



## A cautionary note on synonymization based on mitochondrial data in Orthoptera: a comment of Hochkirch *et al.* 2023

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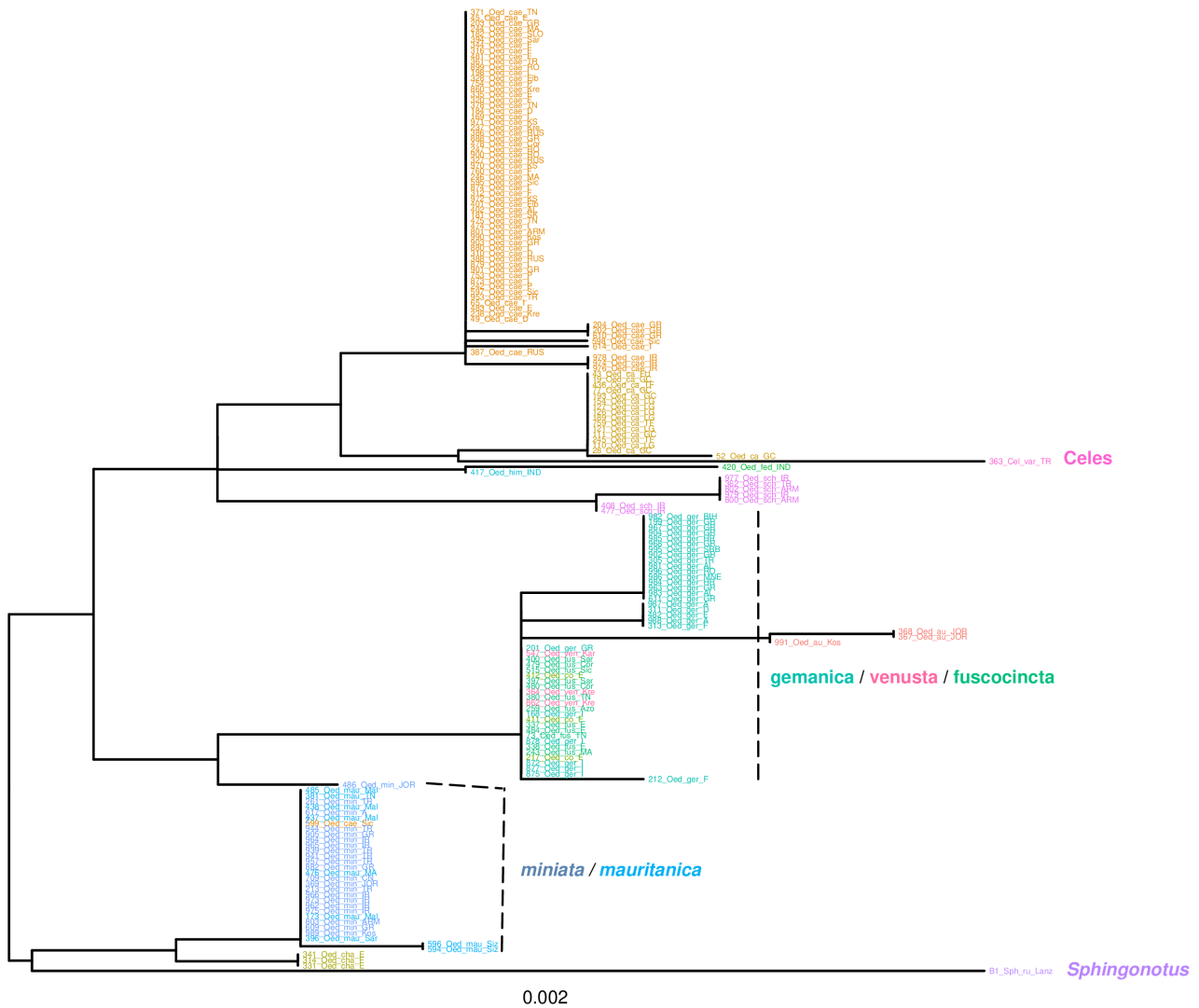
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Hochkirch *et al.* (2023) recently published a phylogeny of the band-winged grasshopper genus *Oedipoda*. Using three mitochondrial and one nuclear loci, they estimated the phylogeny of 177 specimens. This phylogeny offers insights into the evolutionary history and biogeography of the group and demonstrates the usefulness of molecular data to estimated populations and species histories. They also provide valid taxonomical changes, raising two former subspecies and one former synonymy to the species level. However, we believe that taxonomic changes regarding synonymization proposed by the authors are premature given the data utilized. Indeed, we will illustrate cases below where well-established species lack support from mitochondrial data, which is comparable to or even more extensive than that used by Hochkirch *et al.* (2023).

