

DNA Barcodes Combined with Multilocus Data of Representative Taxa Can Generate Reliable Higher-Level Phylogenies

GERARD TALAVERA^{1,2,*}, VLADIMIR LUKHTANOV³, NAOMI E. PIERCE², AND ROGER VILA⁴

¹Institut Botànic de Barcelona (IBB, CSIC-Ajuntament de Barcelona), Passeig del Migdia s/n, 08038 Barcelona, Catalonia, Spain; ²Department of Organismic and Evolutionary Biology and Museum of Comparative Zoology, Harvard University, 26 Oxford Street, Cambridge, MA 02138, USA;

³Department of Karyosystematics, Zoological Institute of Russian Academy of Sciences, Universitetskaya nab. 1, 199034 St. Petersburg, Russia; ⁴Institut de Biologia Evolutiva (CSIC-UPF), Passeig Marítim de la Barceloneta, 08003 Barcelona, Catalonia, Spain;

*Correspondence to be sent to: Institut Botànic de Barcelona (IBB, CSIC-Ajuntament de Barcelona), Passeig del Migdia s/n, 08038 Barcelona, Catalonia, Spain;
E-mail: gerard.talavera@csic.es.

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Abstract.—Taxa are frequently labeled *incertae sedis* when their placement is debated at ranks above the species level, such as their subgeneric, generic, or subtribal placement. This is a pervasive problem in groups with complex systematics due to difficulties in identifying suitable synapomorphies. In this study, we propose combining DNA barcodes with a multilocus backbone phylogeny in order to assign taxa to genus or other higher-level categories. This sampling strategy generates molecular matrices containing large amounts of missing data that are not distributed randomly: barcodes are sampled for all representatives, and additional markers are sampled only for a small percentage. We investigate the effects of the degree and randomness of missing data on phylogenetic accuracy using simulations for up to 100 markers in 1000-tips trees, as well as a real case: the subtribe Polyommantina (Lepidoptera: Lycaenidae), a large group including numerous species with unresolved taxonomy. Our simulation tests show that when a strategic and representative selection of species for higher-level categories has been made for multigene sequencing (approximately one per simulated genus), the addition of this multigene backbone DNA data for as few as 5–10% of the specimens in the total data set can produce high-quality phylogenies, comparable to those resulting from 100% multigene sampling. In contrast, trees based exclusively on barcodes performed poorly. This approach was applied to a 1365-specimen data set of Polyommantina (including ca. 80% of described species), with nearly 8% of representative species included in the multigene backbone and the remaining 92% included only by mitochondrial COI barcodes, a phylogeny was generated that highlighted potential misplacements, unrecognized major clades, and placement for *incertae sedis* taxa. We use this information to make systematic rearrangements within Polyommantina, and to describe two new genera. Finally, we propose a systematic workflow to assess higher-level taxonomy in hyperdiverse groups. This research identifies an additional, enhanced value of DNA barcodes for improvements in higher-level systematics using large data sets. [Birabiro; DNA barcoding; *incertae sedis*; *Kipepeo*; Lycaenidae; missing data; phylogenomic; phylogeny; Polyommantina; supermatrix; systematics; taxonomy]

The impact of missing data in modern phylogenetics is highly debated. It is well accepted that phylogenetic accuracy improves with a greater sampling of taxa and more informative characters. In practice, possible detrimental effects of imbalanced sampling for phylogenetic inference are often circumvented by excluding taxa and/or genes when the former have problematic placements or the latter have been poorly sampled. However, increasing evidence suggests that inclusion of incomplete taxa (that have not been sequenced for all markers) or incomplete markers (that have not been sequenced for all taxa) may increase phylogenetic accuracy, or at worst be inconsequential provided that a sufficient number of informative characters are sampled overall (Wiens 2003; Philippe et al. 2004; Wiens 2006; de Queiroz and Gatesy 2007; Wiens and Morrill 2011; Wiens and Tiu 2012; Roure et al. 2013; Grievink et al. 2013; Jiang et al. 2014). According to this view, complete matrices are not essential for optimal phylogenetic performance, and incomplete taxa can still be placed correctly. Nevertheless, this approach is not without controversy (Lemmon et al. 2009; but see Wiens and Morrill 2011; Simmons 2012a, 2012b).

