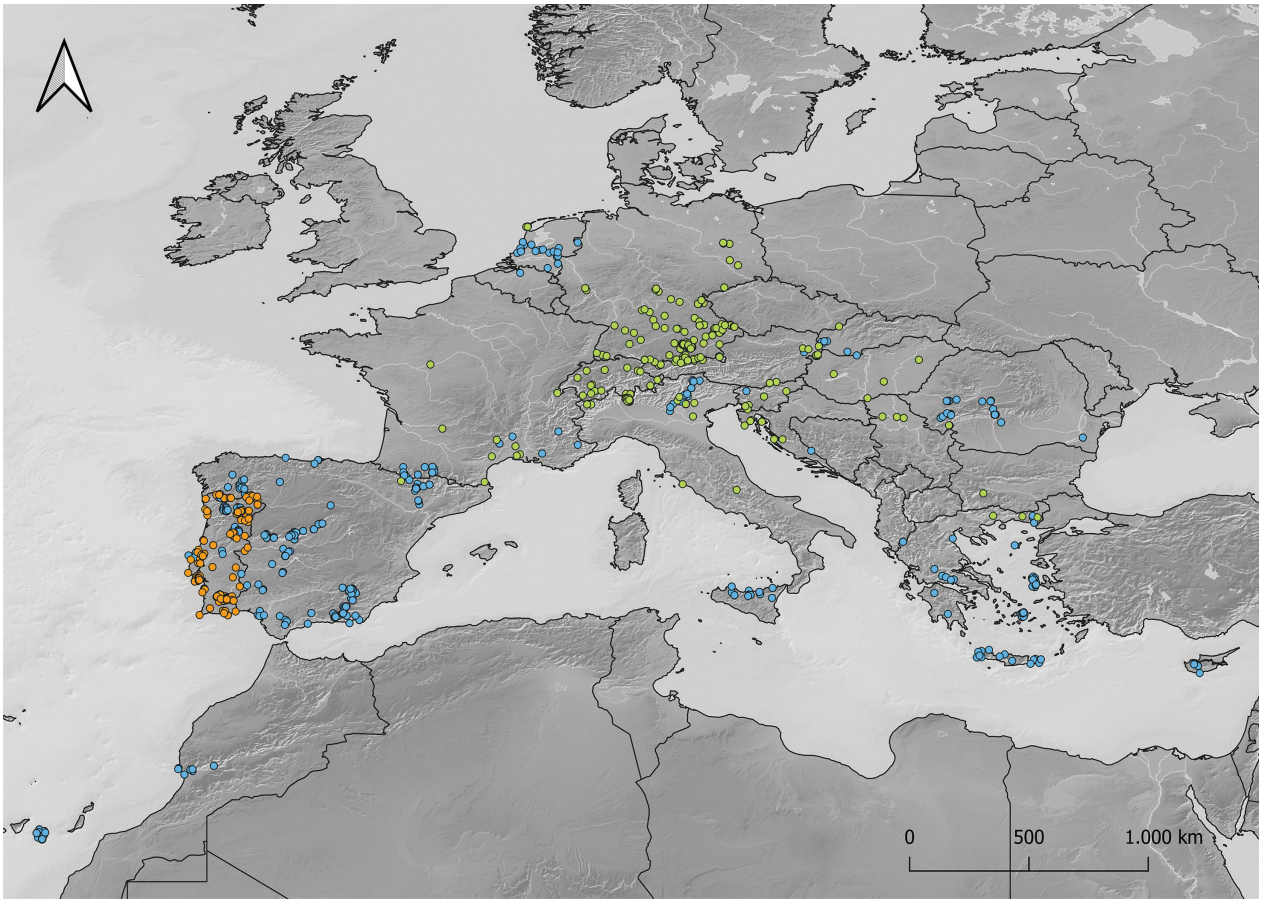


**Figure 1.** Sampling localities of all European samples involved in this study. Color legend: **blue** [MEDOR project], **green** [GBORT-GBOL project (Hawlitschek et al. 2016)], **orange** [IBIOR project (Pina et al. 2024)]. The four samples from Sri Lanka and Saudi Arabia are not included in this map.

