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Article

The efficiency of the *COI* gene as a DNA barcode and an overview of Orthoptera (Caelifera and Ensifera) sequences in the BOLD System

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Abstract

Orthoptera, among the oldest and most numerous insect lineages, is an excellent model for evolutionary studies but has numerous taxonomic problems. To mitigate these issues, the cytochrome *c* oxidase subunit I (*COI*), standardized with the DNA barcode for Metazoa, is increasingly used for specimen identification and species delimitation. We tested the performance of *COI* as a DNA barcode in Orthoptera, using two analyses based on intra- and inter-specific distances, barcode gap, and Probability of Correct Identification (PCI); and estimated species richness through Automatic Barcode Gap Discovery (ABGD) and Assemble Species by Automatic Partitioning (ASAP). We filtered all sequences of Orthoptera available in Barcode of Life Data System (BOLD) and used 11 605 *COI* sequences, covering 1 132 species, 226 genera, and 18 families. The overall average PCI was 73.86%. For 82.2% of genera, the barcode gap boxplots were classified as "good" or "intermediate", indicating that *COI* can be effective as a DNA barcode in Orthoptera, although with varying efficiency depending on the need for more information. ABGD and ASAP inferred species richness similar to labels informed by BOLD for the suborders Caelifera and Ensifera. The representation of Orthoptera in the BOLD database and the results of these analyses are discussed.

Key words: Orthoptera, Caelifera, Ensifera, integrative taxonomy, specimen identification, species delimitation

Résumé

Si les orthoptères, une des lignées d'insectes les plus anciennes et les plus nombreuses, constituent un excellent modèle pour des études sur l'évolution, ils présentent de nombreux problèmes taxonomiques. Pour les atténuer, la sous-unité I de la cytochrome *c* oxydase (*COI*), normalisée avec le code à barres de l'ADN pour les métazoaires, est de plus en plus utilisée pour l'identification de spécimens et la délimitation d'espèces. Nous avons testé la performance de la *COI* comme code à barres de l'ADN chez les orthoptères en utilisant deux analyses basées sur les distances intra- et inter-spécifiques, l'écart des codes à barres et la probabilité d'identification correcte (PCI) et avons estimé la richesse spécifique par découverte automatique de l'écart des codes à barres (ABGD) et assemblage d'espèces par partitionnement automatique (ASAP). Nous avons filtré toutes les séquences d'orthoptères disponibles dans le Barcode of « Life Data System » (BOLD) et utilisé 11 605 séquences de *COI* couvrant 1 132 espèces, 226 genres et 18 familles. La PCI moyenne globale est de 73,86 %. Pour 82,2 % des genres, les diagrammes de quartiles d'écart des codes à barres sont classés comme étant bons ou intermédiaires, ce qui indique que la *COI* peut être un code à barres efficace chez les orthoptères, bien que d'efficacité variable selon l'information supplémentaire requise. Les méthodes ABGD et ASAP infèrent des richesses spécifiques semblables aux étiquettes obtenues à partir du système BOLD pour les sous-ordres des califères et des ensifères. La représentation des orthoptères dans la base de données de BOLD et les résultats de ces analyses sont abordés. [Traduit par la Rédaction]

Mots-clés : orthoptères, califères, ensifères, taxonomie intégrative, identification de spécimens, délimitation d'espèces

Introduction

Orthoptera is one of the oldest lineages of insects (350 million years of diversification) (Song et al. 2020) and comprises around 29 000 described species worldwide, except for the polar regions (Grimaldi and Engel 2005; Cigliano et al. 2022). The order is subdivided into two suborders, Caelifera (grasshoppers and similar), which groups 35 families, 2 525 genera, and almost 12 400 species; and Ensifera (crickets, katydids, and similar), with 41 families, 2 684 genera, and about 16 800 species, differentiated by the number

of antennomeres, tympanum location, and ovipositor shape (Grimaldi and Engel 2005; Sperber et al. 2021). Orthopterans occur in most terrestrial habitats and a few species are semi-aquatic (Bidau 2014*a*). Most species are omnivorous, although strictly predatory and phytophagous species exist (Sperber et al. 2021). Several species are major agricultural pests (Singh and Kumari 2021); for instance, locust and grasshopper swarms are known for their ability to wipe out crop fields in a single day (Zhang and Hunter 2017; Peng et al. 2020).