Given that comprehensive data sets in terms of both taxa and characters are hard to obtain, especially for

hyperdiverse taxon groups, two strategies are commonly used to explore biodiversity: 1) sampling multiple loci (in the hundreds in the case of phylogenomics) for representatives of higher-level taxonomic categories (the “phylogenetic/-omic approach”), which explores deeper relationships but potentially misses recent diversification; or 2) sampling only one or two loci, such as the mitochondrial COI DNA barcode, for as many taxa as possible, and trying to cover the entire group’s biodiversity at the possible expense of accurate inference of deep relationships (the “barcoding approach”). These approaches are analogous to the “bottom up” (many characters, few taxa) and “top down” (many taxa, few characters) analyses described by Wiens (2005). Option 1) should facilitate resolving higher-level relationships. However, while phylogenetic accuracy is improved by the addition of informative characters, this approach can sometimes create model violations if it fails to detect multiple substitutions due to long branches (Poe 2003; Wiens 2005). In addition, other potential issues such as gene tree discordance and “the anomaly zone” may also challenge phylogenetic accuracy (Jeffroy et al. 2006; Degnan and Rosenberg 2006; Galtier and Daubin 2008; Mendes and Hahn 2018). Option 2) has the benefit of breaking long branches and thus improves the detection

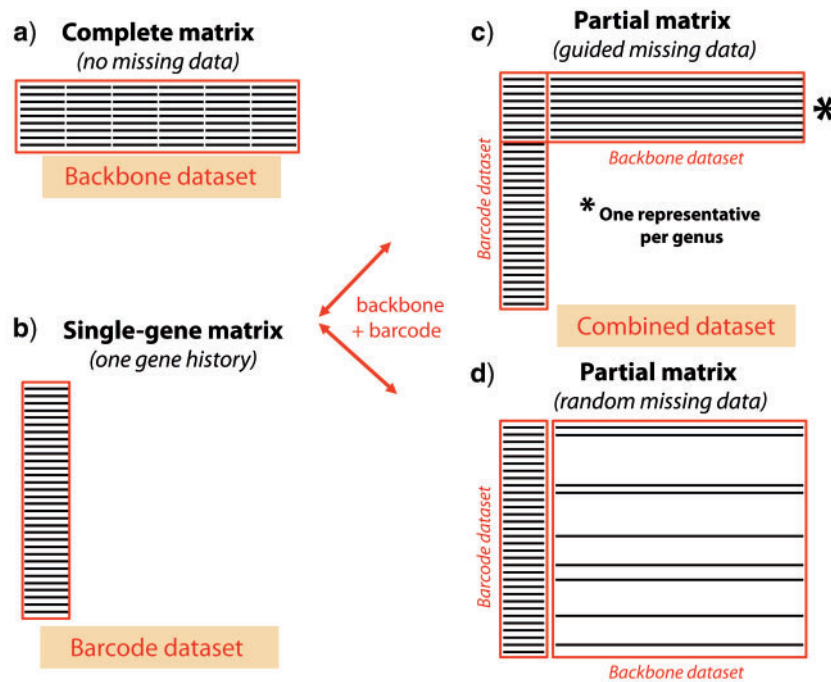


FIGURE 1. Distribution of missing data in molecular matrices. a) A complete matrix, where no missing data are involved (referred as the backbone data set). b) A single-gene matrix, including only one molecular marker and therefore providing information about only one gene history (referred as the barcode data set). c) The combined matrix, the product of merging a backbone and a barcode data set, where one marker (DNA-barcode) is present for all specimens, but other markers are entirely sampled only for a reduced percentage of specimens that have been selected by prioritizing representatives of higher-level taxonomic categories. d) A combined matrix, where the selection of fully sequenced specimens, and therefore the distribution of missing data, is randomly sampled (usually as a result of merging data sets from various sources).