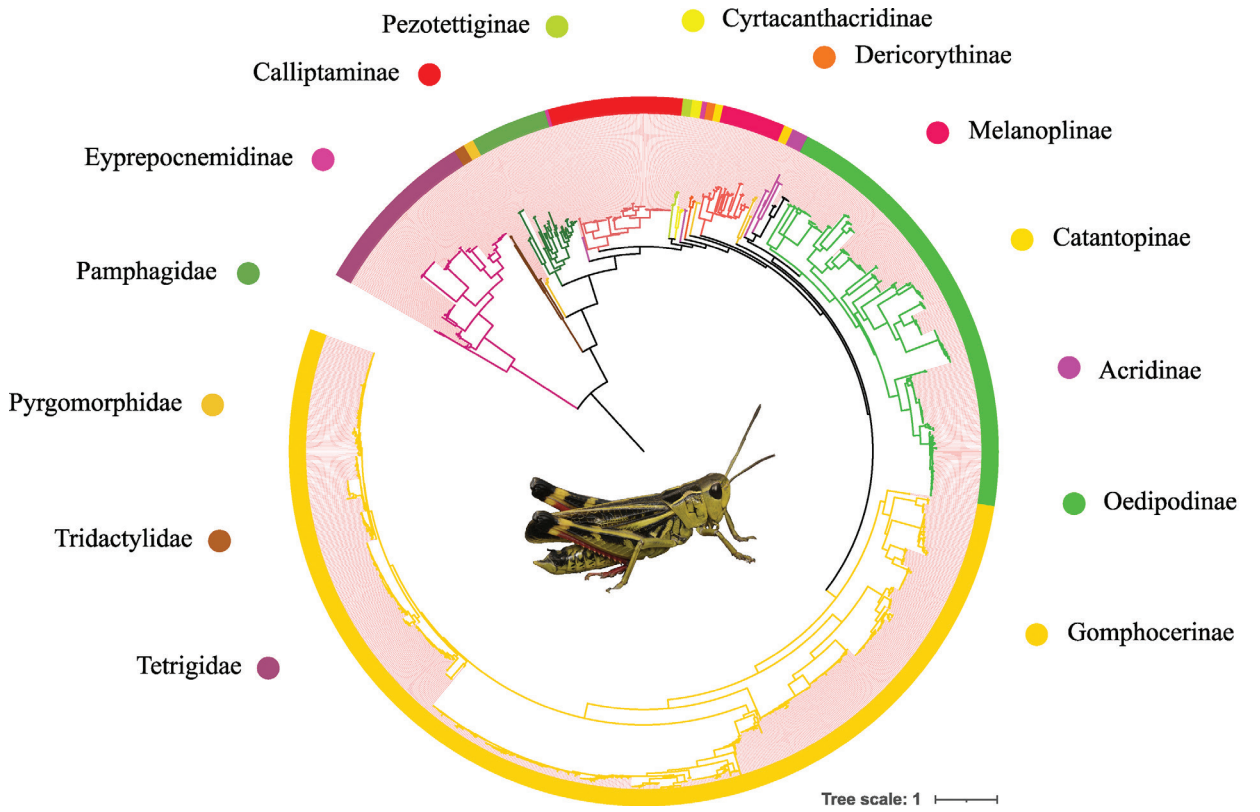


**Figure 2.** Maximum Likelihood tree of Caelifera from Central and Southern Europe. The tree was created with IQ-Tree v.2.2.2.7 on XSEDE (eXtreme Science and Engineering Discovery Environment) through the CIPRES Science Gateway (www.phylo.org). The tree displays 1,443 barcodes of identified (164) and unidentified species. Higher taxonomic levels (all families and the subfamilies of Acrididae) are coloured in the tree. The species depicted inside the tree is *Arcyptera* (*Arcyptera*) *tornosi* Bolívar, 1884.

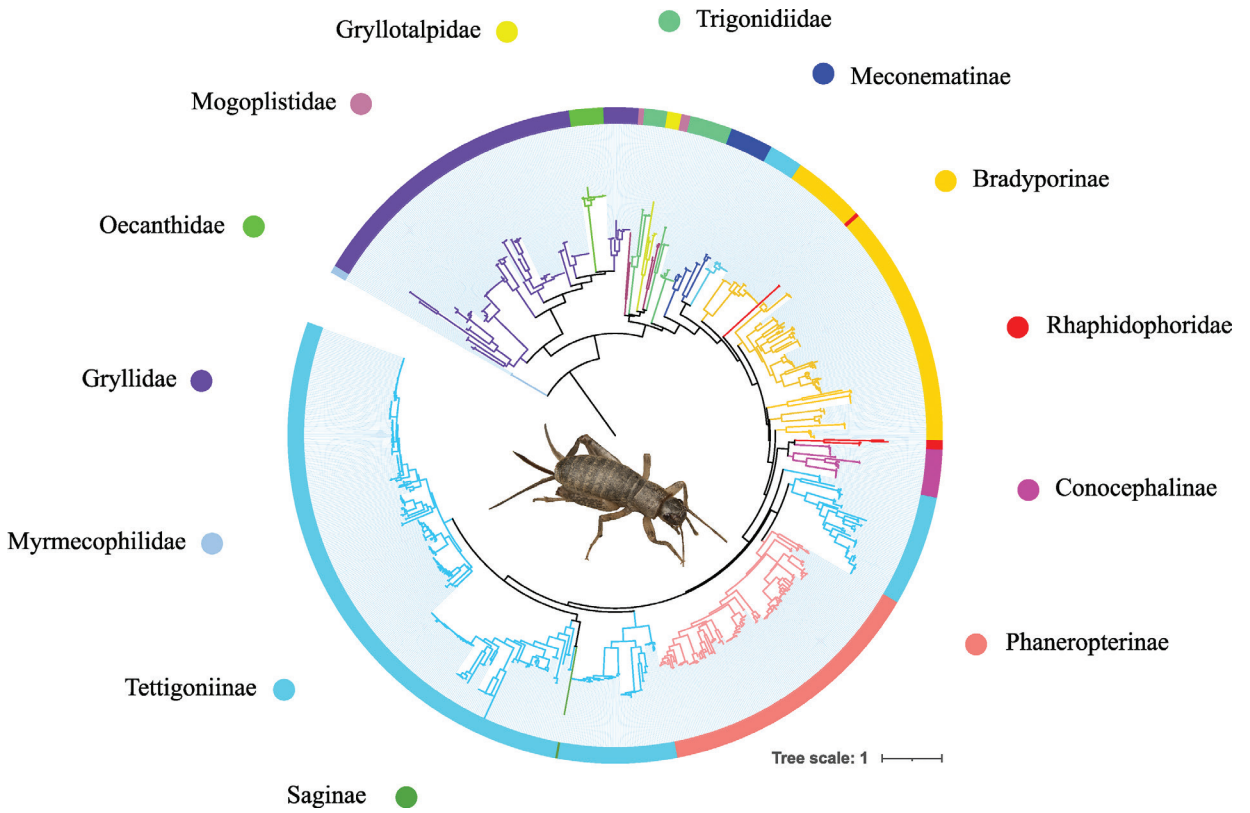


**Figure 3.** Maximum Likelihood tree of Ensifera from Central and Southern Europe. It was created with IQ-Tree v.2.2.2.7 on XSEDE (eXtreme Science and Engineering Discovery Environment) through the CIPRES Science Gateway (www.phylo.org). The tree displays 1,111 barcodes of identified (185) and unidentified species. Higher taxonomic levels (all families and the subfamilies of Tettigoniidae) are coloured in the tree. The species depicted inside the tree is *Pseudomogoplistes vicentae* Gorochov, 1996.

**Table 1.** Barcoding data and putative species clusters, according to all combinations of species delimitation approaches. — * displayed in SPdel summary graph (*I. lemnotica*, *T. caudata*, and *G. obtusus* delimitation features were discussed but not included in the graph).

| Quantity | Caelifera | Ensifera | Total |
|---|---|---|---|
| **Barcordes count** | | | |
| MEDOR dataset | 821 | 620 | 1441 |
| Hawlitschek et al. (2016) | 482 | 263 | 745 |
| Pina et al. (2024) | 175 | 245 | 720 |
| Merged dataset | 1478 | 1128 | 2606 |
| Identified species count | 166 | 185 | 351 |
| Records without BIN | 35 | 17 | 52 |
| **MOTU count*** | | | |
| BINs count | 162 | 283 | 445 |
| ABGD Jukes-Cantor Initial partition | 130 | 237 | 367 |
| ABGD Jukes-Cantor Recursive partition | 145 | 250 | 395 |
| ABGD Kimura two-parameter Initial partition | 116 | 239 | 355 |
| ABGD Kimura two-parameter Recursive partition | 123 | 250 | 373 |
| ASAP Jukes-Cantor Recursive partition | 138 | 218 | 356 |
| ASAP Kimura two-parameter Recursive partition | 130 | 275 | 405 |
| GMYC Yule process and strict clock model | 160 | 304 | 464 |
| GMYC Yule process and relaxed clock model | 160 | 265 | 425 |
| GMYC Coalescent Constant Pop. process and strict clock | 160 | 318 | 478 |
| PTP | 234 | 271 | 505 |