Orthopteran taxonomy is based primarily on adult morphology and the male genitalia plays a prominent role in species delimitation studies (Alexander and Otte 1967; Desutter 1987). However, morphology alone may be misleading, and even expert taxonomists may struggle with species diagnoses. Plastic phenotypes, lack of descriptions for immature stages, and cryptic species complexes also hamper accurate specimen identification in this group (e.g., Song and Wenzel 2008; Guo et al. 2016). Such taxonomic puzzles may benefit from integrative approaches incorporating additional evidence, including acoustic profiling (Riede 2018), cytogenetic characters (Bidau and Martí 2010), and DNA markers (Cigliano and Eades 2010).

DNA barcoding (Hebert et al. 2003), perhaps one of the most emblematic contemporary techniques for molecular taxonomy, since its advent has been applied in a myriad of scientific disciplines (DeSalle and Goldstein 2019). For metazoans, the canonical DNA barcode sequence is a region of 648 base pairs (bp) located at the 5′ end of the mitochondrial gene cytochrome *c* oxidase subunit I (*COI-5P*) (Hebert et al. 2003; Savolainen et al. 2005; Ratnasingham and Hebert 2007). Because it is a rapid and relatively inexpensive method with overall good identification accuracy, DNA barcoding can be employed in large-scale biodiversity surveys (Huang et al. 2013; Hawlitschek et al. 2017), as well as in identification of environmental samples (Hardulak et al. 2020) and even centenarian archived specimens (Françoso and Arias 2013; Raxworthy and Smith 2021). DNA barcoding has also been implemented in species discovery and delimitation, often based on the difference between the maximum intraspecific and the minimum interspecific genetic distance of barcode sequences (the "barcoding gap"; Meyer and Paulay 2005).

The *COI* gene has been increasingly used as a DNA barcode for orthopterans and is proving to be a useful complementary tool in diagnosing and delimiting species (Huang et al. 2013; Zhao et al. 2015; Chapuis et al. 2016; Guo et al. 2016; Chen et al. 2018; Zhou et al. 2019; Kim et al. 2020; Wang et al. 2021). While the number of barcodes is increasing in public databases (such as the Barcode of Life Data System (BOLD)), orthopteran taxonomy suffers from a shortage of taxonomists and lack of sufficient funding for taxonomic studies (Cigliano and Eades 2010). Hence, misidentifications permeate the literature and the material entered in databases and deposited in museums, a scenario further complicated by the significant number of synonymies for some groups (Cigliano et al. 2022). Because correct identification of reference sequences is essential for the success of DNA barcoding (Ekrem et al. 2007), an analysis of the state-of-the-art and the performance of orthopteran *COI* barcodes available in BOLD could be useful to guide future studies with these organisms.

Here, we datamined all the orthopteran *COI* sequences available in BOLD, aiming to (*i*) provide an overview of the sequences available in BOLD; (*ii*) assess the existence of barcoding gaps in the available genera; (*iii*) test the specimen identification efficiency of *COI* through the Probability of Correct Identification (PCI); and (*iv*) point out orthopteran genera with inconsistent taxonomy that may benefit from integrative studies implementing DNA barcodes.

Materials and methods

Data obtention and filtering

We retrieved all sequences of Orthoptera available in BOLD (https://www.boldsystems.org; Ratnasingham and Hebert 2007) in April 2021, using as query the 82 valid orthopteran family names (Cigliano et al. 2022) and generating a separate FASTA file for each family. To ensure the quality of our final data set, sequences were filtered following the downstream workflow described by Gonçalves et al. (2021). Briefly, we retained only sequences labeled as *COI-5P* (the standard DNA barcode of metazoans; Hebert et al. 2003) and identified at species level.

A total of 34 549 sequences were aligned using MAFFT 7.0 (Katoh et al. 2019) with the default parameters. AliView (Larsson 2014) was used to inspect alignments, verify stop codons and reading frames, and remove sequences with insertions and deletions. Then, alignments were trimmed to restrict our analysis to the canonical 658 bp barcode region (Hebert et al. 2003) and we removed sequences shorter than 400 bp. Finally, species labels of the remaining sequences were cross-validated with the Orthoptera Species File (http://orthoptera.speciesfile.org; Cigliano et al. 2022).