### 1) Small amount of genetic data could fail to identify good species

First, it is important to note that the dataset employed by Hochkirch *et al.* (2023) primarily relies on mitochondrial sequences. We reanalyses the datasets focusing only on the nuclear marker (ITS2). A phylogeny was estimated using IQTREE2 (Minh *et al.* 2020) and the best model selected by the model selection procedure. The estimated phylogeny has a limited resolution (Figure 1). It does not support genetic differentiation of *miniata* / *mauritanica* nor *germanica* / *venusta* / *fuscocincta*. It does not even recover the monophyly of *Oedipoda* relative to *Sphingonotus* and *Celes*. This demonstrates that any relevant genetic information regarding recent population differentiation could only come from mitochondrial DNA. However, there are many examples of well accepted species that will not be identified using mitochondrial DNA alone despite clear evidence when a larger amount of nuclear data is used.

The most notable example within Orthoptera is species from the *Gomphocerippus* *gr. biguttulus*. Following Defaut (2022), we refer to this genus rather than *Chorthippus*. Within this group, the species status of *biguttulus*, *mollis*, and *brunneus* has never been questioned on the phenotypic basis and using large scale molecular study. Moreover, they occur frequently in sympatric without extensive hybridization. Nevertheless, the mitochondrial phylogeny obtained with few loci or with the complete mitochondrial genome results in a complete mix of all species (Hawllitschek *et al.* 2017, 2022; Nattier *et al.* 2011; Nolen *et al.* 2020; Vedenina & Mugue 2011). Other closely related species are also frequently intermixed, including *G. eisentrauti*, *G. rubratibialis*, and even *G. rufus* (Hawllitschek *et al.* 2022; Nolen *et al.* 2020; Schmidt *et al.* 2024). The presence of nuclear copies of mitochondrial sequences (NUMTs) is known to be a problem in grasshoppers (*e.g.*, Song *et al.* 2008), but it could be excluded in the latter analyses because the sequences were obtained using transcriptome sequencing (RNAseq data), and NUMTs are expected to be extremely rare in RNAseq data (Nabholz 2023). Interestingly, using a large amount of nuclear data leads to a clear identification of these species (Hawllitschek *et al.* 2022; Nolen *et al.* 2020; Schmidt *et al.* 2024). Nabholz (2023) demonstrated that the lack of phylogenetic separation between *G. biguttulus*, *G. mollis*, and *G. brunneus* could be explained by a recent divergence (on the order of one million years), which did not allow time for complete lineage sorting (*i.e.*, the genetic diversity present in the ancestral population is still present and shared by the current species). Using the dataset of Nolen *et al.* 2020, we identify that 30 unlinked loci or more are needed to confidently identify the genetic differentiation between these closely related species (Figure S1: <https://zenodo.org/records/11070644>).