**Table 2.** Delimitation pattern of the caeliferan taxa. This only includes cases for which less than seven methods out of eleven reflect the traditional taxonomy. Unidentified MOTUs are included too. Unidentified records clustering within species-level identified MOTUs are not included. The first column indicates the taxon name, while the second one represents the relative number of specimens represented in the current database. The third column describes the pattern of consensus delimitation observed, while the fourth one delineates the name and number of methods not supporting the OTUs proposed in the previous field. In the fourth column, in square brackets, the method names are coded as follows: 1 = BIN; 2 = ABGD_JC_INIT; 3 = ABGD_JC_REC; 4 = ABGD_K2_INIT; 5 = ABGD_K2_REC; 6 = ASAP_JC; 7 = ASAP_K2; 8 = GMYC_STRICT_YULE; 9 = GMYC_REL_YULE; 10 = GMYC_STRICT_COAL; 11 = PTP. — (*): taxa for which the delimitation pattern is not clear, due to methods splitting and merging the same clusters simultaneously.

| Taxa | Number of specimens | Consenus delimitation | Methods not supporting the consensus delimitation |
|---|---|---|---|
| **Tetrigidae** | | | |
| *Tetrix bipunctata* | 8 | Merged with *T. kraussii* | — |
| *Tetrix ceperoi* | 11 | Merged in a single MOTU | **5/11** [3,5,6,7,11] |
| *Tetrix depressa* | 13 | Split into two MOTUs | **1/11** [11] |
| *Tetrix kraussii* | 9 | Merged with *T. bipunctata* | — |
| *Tetrix nodulosa* | 2 | Split into two MOTUs | — |
| **Pamphagidae** | | | |
| *Acinipe segurensis* | 5 | Split into two MOTUs | **1/11** [11] |
| *Eumigus monticola* | 12 | Split into three MOTUs | **3/11** [1,3,5] |
| *Eumigus* sp. 1 (MEDOR863-23) | 1 | Independent unidentified MOTU | — |
| *Eumigus* sp. 2 | 3 | Independent unidentified MOTU | — |
| *Ocnerodes* sp. (MEDOR392-23) | 1 | Independent unidentified MOTU | — |
| *Orchamus* sp. (MEDOR081-23) | 1 | Independent unidentified MOTU | — |
| Pamphagidae sp. 1 (MEDOR1128-23) | 1 | Merged with *Glauia* sp. | — |
| Pamphagidae sp. 2 (MEDOR348-23, MEDOR349-23) | 2 | Independent unidentified MOTU | — |
| **Acrididae – Dericorythinae** | | | |
| *Dericorys* sp. | 3 | Independent unidentified MOTU | — |
| **Acrididae – Pezotettiginae** | | | |
| *Pezotettix giornae* | 6 | Split into three MOTUs | **3/11** [4,5,11] |
| **Acrididae – Melanoplinae** | | | |
| *Miramella alpina* | 4 | Forming an independent MOTU, but one record fits with *M. irena* | **2/11** [4,11] |

| Taxa | Number of specimens | Consenus delimitation | Methods not supporting the consensus delimitation |
|---|---|---|---|
| *Odontopodisma schmidtii* | 3 | Split into two MOTUs | **5/11** [2,4,5,6,7] |
| *Odontopodisma* sp. | 6 | Independent unidentified MOTU | **6/11** [1,2,3,4,5,11] * |
| *Peripodisma* sp. | 1 | Independent unidentified MOTU | — |
| **Acrididae – Calliptaminae** | | | |
| *Calliptamus barbarus* | 28 | Split into two MOTUs | — |
| *Calliptamus siciliae* | 13 | Split into two MOTUs | **1/11** [11] |
| **Acrididae – Acridinae** | | | |
| *Acrida* sp. (GBORT781-15) | 1 | Independent unidentified MOTU | — |
| **Acrididae – Oedipodinae** | | | |
| *Acrotylus fischeri* | 5 | Merged with *A. insubricus* and part of *A. patruelis* | — |
| *Acrotylus insubricus* | 11 | Merged with *A. fischeri* and part of *A. patruelis* | — |
| *Acrotylus patruelis* | 11 | Forming an independent MOTU, but two records fit with *A. insubricus* and *A. fischeri* | — |
| *Aiolopus puissanti* | 2 | Merged with *A. thalassinus* | — |
| *Aiolopus thalassinus* | 9 | Forming an independent MOTU, but eight records fit with *A. puissanti* | **1/11** [4] |
| *Bryodemella tuberculata* | 8 | Split into two MOTUs | **3/11** [2,4,11] |
| *Oedaleus decorus* | 11 | Split into two MOTUs | **1/11** [4] |
| *Oedipoda coerulea* | 7 | Merged with *O. fuscocincta* and *O. germanica* | — |
| *Oedipoda fuscocincta* | 2 | Merged with *O. germanica* and *O. coerulea* | — |
| *Oedipoda germanica* | 3 | Merged with *O. fuscocincta* and *O. coerulea* | — |
| *Psophus stridulus* | 5 | Split into two MOTUs | **5/11** [1,2,4,6,11] |
| *Sphingonotus* spp. (except *Sphingonotus guanchus*) | 94 | Divided into three main MOTUs, from different species: *S. azurescens*, *S. almeriense*, *S. sublaevis*, *S. caerulans*, *S. morini*, *S. nodulosus*; *S. caerulans*, *S. rubescens*, *S. lluciapomaresi*, *S. lusitanicus*, *S. azurescens* and *S. azurescens*, *S. almeriense*, *S. imitans* | **10/11** [1-10] * |
| *Thalpomena* sp. | 2 | Independent unidentified MOTU | — |
| **Acrididae – Gomphocerinae** | | | |
| *Arcyptera* spp. | 32 | Merged in a single MOTU | **5/11** [1,8,9,10,11] |
| *Chorthippus dorsatus*, *Chorthippus loratus* and *Chorthippus dichrous* | 27 | Divided into two main MOTUs, one including just *Chorthippus dorsatus* and one including all three species* | **4/10** [2,4,5,7] |
| *Chorthippus jucundus* | 2 | Split into two MOTUs | — |
| *Chorthippus* sp. (MEDOR421-23) | 222 | Merged in a single MOTU | **10/11** [1,2,3,4,5,7,8,9,10,11] * |
| *Chorthippus vagans* | 16 | Merged in a single MOTU, but *C. v. dissimilis* specimens cluster into a different MOTU | **6/11** [2,3,4,5,7,11] * |
| *Euchorthippus* spp. (except *Euchorthippus albolineatus*) | 47 | Merged in a single MOTU | **5/11** [1,8,9,10,11] |
| *Omocestus panteli*, *O. viridulus*, *O. rufipes*, *O. haemorrhoidalis*, *O. femoralis*, *Myrmeleotettix maculatus*, *Stenobothrus festivus*, *S. bolivarii*, *S. stigmaticus*, *S. grammicus*, *S.* sp. | 116 | Merged in a single MOTU | **8/11** [1,4,5,7,8,9,10,11] * |
| *Pseudochorthippus parallelus* and *Pseudochorthippus montanus* | 27 | Divided into five main MOTUs, three ones including just *P. parallelus* and two ones including both species | **10/11** [1,2,3,4,5,7,8,9,10,11] * |
| *Stauroderus scalaris*, *Gomphocerus sibiricus*, *Stenobothrus* sp., *Gomphocerippus rufus*, *Chorthippus yersini*, *Ch. apricarius*, *C. brunneus*, *C. mollis*, *C. jacobsi*, *C. binotatus*, *C. messinai*, *C. nevadensis*, *C. biroi*, *C. acroleucus*, *C. macrocerus*, *C. mollis ignifer*, *C. biguttulus*, *C.* sp., *C. vagans dissimilis*, *C. maritimus maritimus* | 137 | Merged in a single MOTU | **10/11** [1,2,3,4,5,7,8,9,10,11] * |
| *Stenobothrus eurasius*, *S. lineatus*, *S. fischeri*, *S. stigmaticus*, *S. nigromaculatus*, *S. rubicundulus*, *S.* sp. | 43 | Merged in a single MOTU | **4/11** [4,5,7,11] |
| *Stenobothrus grammicus*, *S. lineatus*, *S. festivus*, *S. bolivarii*, *S. stigmaticus*, *S. crassipes*, *S.* sp., *Omocestus bolivari*, *O. minutissimus*, *O. uhagonii*, *O. femoralis*, *O. antigai antigai*, *Myrmeleotettix maculatus*, *M. antennatus* | 236 | Merged in a single MOTU | **5/11** [1,8,9,10,11] |