Data analyses

For the barcoding gap analysis, we generated separate FASTA files for each genus. To allow both intra- and interspecific comparisons, the analyses described below comprise only genera featuring at least two species, with at least one of the species represented by two or more barcodes. For each genus, the R package APE (Paradis and Schliep 2019) was used to estimate intraspecific and interspecific pairwise *p*-distances through the function "dist.dna()". The values generated were visualized as boxplots, which were used to test the performance of *COI* for each genus, following the visual classification proposed by Badotti et al. (2017): good, intermediate, and poor. Performance was considered "good" when there was a clear gap between boxplots of intra- and interspecific distances; "intermediate" when the whiskers of the boxplots overlapped; and "poor" when the boxes of the boxplots overlapped (for more details see Badotti et al. 2017).

DNA barcoding can be useful for specimen identification even in the absence of a barcoding gap (see Collins and Cruickshank 2012). Therefore, we calculated the PCI (Hollingsworth et al. 2009) to measure the discriminative effectiveness of *COI* in specimen identification. We used the R package SPIDER (Brown et al. 2012) to estimate the maximum intraspecific distance and minimum interspecific distance (or nearest-neighbor distance) for each species. If the

maximum intraspecific distance was less than the minimum interspecific distance, identification was considered a success (Hollingsworth et al. 2009). Species with only one sequence were not analyzed, as it would not be possible to obtain intraspecific distances. The PCI values obtained were graphically represented in scatter plots, as suggested by Collins and Cruickshank (2012). All analyses were conducted in R version 4.0.5 (R Core Team 2018).

Finally, we estimated the species richness in the data set compiled for each suborder, using the Automatic Barcode Gap Discovery (ABGD; Puillandre et al. 2012) and the Assemble Species by Automatic Partitioning (ASAP; Puillandre et al. 2021). Both methods cluster barcode sequences into hypothetical species based on pairwise distances. The analyses were run in their respective web interfaces (ABGD: https://bioinfo.mnhn.fr/abi/public/abgd/; ASAP: https://bioinfo.mnhn.fr/abi/public/asap/), using default parameters and simple distances. We interpreted the ABGD results using a prior intraspecific divergence limit of $P = 0.01$ (Puillandre et al. 2012; Gonçalves et al. 2021). The best ASAP partition was selected based on the lowest "asap-score" (Puillandre et al. 2021). We compared the ABGD and ASAP estimates with the number of species labels (species names) according to BOLD.

Results

Of the 82 valid orthopteran families, 47 lack sequence data in BOLD. We started our analysis with 34 549 sequences, encompassing 35 orthopteran families. After the filtering steps to ensure a robust data set (see the Materials and methods), our final data set consisted of 11 605 *COI* sequences, representing 18 families (21.9% of the total valid families), 226 genera (4.3% of the total valid genera), and 1 132 species (3.8% of the total valid species) (Table 1; for a complete list see Supplementary Table S1). We changed the labels of 809 sequences (7% of the data set) belonging to 98 species, due to invalid or misspelled names (Supplementary Table S2). Most of the sequences belong to suborder Caelifera (55.2%). Regarding family coverage, our sample was heterogeneous. Families that contributed most to our data set were Acrididae (46.4%), Tettigoniidae (27.7%), and Gryllidae (7.9%); in contrast, Phalangopsidae and Prophalangopsidae were represented by one genus and two species each. Sequence coverage by species ranged from 1 to 355, with 77% of the species represented by fewer than 10 sequences. The sampled sequences cover roughly 4% of the valid species of Orthoptera.

Among the 226 genera evaluated, *COI* performance was ranked "good" for 139 (61.5%), "intermediate" for 47 (20.8%), and "poor" for 40 (17.7%). The rank system is illustrated in Fig. 1, and an overall classification of each family is provided in Table 1. Data for each genus are appended in Supplementary Table S1. Intra- and inter-specific distances varied considerably among genera (Supplementary Table S1), with an average of 3.78% and 6.06%, respectively.

The average PCI of Orthoptera was 73.86% (Fig. 2), but this value ranged dramatically among families and genera. We found a consistent relationship between the barcoding gap and the PCI results: genera with "good" performance showed a higher PCI, whereas genera with "poor" performance showed a lower PCI (Fig. 3). Detailed PCI values for each family and genus are provided in Supplementary Table S1.