of multiple substitutions, but the smaller number of informative characters, as well as the linkage of those characters in a single mitochondrial gene region, may result in weakly supported phylogenetic hypotheses (DeSalle et al. 2005). Moreover, the resulting single-gene phylogenetic histories are likely to not reflect the species trees (Pamilo and Nei 1988; Maddison 1997; Nichols 2001; Degnan and Rosenberg 2006). The debate regarding the costs and benefits of sampling more taxa on the one hand or more characters on the other has a long history and is still unresolved (GrayBeal 1998; Rannala et al. 1998; Zwickl and Hillis 2002; Poe 2003; Rosenberg and Kumar 2001; Wiens and Morrill 2011; Philippe et al. 2011; Wiens and Tiu 2012; Zheng and Wiens 2016).

In theory, sampling many loci for many taxa would be the best solution, but this remains a costly option for many, often due to the difficulty of obtaining samples with well-preserved DNA. In practice, most phylogeneticists have to cope with the problem of missing data or imbalanced sets of sequences in the attempt to build large phylogenies by merging multiple data sets. The availability of public molecular data increases exponentially, but these data are remarkably heterogeneous. Heterogeneity comes from the varied sampling strategies followed in different studies, from uneven sequencing of various genetic markers and/or from sampling biases involving particular clades or genes.

A number of studies have attempted to evaluate the performance of patchy supermatrices in phylogenetics (Wiens et al. 2005; de Queiroz and Gatesy 2007; Cho et al. 2011; Kawahara et al. 2011; Roure et al. 2013; Hovmöller et al. 2013; Streicher et al. 2015; Philippe et al. 2017). Patchy supermatrices may differ not only in their completeness but also in their randomness, a rarely assessed but possibly important parameter. Randomness in matrix patchiness can vary in both dimensions: taxon or marker. Previous studies tested scenarios with maximum randomness: probabilities of representation are equal for all taxa and characters (i.e., all taxa may equally have 1,2,3... to the maximum number of markers). It is still unclear whether partial matrices with guided missing data (those where only a set of selected samples are fully represented by all genes; see Fig. 1) can benefit phylogenetic accuracy. We here test the effects of missing data on partial matrices where some taxa have a maximum number of markers and some have only one and always the same one, the standard COI mitochondrial DNA barcode. We also test the relevance of the selected taxa having the maximum number of markers by using a random strategy versus a taxonomically guided strategy that prioritizes having at least one representative per higher-level clade (genus in this case). The motivating question is to evaluate whether partial matrices can be strategically designed by combining multiple genes for

relatively few representative taxa (the phylogenetic/omic approach) to resolve higher-level relationships, with barcode data from many taxa mostly informing species-level or shallow relationships (the barcoding approach).

We analyze different scenarios through simulation experiments and use this approach to assess a challenging empirical data set, the Polyommata butterflies. The subtribe Polyommata (Lycaenidae, Polyommatae) is a species-rich group (ca. 480 species) that is the product of one or more radiations (Kandul et al. 2004; Wiemers et al. 2010; Vila et al. 2011; Talavera et al. 2013a; Talavera et al. 2015, Stradomsky 2016). Butterflies in this group are morphologically highly similar and their taxonomy has been unstable. Species diversity in the Polyommata has been classified with 82 formally described generic names in a wide array of taxonomic combinations. Prior to this work, we addressed a higher-level taxonomic revision after reconstructing the first comprehensive molecular phylogeny of the group, based on three mitochondrial genes plus six nuclear markers (Talavera et al. 2013a). This data set included 109 specimens representing nearly all genera and subgenera described within the subtribe. The resulting phylogeny uncovered several polyphyletic genera. We develop objective criteria for a systematic arrangement that could best accommodate pre-existing generic nomenclature to the new phylogenetic framework, and, after applying a flexible temporal scheme, we delimited 32 genera.