**Table 3.** Delimitation pattern of the ensiferan taxa. This only includes cases for which less than seven methods out of eleven reflect the traditional taxonomy. Unidentified MOTUs are included too. Unidentified records clustering within species-level identified MOTUs are not included. The first column indicates the taxa name, while the second one represents the relative number of specimens represented in the current database. The third column describes the pattern of consensus delimitation observed, while the fourth one delineates the name and number of methods not supporting the OTUs proposed in the previous field. In the fourth column, in square brackets, the method names are coded as follows: 1 = BIN; 2 = ABGD_JC_INIT; 3 = ABGD_JC_REC; 4 = ABGD_K2_INIT; 5 = ABGD_K2_REC; 6 = ASAP_JC; 7 = ASAP_K2; 8 = GMYC_STRICT_YULE; 9 = GMYC_REL_YULE; 10 = GMYC_STRICT_COAL; 11 = PTP. — (*): Taxa for which the delimitation pattern is not clear, due to methods splitting and merging the same clusters simultaneously. — (**): See supplementary for discussion on this species' delimitation pattern.

| Taxa | Number of specimens | Consensus delimitation | Methods not supporting the consensus delimitation |
|---|---|---|---|
| **Gryllidae** | | | |
| *Eugryllodes escalerae* | 7 | Split into two MOTUs | **4/11** [7,8,10,11] |
| *Eugryllodes pipiens pipiens* | 7 | Split into two MOTUs | — |
| *Gryllomorpha longicauda* | 12 | Split into three MOTUs | **7/11** [2,3,6,8,9,10,11] * |
| *Modicogryllus frontalis* | 2 | Merged with *M. truncatus*** | — |
| *Modicogryllus* sp. 1 (MEDOR957-23, MEDOR958-23) | 2 | Independent unidentified MOTU | **5//11** [2,4,8,9,11] |
| *Modicogryllus* sp. 2 | 7 | Independent unidentified MOTU | **5//11** [2,4,8,9,11] |
| *Modicogryllus truncatus* | 1 | Merged with *M. frontalis*** | — |
| *Ovaliptila* sp. 1 (MEDOR1122-23, MEDOR169-23) | 2 | Independent unidentified MOTU | — |
| *Ovaliptila* sp. 2 (MEDOR1121-23, MEDOR080-23) | 2 | Independent unidentified MOTU | — |
| *Petaloptila aliena* | 4 | Merged with *P. galaica* | — |
| *Petaloptila galaica* | 13 | Merged with *P. aliena* | — |
| *Petaloptila* sp. 1 (MEDOR429-23) | 1 | Independent unidentified MOTU | **4/11** [1,2,4,6] |
| *Petaloptila* sp. 2 | 12 | Independent unidentified MOTU | — |
| **Oecanthidae** | | | |
| *Oecanthus pellucens* | 15 | Split into two MOTUs | **8/11** [2,3,4,5,6,8,10,11] * |
| *Mogoplistidae* | | | |
| *Paramogoplistes dentatus* | 3 | Split into two MOTUs | **2/11** [6,9] |
| *Gryllotalpidae* | | | |
| *Gryllotalpa vineae* | 3 | Split into two MOTUs | **2/11** [6,9] |
| *Trigonidiidae* | | | |
| *Nemobius sylvestris* | 16 | Split into three MOTUs | **6/11** [1,2,3,6,9,11] * |
| *Pteronemobius* sp. | 2 | Independent unidentified MOTU | — |
| **Rhaphidophoridae** | | | |
| *Dolichopoda* (MEDOR276-23) | 1 | Independent unidentified MOTU | — |
| **Tettigoniidae – Meconematinae** | | | |
| *Cyrtaspis scutata* | 6 | Split into two MOTUs | — |
| *Meconema meridionale* | 6 | Split into two MOTUs | — |
| **Tettigoniidae – Phaneropterinae** | | | |
| *Barbitistes serricauda* | 7 | Split into two MOTUs | — |
| *Isophya brevicauda* | 4 | Merged in a single MOTU | **6/11** [2,3,4,5,6,9] * |
| *Isophya kraussii* | 8 | Merged in a single MOTU | **8/11** [2,3,4,5,6,8,9,10] * |
| *Isophya modestior* | 6 | Split into three MOTUs | **3/11** [6,9,11] |
| *Leptophyes albovittata* | 6 | Split into three MOTUs | **5/11** [1,6,7,8,10] |
| *Odontura* sp. 1 (MEDOR1009-23, MEDOR1010-23, MEDOR054-23) | 3 | Independent unidentified MOTU | — |
| *Odontura* sp. 2 (MEDOR1016-23) | 1 | Independent unidentified MOTU | **5/11** [2,3,4,5,6] |
| *Odontura* sp. 3 (MEDOR1017-23, MEDOR1020-23, MEDOR1021-23) | 3 | Independent unidentified MOTU | **5/11** [2,3,4,5,6] |
| *Odontura* sp. 4 (MEDOR1018-23, MEDOR1019-23, MEDOR1022-23, MEDOR531-23) | 4 | Independent unidentified MOTU | **6/11** [2,3,4,5,6,11] |
| *Phaneroptera nana* | 22 | Split into two MOTUs | — |
| *Phaneroptera sparsa* | 8 | Split into two MOTUs | — |
| *Poecilimon* (MEDOR1253-23) | 1 | Independent unidentified MOTU | — |
| *Poecilimon cretensis* | 3 | Split into two MOTUs | — |
| *Poecilimon fussii* | 7 | Split into two MOTUs | **3/11** [1,7,10] |