The number of hypothetical species estimated by ABGD and ASAP was higher than the number of species labels in our data set, for both suborders. For Caelifera, our data set comprised 546 species labels, but ABGD and ASAP estimated 1173 and 584 hypothetical species, respectively. For Ensifera, our data set comprised 586 species labels, but ABGD and ASAP estimated around 607 and 570 hypothetical species, respectively. These results suggest potential cryptic diversity among the *COI* sequences of BOLD, particularly for Caelifera.

Discussion

Database

The standard DNA barcode for metazoans is *COI*, but validating its efficacy and searching for appropriate threshold values is mandatory for accurate specimen identification and species delimitations (Hebert et al. 2003). Here, we aimed to test the efficiency of *COI* as a DNA barcode for Orthoptera, using the barcoding gap and PCI analyses. Our results showed that *COI* performs well and that BOLD displays a taxonomic consistency, because we found overall high PCI values and "good" barcoding gap performance for most of the genera analyzed. Low PCI values and overlapping intra- and interspecific distances for genera indicate potential mislabeling in reference databases or complex biological scenarios, such as non-monophyletic taxa, which should be assessed with more detail in future studies.

We started our filtering with 34 549 sequences. However, almost 23 000 sequences were discarded. Most of the sequences removed lack species-level identification, showing that these data were generated and published without a strong taxonomic basis. As a result, such sequences with incomplete taxonomic labels affect the efficacy of DNA barcoding. We emphasize the importance of depositing sequences obtained from rigorously identified specimens to increase the efficiency and accuracy of the method, besides improving studies using molecular data such as phylogenetic (Gu et al. 2020; Shen et al. 2020; Wang et al. 2021) and phylogeographic (Ma et al. 2012) in their analyses.

The use of molecular data in taxonomic studies of Orthoptera is still incipient for many families. So far, only eight genomes have been sequenced and about 250 complete mitogenomes are known (Song et al. 2020). More extensive work has recently emerged, which focused on studies of genome-size evolution (Mao et al. 2020; Ylla et al. 2021; Yuan et al. 2021), mitogenomics (Li et al. 2019; Ma et al. 2019), and phylogenomics associated with the evolution of acoustic communication (Song et al. 2020). However, these studies have investigated only a tiny fraction of orthopteran diversity.

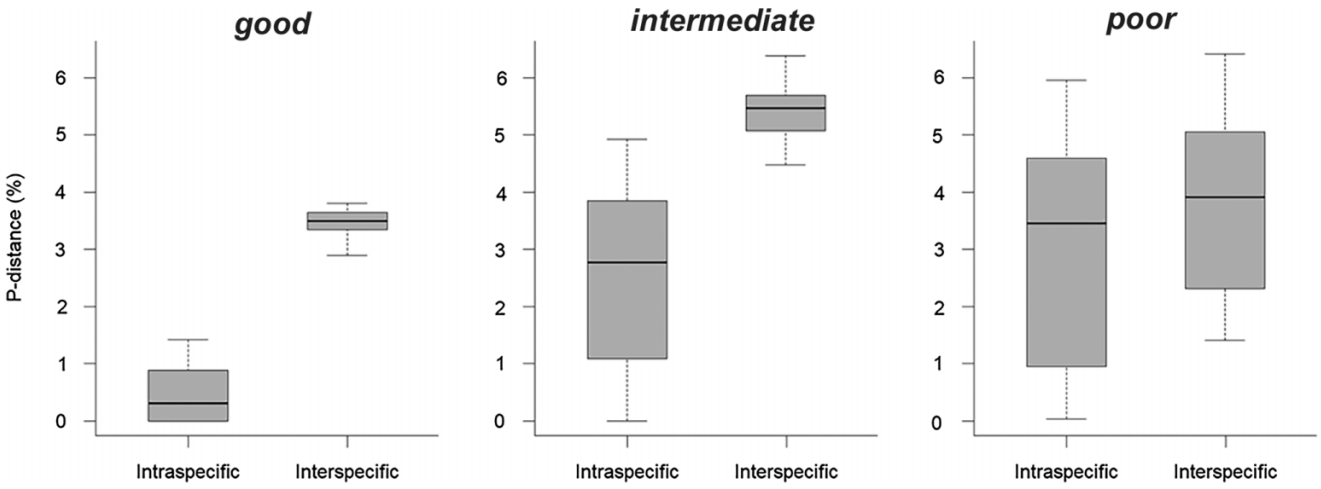
For some families, a large number of sequences were available, such as Acrididae (5386), Tettigoniidae (3221), and Gryllidae (913), whereas other families lack sequence data in BOLD. We somewhat expected this contrast because these families attract more researchers due to their economic and social importance (Bidau 2014*b*; Zhang and Hunter 2017; Song