The controversial taxonomy of this group mirrors the high evolutionary lability of most morphological characters. It's possible that the group contains cryptic diversity, and that taxa not characterized genetically so far might be assigned to the wrong genus. In fact, a remarkable number of species in this group have been assigned to multiple genera by different authors. For example, the North American taxon *acmon* Westwood, [1851] (originally described as *Lycaena acmon*) has been placed in the genera *Plebejus* (Pelham 2008), *Aricia* (Bálint and Johnson 1997), and *Icaricia* (Layberry et al. 1998, Talavera et al. 2013a). The enigmatic and morphologically distinct taxon *avinovi* Stshetkin 1980 has been placed in the genera *Polyommatus* (Bálint and Johnson 1997), *Rimisia* (Zhdanki 2004; Eckweiler and Bozano 2016) and *Afarsia* (Shapoval and Lukhtanov 2016). This situation is not unique to the Polyommata but extends to many other insect groups where rare or morphologically similar taxa provide challenging taxonomic assignments due to difficulties in finding diagnostic synapomorphies.

In this study, we increase taxon sampling for the Polyommata to 1360 specimens, comprising about 80% of putative species. We combine DNA barcodes with the genus-level phylogenetic backbone in a supermatrix where specimens having only COI barcodes (658 bp) represent ca. 92% of the total matrix, and specimens with multiple markers (6666 bp) represent ca. 8%. With this approach, we aim to screen the phylogenetic diversity of the group, assign species or subspecific taxa to genera,

identify unrecognized major clades and re-evaluate the phylogenetic history of the group with a nearly complete taxon sampling.

We also design a battery of simulations to evaluate phylogenetic accuracy for partial matrices, with particular emphasis on testing whether strategic selections of fully sequenced representatives improve accuracy over random selections. Our simulations test two scenarios: 1) a phylogenetic data set resembling our empirical data and 2) a phylogenomic data set (sampling 100 genes per taxon) to test the possible effect of backbone-barcode imbalances in large-scale studies. We propose a systematic workflow to assess higher-level taxonomy in hyperdiverse groups. In so doing, we also reinforce the value of the COI DNA barcode in higher systematics when combined with a minimal, but a well-designed, multilocus framework.

MATERIALS AND METHODS

Empirical Molecular Data Sets

We gathered molecular data for as many taxa as possible within the Polyommata butterflies (genera, subgenera, species, and subspecies), sampling as many populations as possible within the distribution range of each taxon (Supplementary Table S1 available on Dryad at <http://dx.doi.org/10.5061/dryad.m0cfxpp0d>). Our phylogenetic approach involved building two different molecular data sets. First, we took advantage of a multilocus matrix assembled for an earlier study (Talavera et al. 2013a), that included a mitochondrial DNA fragment containing three gene regions, plus six nuclear markers (6666 bp, hereafter referred to as the backbone data set, Fig. 1a). This data set included 109 specimens with at least one representative of each of the 82 formally described genera in Polyommata (with the exception of *Xinjiangia* Huang and Murayama 1988 and *Grumiana* Zhdanki 2004). The markers included in the backbone data set were mitochondrial *cytochrome oxidase I* (COI), *leucine transfer RNA* (leu-tRNA) and *cytochrome oxidase II* (COII), and nuclear *elongation factor-1 alpha* (EF-1a), 28S ribosome unit (28S), *histone H3* (H3), *wingless* (*wg*)—*carbamoyl-phosphate synthetase 2/aspartate transcarbamylase/dihydroorotase* (CAD) and *internal transcribed spacer 2* (ITS2).