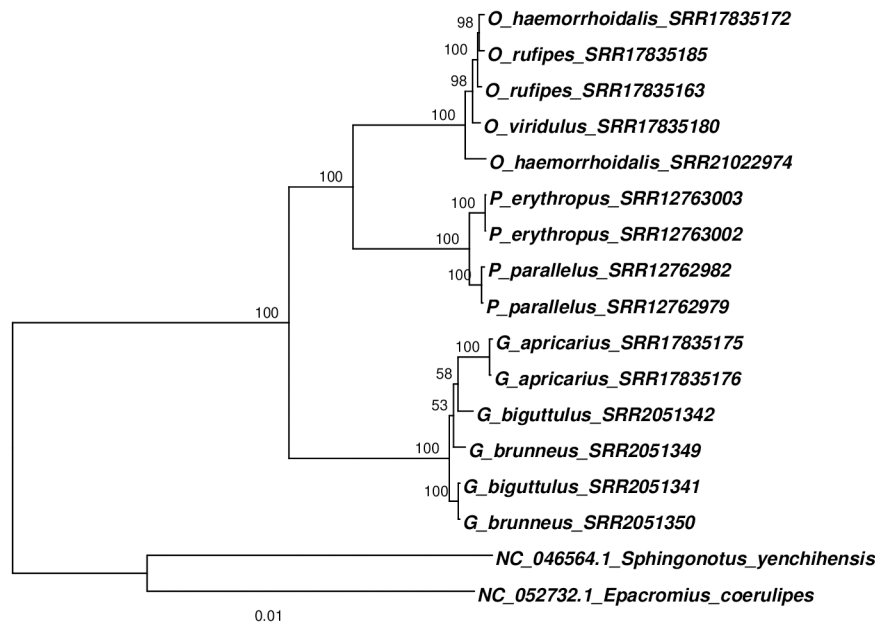


**FIGURE 1.** Molecular phylogeny estimated using IQTREE2 (Minh *et al.* 2020) and ITS2 loci. Colours represent species according to Supplementary Table 1 of Hochkirch *et al.* 2023. Dotted lines indicate species not supported by the phylogenetic estimation.

Another example arises from three species of the genus *Omocestus* : *O. haemorrhoidalis*, *O. rufipes* and *O. viridulus*. These species exhibit distinct phenotypes, both morphologically and in their songs, making them easily identifiable in fields. However, mitochondrial loci have failed to support their species status (Hawlitsek *et al.* 2022). Since this case has received less attention than that of *Gomphocerippus*, we conducted a mitochondrial phylogeny incorporating an additional specimen of *O. haemorrhoidalis* assembled from transcriptome data (Accession SRS14623658) (Figure 2). Nuclear data obtained from transcriptome sequencing confirm the morphological species delimitation (Hawlitsek *et al.* 2022). Here, however, the cause of the lack of mitochondrial resolution remains to be investigated and could be attributed to factors such as introgression or incomplete lineage sorting.

There is little doubt that these species would not be distinguished by the mitochondrial genes used by Hochkirch *et al.* (2023). Beyond the Acrididae, further examples can be found in birds such as the Yellowhammer (*Emberiza citrinella*) and the Pine Bunting (*E. leucocephalos*), where hybridization has been proposed as the cause of the absence of any mitochondrial differentiation (Nikelski *et al.* 2023) or in the *Saturnia* moth, where the mitochondrial sequences of *S. pavonia* and *S. pavoniella* are completely mixed, but a structure emerges using a large number of nuclear sequences (Khan *et al.* 2024). Finally, we can consider cicadas in the genus *Cicadetta*, where the molecular phylogeny based on a few markers does not precisely identify the different species, despite characteristic sound emissions associated with well-defined distribution areas (Hertach *et al.* 2015, 2016).

Importantly, reviewing these cases is not intended to convey the message that greater emphasis should be placed on morphology against molecular data in taxonomy. A population with clear genetic differentiation but lacking morphological differentiation could still warrant taxonomic description. In contrast, a population that is morphologically differentiated and geographically separated but lacks mitochondrial differentiation should be subject to further investigation rather than automatic synonymization. We believed that this approach would increase taxonomic stability.



**FIGURE 2.** Molecular phylogeny estimated using IQTREE2 (Minh *et al.* 2020) and the complete mitochondrial genome showing the paraphyly of the species within the genus *Omocestus* and *Gomphocerippus*. Node supports are ultra-fast bootstrap supports. The code to the right of the species name indicates SRA accession number. All data are from Hawlitschek *et al.* 2022 except *Omocestus haemorrhoidalis* accession SRR21022974. Abbreviation: O. = *Omocestus*, P. = *Pseudochorthippus* and G. = *Gomphocerippus*.