Table 1. Families of Orthoptera with the number of genera, species, and sequences (percentage shows the proportion of each family of the total sequences), number of genera with the barcode gap classification according to Badotti et al. (2017), and Probability of Correct Identification (PCI) values.

				Barcode gap boxplot classification			
Family	Genera	Species	Sequences (%)	Good	Intermediate	Poor	PCI (%)
Caelifera							
Acrididae	95	477	5 386 (46.41)	44	30	21	46.43
Dericorythidae	1	5	8 (0.06)	1	—	—	100
Morabidae	2	4	179 (1.54)	1	1	—	75
Pamphagidae	3	13	22 (0.18)	2	—	1	85.71
Pyrgomorphidae	4	15	229 (1.97)	3	1	—	84.61
Romaleidae	1	11	335 (2.88)	1	—	—	27.27
Tetrigidae	3	16	222 (1.91)	3	—	—	38.46
Tridactylidae	2	5	23 (0.19)	1	—	1	33.33
Ensifera							
Anostostomatidae	3	7	73 (0.62)	3	—	—	100
Gryllidae	17	122	913 (7.86)	13	1	3	72.72
Gryllotalpidae	2	7	42 (0.36)	1	1	—	71.42
Mogoplistidae	1	13	41 (0.35)	1	—	—	88.88
Myrmecophilidae	1	3	76 (0.65)	1	—	—	66.66
Phalangopsidae	1	2	9 (0.07)	–	—	1	50
Prophalangopsidae	1	2	43 (0.37)	–	—	1	0
Rhaphidophoridae	9	88	533 (4.59)	8	1	—	82.19
Tettigoniidae	69	305	3 221 (27.75)	48	10	11	75.39
Trigonidiidae	11	37	250 (2.15)	8	2	1	75.75
Total	**226**	**1 132**	**11 605**	**139 (61.5%)**	**47 (20.7%)**	**40 (17.7%)**	**73.86**

Note: Total values are provided for the Orthoptera.

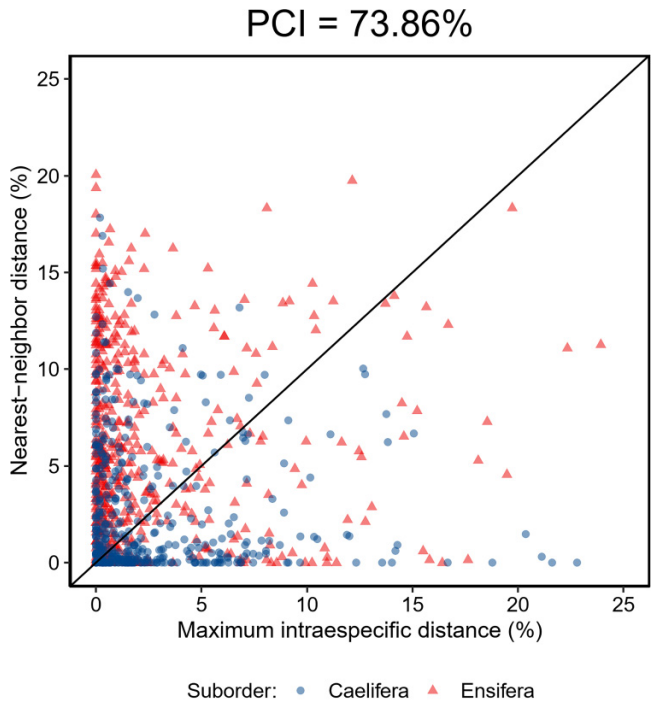
Fig. 1. Examples of the barcode gap classification according to Badotti et al. (2017) used in this study. Classification was "good" when boxplots showed a gap between intra- and inter-specific distances; "intermediate" when the whiskers of intra- and inter-specific distances overlapped; and "poor" when the boxes of intra- and inter-specific distances overlapped.