A second data set (hereafter referred to as the barcode data set, Fig. 1b) was generated by assembling a single-gene matrix (658 bp) for the universal barcode fragment of mitochondrial COI. This data set exemplifies a molecular matrix with a one-gene phylogenetic history, often involving a limited number of informative characters. A total of 1365 barcodes were retrieved from multiple sources: 109 from the backbone data set, 1100 from the public repositories GenBank and BOLD, and 156 from specimens collected in the field or obtained from collections and sequenced specifically for this research. New collection efforts specifically targeted taxa and populations that are difficult to

obtain and/or are not sampled in previous studies (collection data in [Supplementary Table S1](#) available on Dryad). The barcode data set included representatives of approximately 80% of the roughly 480 species of Polyommata currently recognized ([Bálint and Johnson 1997](#); [Talavera et al. 2013a](#)). Both backbone and barcode data sets included as outgroup taxa four representatives for the sister subtribe Everina and one for Leptotina based on [Talavera et al. \(2013a\)](#). All specimens used in this study are listed in [Supplementary Table S1](#) available on Dryad.

Based on unexpected taxonomic placements or divergences observed from preliminary phylogenetic inspections of the barcode data set, we increased sequencing coverage by sequencing multiple markers for four additional taxa (*Chilades kedonga*, *Chilades elicola*, *Kretania psyorita*, and *Neolysandra corona*) ([Supplementary Table S1](#) available on Dryad), thus increasing the backbone data set to 113 specimens.

A matrix merging barcode and backbone data sets (hereafter referred to as the “combined” data set) was also built for downstream analyses. This consisted of a matrix of 1365 specimens, where approximately 8% (113 specimens) were completely sequenced for all markers, and 92% (1252 specimens) were represented by COI barcodes uniquely. In this asymmetric matrix of characters only one leading marker is complete, and the presence/missing data of other markers is intentionally guided towards particular taxa (Fig. 1c). This model contrasts with that of a partial matrix where the presence/missing data of other markers is randomly distributed across taxa (Fig. 1d).

DNA extraction, amplification, and sequencing for both barcode and backbone data sets followed standard protocols used for Lycaenidae ([Vila et al. 2011](#); [Talavera et al. 2013a](#)). Newly sequenced specimens are stored in the DNA and Tissues Collection of the Institut de Biologia Evolutiva (CSIC-UPF) in Barcelona and the sequences obtained were submitted to GenBank ([Supplementary Table S1](#) available on Dryad).

Phylogenetics and Divergence Times (Empirical Data Set)

Both barcode and backbone data sets were realigned based on available matrices from [Talavera et al. \(2013a\)](#), using Geneious 10.0.3. The barcode matrix consisted of 1365 sequences of 658 bp. The final backbone matrix consisted of 113 tips and 6672 bp: 2172 bp of COI + leu-tRNA + COII, 1171 bp of EF-1a, 745 bp of CAD, 811 bp of 28S, 370 bp of Wg, 1075 bp of ITS2, and 328 bp of H3. Three data sets, backbone alone, barcode alone, and backbone and barcode combined, were used for phylogenetics.

Bayesian inference was used to simultaneously infer evolutionary relationships and divergence times with the software BEAST 1.8.0 ([Drummond et al. 2012](#)). Data in the backbone and combined data sets were partitioned by six markers, considering COI + leu-tRNA + COII a single evolutionary unit in the mitochondrial genome. Models for DNA substitution for each marker were chosen according to the Akaike information criterion in

JModeltest ([Guindon and Gascuel 2003](#); [Darriba et al. 2012](#)). As a result, the HKY model was used for H3, the TN model for CAD, and a GTR model for the rest of the markers, in all cases with a gamma distribution (+G) and a proportion of invariants (+I) to account for heterogeneity in evolutionary rates among sites. The gamma distribution was estimated automatically from the data using six rate categories. Normally distributed tmrca priors including maximum and minimum ages within the 95% HPD distribution were established on four well-supported nodes according to [Talavera et al. \(2013a\)](#). The uncorrelated relaxed clock ([Drummond et al. 2006](#)) and a constant population size under a coalescent model were established as priors. The rest of the settings and priors were set by default. Two independent chains were run for 50 million generations each, sampling values every 1000 steps. All parameters were analyzed using the program Tracer ver. 1.7 to check for stationarity and convergence between runs. Burn-in values were applied accordingly. Independent runs were combined in LogCombiner ver. 1.6.0 and tree topologies were assessed in TreeAnnotator ver. 1.6.0 to generate a maximum clade credibility tree of all sampled trees with median node heights.