| Taxa | Number of specimens | Consenus delimitation | Methods not supporting the consensus delimitation |
|---|---|---|---|
| *Poecilimon schmidtii* | 4 | Split into two MOTUs | **1/10** [10] |
| **Tettigoniidae – Conocephalinae** | | | |
| *Conocephalus* sp. (MEDOR778-23, MEDOR779-23) | 2 | Independent unidentified MOTU | — |
| **Tettigoniidae – Bradyporinae** | | | |
| *Ephippiger diurnus* | 4 | Split into two MOTUs | **2/11** [6,9] |
| *Ephippiger* sp. (MEDOR820-23, MEDOR821-23) | 2 | Independent unidentified MOTU | — |
| *Ephippigerida diluta* | 5 | Split into two MOTUs | **1/11** [9] |
| Ephippigerini sp. (MEDOR223-23, MEDOR441-23) | 2 | Independent unidentified MOTU | **1/11** [9] |
| *Lluciapomaresius anapaulae* | 1 | Merged with *L. asturiensis* | **1/11** [6] |
| *Lluciapomaresius asturiensis* | 8 | Split into three MOTUs, one of which includes one specimen of *L. anapaulae* | **5/11** [1,6,8,10,11] |
| *Lluciapomaresius* sp. 1 (MEDOR233-23) | 1 | Independent unidentified MOTU | **5/11** [2,3,6,9,11] |
| *Lluciapomaresius stalii* | 15 | Merged in a single MOTU | **5/11** [1,7,8,9,10] |
| *Neocallicrania lusitanica* | 3 | Split into two MOTUs, one of which includes one specimen of *N. miegii* | **3/11** [7,8,10] |
| *Neocallicrania miegii* | 9 | Split into two MOTUs, with one single specimen merged with *N. lusitanica* | **3/11** [7,8,10] |
| *Neocallicrania selligera* (except *N. s. selligera* and *N. s. meridionalis*) | 6 | Split into three MOTUs | **5/11** [6,7,8,10,11] |
| *Parasteropleurus martorellii* | 9 | Split into two MOTUs | **1/11** [10] |
| *Platystolus martinezii* | 9 | Split into three MOTUs | **5/11** [2,3,4,5,6] |
| *Pycnogaster cucullatus* | 2 | Split into two MOTUs | — |
| *Steropleurus brunnerii* | 4 | Split into two MOTUs | — |
| *Steropleurus flavovittatus* | 6 | Split into two MOTUs | **2/11** [6,11] |
| *Steropleurus* sp. (MEDOR046-23) | 1 | Independent unidentified MOTU | — |
| **Tettigoniidae – Tettigoniinae** | | | |
| *Antaxius difformis* | 6 | Split into two MOTUs | — |
| *Antaxius kraussii* | 9 | Split into three MOTUs | **7/11** [4,5,6,8,9,10,11] * |
| *Bicolorana bicolor* | 7 | Split into two MOTUs | **3/11** [6,8,10] |
| *Decticus verrucivorus* | 4 | Split into two MOTUs | **4/11** [2,4,6,9] |
| *Eupholidoptera schmidti* | 10 | Merged in a single MOTU | **5/11** [1,3,5,8,10] |
| *Eupholidoptera smyrnensis* | 8 | Split into two MOTUs | **2/11** [6,9] |
| *Eupholidoptera* sp. 1 (MEDOR128-23, MEDOR129-23) | 2 | Independent unidentified MOTU | **5/11** [2,3,4,6,9] |
| *Eupholidoptera* sp. 2 (MEDOR127-23, MEDOR282-23) | 2 | Independent unidentified MOTU | **5/11** [2,3,4,6,9] |
| *Incertana decorata* | 2 | Merged with *I. drepanensis* | — |
| *Incertana drepanensis* | 1 | Merged with *I. decorata* | — |
| *Montana* sp. (MEDOR537-23, MEDOR059-23, MEDOR058-23) | 3 | Independent unidentified MOTU | — |
| *Parnassiana* sp. (MEDOR292-23) | 1 | Independent unidentified MOTU | — |
| *Pholidoptera fallax* | 3 | Split into two MOTUs | — |
| *Platycleis affinis* | 19 | Split into three MOTUs, one of which includes one specimen of *P. falx* | **2/11** [8,10] |
| *Platycleis albopunctata* | 22 | Split into two MOTUs, one of which includes specimens of *P. sabulosa*, while the second one is merged with *P. concii*, *P. grisea*, *P. intermedia* | **3/11** [8,9,10] |
| *Platycleis concii* | 4 | Merged with *P. albopunctata*, *P. grisea*, *P. intermedia* | **3/11** [8,9,10] |
| *Platycleis falx* | 1 | Merged with *P. affinis* | — |
| *Platycleis grisea* | 2 | Merged with *P. albopunctata*, *P. concii*, *P. intermedia* | **3/11** [8,9,10] |
| *Platycleis intermedia* | 6 | Merged with *P. albopunctata*, *P. grisea*, *P. concii* | **3/11** [8,9,10] |
| *Platycleis sabulosa* | 33 | Split into two MOTUs, one of which includes specimens of *P. albopunctata* | **3/11** [8,9,10] |
| *Pterolepis lusitanica* | 2 | Merged with *P. spoliata* | — |
| *Pterolepis spoliata* | 12 | Split into three MOTUs, one of which includes two specimens of *P. lusitanica* | **1/11** [11] |
| *Rhacocleis annulata* | 6 | Split into three MOTUs | **1/11** [9] |