2018; Singh and Kumari 2021), besides being easier to collect (Sperber et al. 2021). Surprisingly, groups such as Phalangopsidae had few sequences even though they occur across the Neotropical region (Cigliano et al. 2022). We believe that this reflects the difficulty of collecting specimens (Desutter-Grandcolas 1995).

Although orthopteran diversity is higher in Neotropical regions (Song 2018; Cigliano et al. 2022), many of the available sequences are from species of the Oriental region. The Acrididae (suborder Caelifera), considered by several authors as the family with the most orthopteran crop pests (Bidau 2014*b*; Zhang and Hunter 2017; Peng et al. 2020; Singh and Kumari 2021), is the best represented in BOLD. Their reputa-

Fig. 2. Probability of Correct Identification (PCI) of Orthoptera. Each point represents a species and is coded by suborder (Caelifera, blue dots; Ensifera, red triangles). For each species, the maximum intraspecific distance was compared with the minimum interspecific distance (nearest-neighbor distance). Species above the central diagonal line were successfully identified; species below the line were identification failures. [Color online.]



tion as "agricultural pests" is caused mainly by the outbreaks that result in devastated crops, lending this family high economic importance (Singh and Kumari 2021; Chatterjee 2022). Additionally, the ancient cultural practices in Asia revolving around Orthoptera (Jin and Yen 1998; Costa-Neto 2003; Bidau 2014*b*) explain the high abundance in sequences from native Asian families and the interest of local researchers in using these organisms as study models.

Barcoding gap

The overall "good" performance found for the genera analyzed shows that *COI* is an effective marker for studying the diversity of Orthoptera. Intra- and inter-specific distances varied considerably. The majority of genera showed interspecific distances ranging from 7% to 15% and intraspecific distances less than 5%. Previous studies suggested that a threshold value of 3% could be used to delimit arthropod species (Hebert et al. 2003; Huang et al. 2013), and this value has been much used for Orthoptera (Huang et al. 2013; Zhao et al. 2015; Wang et al. 2019; Zhou et al. 2019; Gu et al. 2020; Kim et al. 2020; Kundu et al. 2020). However, we must consider that this is an average value for a broad taxonomic scope, which could well have a wider genetic diversity. Moreover, evolution rates and coalescence times may vary among taxa (Wiemers and Fiedler 2007; Meier et al. 2008). Studies with other arthropod groups show that there is no universal threshold to distinguish between intra- and inter-specific distances (Zimmermann et al. 2015; Poppe et al. 2017, 2019), and that a specific value should be assessed whenever possible.

The best-represented families in terms of sequence abundance (Acrididae, Tetiigoniidae, and Gryllidae) displayed a "good" barcoding performance (see Table 1). Furthermore, our analysis at genus level agrees with the results from previous studies that validated the efficiency of the method (Huang et al. 2013, for *Spathosternum*; Wang et al. 2019, for *Sinocyrtaspis*; Gu et al. 2020, for *Fruhstorferiola*; Kim et al. 2020, for *Tettigonia* and *Paratlanticus*; Wang et al. 2021, for *Tonkinacris*). However, for groups with few sequences or inadequate sampling, DNA barcoding is less accurate, as reported by Trewick (2008) for *Sigaus*.

Biological factors can also affect the efficacy of DNA barcoding (Song et al. 2008; Moulton et al. 2010), such as heteroplasmy (White et al. 2008) and nuclear-mitochondrial pseudogenes (numts) (Song et al. 2008). Moreover, incomplete lineage sorting, hybridization between closely related species, and *Wolbachia* infections are also reported as interfering with DNA barcoding efficacy in orthopteran specimen identification (Hawlitschek et al. 2017). A solution to these biases is to obtain several barcode sequences from the same specimen to determine a dominant haplotype and assume that it is representative of the species (Kang et al. 2016).