Maximum likelihood (ML) tree inference was performed using two methods, RAxML v.8.2.12 ([Stamatakis 2014](#)) and IQtree v.2 ([Minh et al. 2020](#)). For RAxML, a general GTRCAT substitution model for all genes was chosen and 100 rapid bootstrap inferences were executed. For IQtree inference, a general best-fit model for all genes was automatically selected by ModelFinder ([Kalyaanamoorthy et al. 2017](#)) and clade support was assessed using ultrafast likelihood bootstrap with 1000 replicates ([Hoang et al. 2018](#)). To test for possible effects of different modeling approaches and partitioning schemes, we also inferred ML trees for the combined data set partitioning characters by codon position, where best substitution models were selected by ModelFinder in IQtree and by PartitionFinder 2.1.1 ([Lanfear et al. 2017](#)) for RAxML.

For the resulting BEAST trees, nodes for genera (as reviewed in [Talavera et al. 2013a](#)) were collapsed into a single branch, producing a genus level tree for subsequent topological comparisons of intergeneric cladogenetic events between the three different data sets. Genus-level trees were produced to discriminate between topological differences belonging to inter- or intrageneric relationships, which are not possible to evaluate from the whole trees. The resulting backbone phylogeny, improved in four relevant taxa, was taken as a reference to re-evaluate generic classifications in Polyommata by applying the flexible temporal scheme (4–5 Myr) proposed in [Talavera et al. \(2013a\)](#).

Simulations

We designed simulations to test the performance of combined data sets in both resolving higher-level