## 2) On the cases of *Oedipoda fuscocincta morini* Defaut 2006 and *Oedipoda caerulescens sardeti* Defaut 2006

We would like to discuss the taxa *Oedipoda fuscocincta morini* Defaut 2006 and *Oedipoda caerulescens sardeti* Defaut 2006, for which we have taxonomic expertise. Hochkirch *et al.* (2023) include two specimens of *O. fuscocincta morini* Defaut 2006. This subspecies from Corsica and Sardinia is slightly smaller and has blue-green hind wings compared to the yellow wings of the nominate *fuscocincta* (Defaut 2006). These morphological differences, along with clear allopatry, meet the criteria for subspecies designation (Mayr 1963). As for *sardeti*, it is endemic to Corsica with clear morphological differences from continental populations. Particularly, the black stripe bordering the costal edge of the hind wings is very short (compared to reaching the middle of the costal edge in the nominate subspecies), the hind tarsi are red or reddish-brown (as opposed to brown to yellowish), and there are differences in morphometric ratios on the head (Defaut 2006). Hochkirch *et al.* (2023) suggest extending the name *sardeti* to continental Spain because two specimens from Corsica are within a clade with Spanish individuals. The lack of genetic differentiation observed by Hochkirch *et al.* demonstrates that this population recently became isolated, yet it is still largely compatible with a subspecies status.

## 3) Additional notes

The four specimens of the subclade named “*O. coerulea*” were collected in the Iberian Pyrenees (specimens 217, 411, 412, 482) and form a separate clade from the *fuscocincta* clade. The other two were collected in Sierra Nevada (337: Pampaneira, ≈ 1,060 m, and 338: Hotel Santa Cruz, 1,540 m) and are present in a separate subclade within the *fuscocincta* clade. However, it should be noted that the Sierra Nevada is not connected to the Pyrenees, and the possibility that these specimens belong to a different taxon should be considered. Nevertheless, as the authors point out, the type locality of *O. coerulea* remains to be defined, as its describer stated that he did not know it (Saussure, 1884, p. 150: “Patria?”). We also

note that the types are not present, at least in their original form, in the collection of the Geneva museum, but one could search for them among *O. miniata* and *O. fuscocincta* specimens (boxes V19 and V20: see Hollier 2012, 2012, 241).

Next, out of the four Pyrenean specimens that fall into the *coerulea* clade, three had blue wings, as expected, but one had red wings (specimen 482). It was identified as *germanica* by its collector (A. Hochkirch *in litt*). Morphological identification would have been possible because the black band on the costal edge of the hindwings is very short in *coerulea* and very long in *germanica* (of intermediate length in hybrids). It could well be a hybrid, but as the Pyrenean collector only provided a postfemur, it is not possible to be certain. Finally, one specimen (484) belonging to the *germanica* clade has blue wings. Once again, a hybrid between *germanica* and *coerulea* should be considered.

#### 4. Conclusions

Hochkirch *et al.* (2023) provide a very comprehensive sampling across the Western Palearctic species of the genus *Oedipoda*. The phylogeny estimated yields interesting insights and was useful to take taxonomic decisions to raise subspecies at species. Mitochondrial DNA, including barcoding methods, have proven effective in identifying cryptic lineages or demonstrating significant genetic differentiation among populations previously considered subspecies. However, rapid speciation and gene flow between closely related species limits the accuracy of analyses based on few markers in representing species history. Therefore, we argue in avoiding synonymization based on a limited number of markers in order to increase taxonomic stability. Instead, we advocate that integrative taxonomy approach should be favored whenever possible (Schlick-Steiner *et al.* 2010), and suggest refraining from implementing the synonymization proposed by Hochkirch *et al.* (2023) at this time.

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