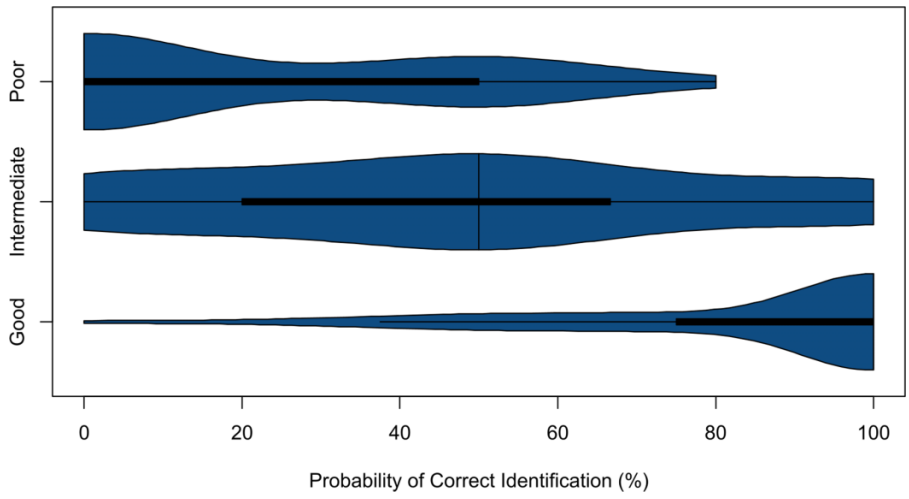
Several genera studied here had boxplots with a prominent number of outlier comparisons. These atypical values may be related to the above scenarios, resulting in molecular distances outside the average for their species. Outliers can also result from population under-sampling, cryptic species complexes, or operational biases such as mislabeling.

Probability of Correct Identification (PCI)

The global PCI value for Orthoptera was 73.86% and PCI values were generally positively related to the barcoding gap rank system. Genera with higher PCI values overall were classified as "good", whereas those with low PCI values received a "poor" classification. Previous studies using the same metrics to evaluate barcodes available in public databases found similar values for nematodes (72.72%; Gonçalves et al. 2021), true bugs (74.33%; Bianchi and Gonçalves 2021), and apid bees (77.02%; Gonçalves et al. 2021). For other taxonomic groups, identification success was closer to 100%, such as in spiders (Blagoev et al. 2009), lepidopterans (Pérez-Asso et al. 2016), and dipterans (Bakhoum et al. 2018).

Considering Acrididae and Tettigoniidae, the two best-represented families in terms of sequence abundance, we observed PCI values of 46.43% and 75.39%, respectively. Although well-characterized families in general yield more robust and reliable results, the results for Acrididae were not expected. Although the abundance of studies minimizes taxonomic errors, more extensive specimen sampling by multiple research groups may actually increase the number of errors. Half of the sequences sampled here belong to this family, and as seen from the barcode gap boxplots, most genera (62%) showed several outliers. These outliers may account for the low PCI values (Badotti et al. 2017).

Fig. 3. Violin plot showing the correlation between barcode gap classification and Probability of Correct Identification (PCI) values in our analysis. [Color online.]



Hawlitschek et al. (2017) analyzed sequences from 127 orthopteran species from Central Europe and found 100% identification success for species of Ensifera, whereas for Caelifera only 59.1% of species were successfully identified. Our results agree with these findings when considering only the best-represented families for species and sequences (Gryllidae + Tettigoniidae for Ensifera, with an average PCI of 74%; Acrididae for Caelifera, with only 46.43%). We believe that the comprehensive sampling lowered our PCI values due to the increase in errors, but the general context is proportionally similar. We emphasize that there is no consensus about the definition and calculation of PCI (Martín et al. 2020) or about threshold values for splitting intra- and inter-specific distances (Will and Rubinoff 2004; DeSalle et al. 2005), and therefore these metrics will vary among different taxa and approaches.

ABGD and ASAP

ABGD and ASAP species estimates were incongruent with the number of species labels in our data set, especially considering the ABGD estimates for Caelifera. Because ABGD is heavily affected by the sequences used as input (Puillandre et al. 2012), these inconsistencies may be due to the barcode gap performance of Caelifera, in which 50% of the genera showed "intermediate" or "poor" results. In contrast, around 75% of the genera of Caelifera displayed a "good" barcode gap performance, which could explain the higher congruency of ABGD and ASAP estimates with the number of species labels. Further research may investigate possible cryptic species complexes in Orthoptera, especially in Caelifera.