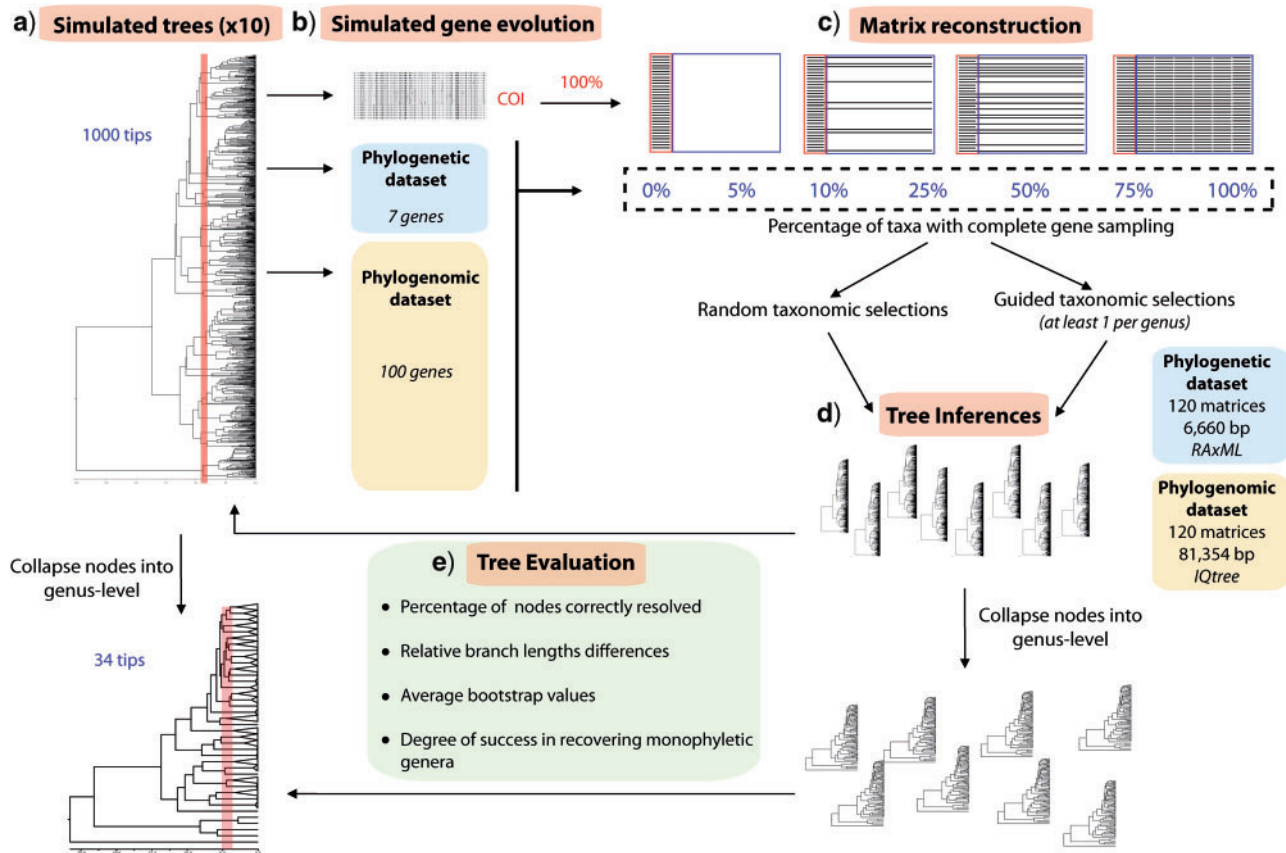


FIGURE 2. Diagram representing the designed simulation experiments. a) Ten reference trees of 1000 tips were simulated using TreeSim. b) Sequence evolution for independent markers were simulated along trees with SeqGen to generate two data sets: phylogenetic data sets including 7 markers + 1 barcode, and phylogenomic data sets including 100 markers + 1 barcode. c) A matrix reconstruction procedure produced matrices including different fractions of nonbarcode fully sequenced tips (0%, 5%, 10%, 25%, 50%, 75%, and 100%). Two strategies in selecting these tips were tested: random selections versus guided selections, where new additions of fully sequenced tips prioritized representatives for each genus. All matrices included barcode data for all tips. d) Tree inference for all generated matrices were performed, using RAxML for phylogenetic data sets (120 matrices, 6660 bp each), and IQtree for phylogenomic data sets (120 matrices, 81 354 bp each). Trees were collapsed into single branches at nodes defining genera, thus generating genus-level trees. e) Phylogenetic performance for both species-level and genus-level trees were evaluated against the originally simulated reference trees. Tree evaluation metrics included the proportion of correctly resolved nodes, relative branch-length differences, averaged bootstrap values and degree of success in recovering monophyletic genera (for species-level trees only).

relationships and placing barcodes within the correct genera. A schematic experimental design is shown in Figure 2. Ten reference trees were first simulated using the function “sim.bd.taxa.age” in the R package TreeSim (Stadler 2011). Parameters were set using information from the Polyommata phylogeny, including number of tips, evolutionary time and the flexible temporal scheme delimiting the number of genera. With these parameters, trees were simulated to generate 1000 tips evolving in 15 Myr, λ was set to 0.9 and μ to 0.05. An approximate stem age interval between 4 and 5 Myr was then used to delimit 34 monophyletic clades or hypothetical genera, each of which randomly included a number of tips, ranging from 1 to 166.