| Taxa | Number of specimens | Consenus delimitation | Methods not supporting the consensus delimitation |
|---|---|---|---|
| *Rhacocleis* sp. 1 (MEDOR070-23) | 1 | Independent unidentified MOTU | — |
| *Rhacocleis* sp. 2 (MEDOR1298-23) | 1 | Independent unidentified MOTU | **4/11** [2,3,6,9] |
| *Rhacocleis* sp. 3 (MEDOR1297-23, MEDOR069-23) | 2 | Independent unidentified MOTU | **4/11** [2,3,6,9] |
| *Roeseliana oporina* | 2 | Merged with *R. r. roeselii* | **1/11** [10] |
| *Roeseliana roeselii roeselii* | 8 | Merged with *R. oporina* | **1/11** [10] |
| *Sporadiana sporadarum* | 5 | Split into two MOTUs | **3/11** [6,9,11] |
| *Tessellana lagrecai* | 3 | Merged with *T. tessellata* | **8/11** [1,2,3,6,8,9,10,11] * |
| *Tessellana* sp. (MEDOR1449-23) | 1 | Independent unidentified MOTU | **4/11** [2,3,6,11] |
| *Tessellana tessellata* (except *T. t. tessellata*) | 15 | Merged with *T. lagrecai* | **8/11** [1,2,3,6,8,9,10,11] * |
| *Tessellana tessellata tessellata* | 1 | Independent MOTU | **4/11** [2,3,6,11] |
| *Thyreonotus bidens* | 13 | Split into four MOTUs | **7/11** [2,3,4,5,6,7,11] * |
| *Thyreonotus corsicus* | 4 | Split into two MOTUs | **1/11** [9] |

apart from the initial partition for caeliferans. Recursive partitions consistently indicated more species than initial partitions. Contrary to ABGD findings, the ASAP method with Kimura two-parameter resulted in larger partitions compared to those from Jukes-Cantor.

The GMYC method, applied with four different parameter combinations, produced a consistent number of species clusters for both ensiferans and caeliferans. However, the application of the Coalescent Constant Population process combined with a relaxed clock model resulted in an anomalously high species count for this dataset, leading to its results being set aside due to poor fit. The outcome of this specific combination, indicating an unexpectedly large number of species, suggests it may not be suitable for this particular dataset analysis. The PTP output in caeliferans surpassed the average species count across BIN, ABGD, ASAP, and GMYC methods, while in ensiferans, the 271 clusters of PTP aligned closely with the averages from other methods. The bPTP analysis, despite extensive generation counts, failed to converge, leading to the exclusion of its results in favour of maximum likelihood outcomes, as recommended by Zhang et al. (2013).

The graphic outputs from SPdel are depicted in File S3 [graphs a, b], showcasing delimitation bars in the order of 11 method and parameter combinations, culminating in a final consensus.

### 3.3. Delimited putative species clusters in Caelifera

In the maximum likelihood tree, while barcodes are correctly assigned in the absence of BIN sharing, higher taxonomic groups are often not retrieved as monophyletic. This suborder's species delimitation methods generally concur on the number of putative species clusters, except for PTP, which delineates a higher species count. The hypothetical species range from 118 to 251. For further details see Table 2.

### 3.4. Delimited putative species clusters in Ensifera

In the maximum likelihood tree, while barcodes are correctly assigned in the absence of BIN sharing, higher taxonomic groups are often not retrieved as monophyletic. In Ensifera, the different delimitation methods show an overall agreement on the number of putative species clusters, with the only exception of PTP, showing a higher number of delimited species. The hypothetical species range from 118 to 251. For further details see Table 3.

## 4. Discussion

### 4.1. Influence of species richness on barcoding efficiency

The percentages of DNA barcoding effectiveness in species identification are somewhat lower than those reported by Hawlitschek et al. (2016) (78.2% for Caelifera + Ensifera, 59.1% for Caelifera, and 100% for Ensifera), but the current findings are based on a substantially larger dataset. Furthermore, in Hawlitschek et al. (2016), each ensiferan genus was often represented by only one species, making it less likely to include any closely related species that might share barcodes. The datasets for ensiferans and caeliferans also highlight differences in the species richness of the respective genera. This significant difference suggests that the higher species richness within each caeliferan genus could contribute to the lower barcoding effectiveness observed, as genera with many species are more likely to exhibit BIN sharing and overlapping barcode clusters.

As expected from a barcode tree (Vences et al. 2005; Hawlitschek et al. 2016), the inferred ML tree showed the taxonomic relations above the generic level as poorly adherent to the recognized taxonomy.

## 4.2. Discussion of delimited putative species clusters by taxonomic families

The detailed discussion of the delimited putative species clusters is organized according to traditional taxonomic families and can be found in File S7 [part 1] for caeliferans and File S7 [part 2] for ensiferans.

## 4.3. Overall patterns of cryptic diversity and recent radiation

The employment of multiple species delimitation methods, each with distinct parameter combinations, on a comprehensive dataset has facilitated the identification of various patterns of diversity, some of which are clearly delineated, while others remain unresolved. In total, 12 cases for caeliferans and 42 cases for ensiferans were identified as potentially harboring more than one cryptic lineage. Additionally, there were 10 cases for caeliferans and 26 for ensiferans of unidentified independent MOTUs. The detected instances of barcode sharing (12 cases for caeliferans and nine cases for ensiferans) can either be attributed to intraspecific variability, suggesting a need for synonymization among these taxa, or may highlight the ineffectiveness of using a single genetic marker for accurate species delimitation, like the case of *Chorthippus* spp.