Comments on certain genera

Some of the genera studied here showed conflicting results between the barcoding gap and PCI analyses. The overlapping of intra- and inter-specific distances (the lack of a barcode gap) does not necessarily limit the use of DNA barcoding for specimen identification (see Collins and Cruickshank 2012), and these patterns may reflect the differences among species in coalescence times (Virgilio et al. 2010). Thus, the intraspecific distances of a species can often overlap the interspecific distances of other species without compromising the success of identification, here evaluated using the PCI.

We have demonstrated the efficiency of *COI* as a DNA barcode in 226 genera (see Supplementary Table S1). Our sample encompassed genera with hundreds of described species, such as *Anaxipha*, as well as others subdivided into subgenera or species groups such as *Schistocerca*, and even synonymies such as *Neoconocephalus* (Cigliano et al. 2022). The large number of species, as well as the emergence of new morphological characters used to designate groups as new species are discovered, has made the taxonomy of certain genera increasingly complex.

Anaxipha, for instance, although under-sampled, showed a PCI of 100% and a "good" barcoding gap performance, showing that DNA barcoding may aid in the resolution of taxonomic problems concerning this group. Similarly, for *Gryllus*, which consists of 102 valid species (Cigliano et al. 2022), many of them with outdated descriptions based solely on body color and external morphology, we found a PCI of 75% and a clear barcoding gap. Richness estimates for both genera were largely congruent with the taxonomic labels of BOLD.

On the other hand, the performance of *COI* for *Schistocerca* was insufficient. Its PCI was only 5% and the barcode gap was ranked as "intermediate", showing several outliers with intra- and inter-specific distances above 25%. Moreover, the ABGD and ASAP estimates for this genus were highly incongruent with the number of BOLD species labels. This wide variation shows that there are probably several identification errors in the sequences, possibly caused by the phenotypic plasticity found for the genus (Song and Wenzel 2008), the presence of nuclear mitochondrial pseudogenes (numts) (Moulton et al. 2010), and (or) operational biases, such as inefficient taxonomic reference, alignment errors, and sample contamination, among others (Mutanen et al. 2016), given the relatively large number of sequences available in BOLD.

Hawlitschek et al. (2017) highlighted hybridization and incomplete lineage sorting as the main factors that reduce the DNA barcode efficiency, especially in Caelifera. For instance, hybridization has been reported and speciation is still recent for some genera, such as *Chorthippus*, *Stenobothrus*, *Omocestus* (Caelifera), and *Teleogryllus* (Ensifera). On the other hand, the genus *Gryllus*, for which hybridization has also been studied, had a PCI of 75% and "good" barcode gap performance, besides having several outliers. In this case, the outliers may have occurred because of misidentifications rather than hybridization.

Heteroplasmy and high prevalences of numts have been reported in previous studies with Orthoptera. *Ognevia* (Acrididae), *Podisma* (Acrididae) (Bensasson et al. 2000), *Anaposdisma* (Acrididae) (Kang et al. 2016), *Ellipes* (Tridactylidae), and *Taeniopoda* (Romaleidae) (Jesús-Bonilla et al. 2017) showed here a poor resolution, with PCI values lower than 20%. These low values may be related to fluctuation in intraspecific distances due to erroneous numt amplification. On the other hand, genera with previously reported numts, such as *Arcyptera* (Acrididae) (Bensasson et al. 2000), *Anabrus* (Tettigoniidae), and *Myrmecophilus* (Myrmecophilidae) (Moulton et al. 2010) showed an "intermediate" *COI* performance and PCI around 50%, with well-defined intra- and inter-specific distances.

Final comments

We have shown here that DNA barcoding is, overall, a valuable tool for orthopteran specimen identification and species delimitation. Genera with "poor" barcode gap performance and (or) low PCI values should be further investigated: are these results a consequence of operational biases, or is DNA barcoding not sufficient for these groups? We advocate for the use of *COI* together with other taxonomic characters (e.g., morphology, bioacoustics, behavior, ecology, and other genetic data) to study the Orthoptera under an integrative taxonomy framework.

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Data availability

All data used for analyses are available at BOLD (Barcode of Life Data System; https://www.boldsystems.org).

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The authors declare that there are no competing interests.

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Supplementary information

Supplementary tables are available with the article at https://doi.org/10.1139/cjz-2022-0041.

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