Consistent with prior studies, delimitation patterns within the Gomphocerinae (Hafayed et al. 2023) and Oedipodinae (Kock et al. 2024) subfamilies (Acrididae) are predominantly influenced by recent radiation events. Species emerging from such events often evade detection through DNA barcoding, resulting in shared BINs and indistinguishable barcodes across all delimitation algorithms. Notably, analogous patterns are also evident in some Tettigoniinae, particularly within the genera *Platycleis* Fieber, 1853 and *Tessellana* Zeuner, 1941 (Platycleidini), which are believed to have undergone recent divergence (Mugleston et al. 2018).

Among well-represented taxa of Caelifera, instances of potential cryptic diversity are notably prevalent within the Pamphagidae and Tetrigidae families, as well as the Calliptaminae and Melanoplinae subfamilies of Acrididae. More distinct and frequent occurrences of potential cryptic diversity are observed within Ensifera, especially among Gryllidae, and also Tettigoniinae, Bradyporinae, and Phaneropterinae (Tettigoniidae). Ensiferans encompass a greater number of taxonomically recognized species (185 vs. 164) and the highest number of putative species clusters identified by each delimitation method, despite a smaller initial specimen count (1128 vs. 1478).

Among all the delimitation methods compared, ABGD, particularly when using the combination of Kimura two-parameters and the initial partition, produced results most consistent with traditional taxonomy. On the other hand, the PTP method was the least consistent, oversplitting the lineages into a large number of clusters.

Geographical regions partially reflect genetic diversification. Various potential cryptic taxa have indeed been identified in isolated localities, such as islands and glacial refugia. Key examples are represented by *Anterastes* supersp. *serbicus* Brunner von Wattenwyl, 1882 in Anatolia and the Balkans (Çiplak et al. 2010a), but also the genus *Oedipoda* Latreille, 1829 in the Mediterranean (Hochkirch et al. 2023), both showing correlation between the radiation of the group and the topography of the species' ranges. The diversification and frequency of these taxa are intimately linked to climatic and geological events, such as Pleistocene glacial cycles, which fragmented populations and created ecological niches for allopatric speciation (Allegrucci et al. 2017). This dynamic has been particularly well-documented in orthopterans with low dispersal capabilities, where repeated isolation and reconnection events, driven by glaciation cycles, have resulted in speciation bursts, especially in geographically complex regions such as the Mediterranean and Anatolia (Ortego et al. 2024).

Widespread European genera, like *Calliptamus* Serville, 1831, a member of the subfamily Gomphocerinae, feature morphologically similar species but display distinct delimitation patterns, including probable cryptic taxa (e.g., within *C. siciliae* Ramme, 1927 and *C. barbarus* (Costa, 1836)). However, these patterns within *Calliptamus* diverge from those observed across other genera in the Acrididae family, despite their similarly broad distribution across Europe.

Another factor impacting geographical diversification patterns is anthropogenic transport and the introduction of non-endemic species. A well-documented case involves *Rhacocleis annulata* Fieber, 1853, native to Sicily but subsequently transported across large areas of continental Europe through the ornamental plant trade (Barataud 2018). A similar scenario has been proposed for *Eupholidoptera smyrnensis* (Brunner von Wattenwyl, 1882) in the Aegean islands (Çiplak et al. 2010b) and may have affected numerous other species, both in recent times and historically, altering distribution ranges and complicating phylogeographic reconstructions (Jesse et al. 2011).

## 4.4. Potential reasons for barcoding and delimitation failure

The BIN discordance analysis and observed delimitation patterns have revealed several cases of incongruency with currently accepted taxonomy. In order to exclude the misidentification of specimens and the use of obsolete taxonomic units as reasons for these incongruencies as far as possible, MEDOR was initially based exclusively on specimens collected in the field over the last decade (between 2013 and 2022) which were identified by experts directly after collection. The identification was verified after analysing the ML trees using the most recently published dichotomous keys. As taxonomic revisions of orthopterans are continuously being published (e.g., Barat 2007, 2012, 2013; Massa et al. 2023), further updates for

many groups were required. Consequently, this dataset conforms to the most up-to-date taxonomy.

The quality control protocol, involving sequence alignment assessment, phylogenetic reconstruction, chromatogram analysis, and sequence identification using BLASTn, has excluded most suspect records potentially representing numts to the greatest extent possible. Additionally, the improvement in PCR primers has contributed to reducing numts. Furthermore, Moulton et al. (2010) noted that the proportion of amplified numts clustering with an incorrect species is minimal compared to the number of orthologs and paralogs that form independent clusters or no clusters at all, suggesting numts occurrence as a minor concern in this analysis.

The influence of *Wolbachia* bacteria was also examined by analysing each sequence with BLASTn. However, merely detecting the presence of this endosymbiont does not necessarily indicate its impact on the host genome or the occurrence of horizontal gene transfer (HGT) (Hawlitschek et al. 2016). HGT signatures are typically identified and removed from data based on phylogenetic incongruence, patchy distribution, or compositional anomalies (Zhaxybayeva and Doolittle 2011), with this project only adopting the first approach due to the limitations of genome-wide strategies.

Signatures of potential hybridization and introgression have been confirmed in several taxa within this dataset, with notable cases reported among both caeliferans and ensiferans. Hybrids among *Chorthippus biguttulus* (Linnaeus, 1758) and related species have been generated under laboratory conditions, but hybrid males were found to be behaviorally sterile due to their intermediate courtship songs rejected by all females (Gottsberger and Mayer 2007), suggesting HGT in the wild to be minimal or absent. On the other hand, as reported by Korsunovskaya (2016), all intermediate morphological forms between *Platycleis albopunctata* (Goeze, 1778) and *P. intermedia* (Serville, 1838) emitted calling signals that match the temporal pattern of *P. albopunctata*, indicating that hybrid males may reproduce. DNA barcoding cannot detect genetic admixture, as the mitochondrial COI is exclusively inherited through the maternal lineage. Therefore, the presence of hybrid specimens in the current dataset cannot be ruled out, requiring further evidence for confirmation (Zhaoke et al. 2021).

Incomplete lineage sorting (ILS), the sharing of ancestral haplotypes among related species, is identified as a primary cause of barcoding failure in numerous groups. Rapid and recent speciation, particularly within the Gomphocerinae subfamily of Acrididae, which diverged approximately in the last 6.44 million years (Hawlitschek et al. 2022), and specifically within the genera *Chorthippus* and *Stenobothrus* Fischer, 1853 (Vedenina and Mugue 2011), exemplifies this issue. Identical mitochondrial haplotypes and barcodes can be obtained between populations isolated for ~1 million years (Nabholz 2023). Additionally, post-glacial re-colonization has been suggested as the origin for Central European populations of some Acrididae species, potentially leading to recent secondary contacts and narrow hybrid zones (Lunt et al. 1998; Nolen et al. 2020).

The current delimitation analyses underscore the limitations of barcoding in distinguishing extremely recently diverged species, while also highlighting interesting patterns in less recently diverged lineages that may conceal cryptic taxa, due to nearly identical morphological and bioacoustic characteristics among different species.

A prime example is provided by the insular Greek populations of *Eupholidoptera* Maran, 1953 and *Poecilimon* Fischer, 1853, particularly species such as *E. smyrnensis* and *P. cretensis* Werner, 1903. Despite being classified as a single taxon, populations from different islands or even different regions within the same island (e.g., Crete) exhibit clear genetic divergence. This divergence is influenced by the mutation rate of the marker in question (COI), which dictates the expected number of mutations diagnostic of a species. The mutation rate determines the pace at which neutral divergence accumulates, potentially enlarging the barcoding gap in rapidly evolving species (Allio et al. 2017).

## 4.5. Perspectives

As previously noted, several evolutionary processes, predominantly hybridization and incomplete lineage sorting (ILS), significantly influence the performance of species delimitation based on barcode sequences. Utilizing methods based on diverse theoretical frameworks helps mitigate biases associated with the limitations of each algorithm.

Taxonomically problematic groups, including Platycleidini, Gomphocerinae, and Stenobothrinae, could be more accurately delimited and phylogenetically resolved through the adoption of more rigorous protocols. Schmidt et al. (2024) have shown RADseq to be an effective method for elucidating the phylogeny of the *C. biguttulus* group and other species within the genera *Chorthippus* and *Pseudochorthippus* Defaut, 2012. The species clusters derived from this approach closely correspond with traditional taxonomy, notwithstanding the complexities introduced by their recent evolutionary histories and frequent occurrences of hybridization. Moreover, significant disparities between nuclear and mitochondrial phylogenies were noted, rendering COI alone insufficient for comprehensive phylogenetic analysis and the detection of recent radiative events.

High-throughput genomic techniques promise to effectively identify divergent lineages among both widespread and endemic species. Esquer-Garrigos et al. (2019) uncovered a surprisingly intricate level of biogeographical complexity at a localized scale in populations of *Ephippiger diurnus* Dufour, 1841 in the Pyrenees. Similarly, the current study has revealed potential divergent lineages within endemic and geographically restricted species, notably among ensiferans, with *Antaxius difformis* (Brunner von Wattenwyl, 1861) and *Thyreonotus bidens* (Bolívar, 1887) serving as prime examples.

Future investigations should leverage high-throughput genetic methodologies not only to address existing taxonomic uncertainties but also to explore the biogeographical characteristics of endemic orthopteran species, which

may demonstrate greater (rather than less) variation and divergence compared to their more ubiquitous counterparts.

## 5. Conclusion

Our barcoding data, thanks to rigid quality protocols and a representative set of assessed species, will be useful for a wide range of applications in taxonomy, conservation management and ecology. The well-known advantages of DNA barcoding will allow a reliable identification of many Mediterranean orthopterans, even just basing on biological fragments and soil eDNA. This becomes crucial for the detection of the presence of endangered species, both globally and locally, in a certain area. Barcodes of IUCN critically endangered species like *Isophya harzi* Kis, 1960, *Zubovskya banatica* Kis, 1965 and *Chorthippus acroleucus* (Müller, 1924) were here published for the first time on BOLD systems (IUCN 2023).

To minimize misidentification and taxonomic confusion, species exhibiting BIN sharing should not be fully considered in species delimitation conclusions, as resolving such cases requires more robust investigations using multi-locus or genomic approaches. However, these species delimitation results provide key insights, including evidence of potential cryptic undescribed taxa, instances of synonymy requiring taxonomic revision, and geographically structured divergent lineages. This comprehensive preliminary assessment of orthopteran biodiversity lays the groundwork for future in-depth taxonomic studies, population genomic analyses, and expanded sampling programs, ultimately advancing the understanding of the evolutionary and biogeographic dynamics of orthopterans in the Mediterranean basin.

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## 7. Disclosure statement

The authors report there are no competing interests to declare.

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# Supplementary Material 1

## Files S1–S8

**Authors:** Ragazzini M, Kleukers R, Willemse L, Baudewijn O, Dey L-S, Hawlitschek O (2025)

**Data type:** .zip

**Explanation notes:** **File S1.** notes on the protocols involved in PCR at LIB (Protocol a), PCR at Naturalis (Protocol b) and DNA sequencing (Protocol c). — **File S2.** Maximum Likelihood trees inferred for caeliferans (Graph a) and ensiferans (Graph b). — **File S3.** Graphic outputs from SPdel (Ramirez et al. 2023), showcasing the species delimitation patterns and consensus inferred for caeliferans and ensiferans. — **File S4.** Species delimitation and consensus patterns from SPdel (Ramirez et al. 2023) for caeliferans and ensiferans. — **Table S5.** Table showing, the first sheet, detailed specimen data, including Project, BIN code, Sample ID (accession number to the BOLD PROJECT "MEDOR Barcoding of Mediterranean Orthoptera"), Sequence length, Species, Author, Voucher deposit, Collector, Collection date, Country, Exact site, Latitude, and Longitude. Additional sheets show the percentage of Orthoptera biodiversity captured by this study across five key countries: Spain, Germany, Portugal, Italy, and Greece. — **File S6.** Table presenting the results of BIN discordance analyses conducted on the BOLD System. It includes counts of concordant, discordant, and singleton BINs for both caeliferans and ensiferans, with a total of six separate sheets. — **File S7.** A concise discussion of species delimitation results, for both caeliferans (Part 1) and ensiferans (Part 2). This includes biogeographical factors explaining geographic isolation, taxonomic comparisons highlighting matches or conflicts with existing classifications, and references to previous studies to provide context for the findings. — **File S8.** A comprehensive description of the command, input files, and options used for the SPdel.py analysis.



**Link:** https://doi.org/10.3897/asp.83.138238.suppl1