Next, DNA sequence evolution was simulated along with the 10 generated trees. We simulated two scenarios: 1) a phylogenetic data set resembling our empirical data and 2) a phylogenomic data set to test the backbone-barcode imbalances in large-scale studies. We used

the software Seq-Gen (Rambaut and Grassly 1997) to simulate evolution across molecular markers. For the phylogenetic data set, Seq-Gen was run independently eight times to simulate evolution in the molecular markers commonly used in Polyommata, *COI*, *COII*, *EF*, *CAD*, *Wg*, *H3*, *ITS2*, and *28S* (with the exception of the short mitochondrial leu-tRNA fragment). Parameters for each marker were extracted from likelihood estimations in JModeltest in the empirical data set and are shown in the Supplementary Table S2 available on Dryad. The eight generated alignments per tree were then concatenated in matrices of 6660 bp, as a complete (backbone) matrix model (Fig. 1a). Barcode data sets were also generated with *COI* alignments, as a single-gene (barcode) matrix model (Fig. 1b). For the phylogenomic data set, we simulated evolution in 100 genes, where values for Seq-Gen parameters for each marker were randomly assigned to values within the range of those used in the empirical data set. The

concatenation of the 100 generated alignments resulted in backbone matrices of 81,354 bp.

In order to test for effects of nonbarcode presence/missing data on phylogenetic (-omic) performance, we also built five data sets where we progressively increased the percentage of representation by additional (nonbarcode) markers by 5%, 10%, 25%, 50%, and 75% (Fig. 2). The selection of tips represented by these markers followed two strategies: 1) a random selection per each percentage and 2) a guided selection per each percentage prioritizing one addition per genus, thus discarding already represented genera (until all genera were represented). Thus, for each of the 10 simulated trees, we produced 12 matrices ranging from 0% to 100% of nonbarcode data. Specifically, we generated one barcode matrix, one complete matrix, five matrices with a random selection of tips with multigene data and five matrices with guided selection of tips with multigene data. Overall, this procedure generated a total of 120 simulated molecular matrices for the phylogenetic data set, and 120 for the phylogenomic data set.

Phylogenetic inference for all matrices in the simulated phylogenetic data set was conducted using ML in RAxML v.8 (Stamatakis 2014). We used the GTRCAT model of nucleotide evolution and conducted a rapid bootstrap analysis with 100 iterations and a search for the best-scoring tree in a single run (-f a). For the phylogenomic data set, ML phylogenetic inference was conducted using IQtree v.2 (Minh et al. 2020), as described for the empirical data set. All resulting trees were also posteriorly collapsed into genus-level trees (where intrageneric tips were collapsed into a single branch) according to each of the reference simulated trees.

Tree Evaluation (Empirical and Simulated)

The resulting phylogenetic trees, both from empirical and simulated data sets, were evaluated along four different axes: 1) percentage of nodes correctly resolved, 2) relative branch lengths differences using the K tree score (K) (Soria-Carrasco et al. 2007), 3) bootstrap values as an average of all nodes (for simulations only) and 4) degree of success in recovering monophyletic genera.

For the empirical data sets, we scored the percentage of matching nodes and K score between the combined and barcode trees. We also scored these metrics for the genus-level barcode tree and for the genus-level combined tree, always taking the genus-level backbone tree as a reference. Values for both genus-level and species-level trees allowed us to discern higher-level (between genera or deeper) and lower-level (intrageneric) topological differences. The degree of success in recovering monophyletic genera was also compared between the combined and barcode trees, using the function “AssessMonophyly” in the R package MonoPhy (Schwery and O’Meara 2016). For the battery of simulations, the percentage of nodes correctly

resolved and K were also retrieved for both genus-level and species-level trees, taking each corresponding simulated tree as a reference (Fig. 2).

RESULTS

Empirical Phylogenetics

At the genus-level, the percentage of nodes matching the backbone tree was higher for the combined trees (81.25% in BEAST, 71.87% in IQtree, and 43.75% in RAxML) than for the barcode trees (12.5% in BEAST, 25% in IQtree, and 21.87% in RAxML) (Supplementary Table S3 available on Dryad). Assuming that the backbone tree provides the best phylogenetic hypothesis, these results indicate a substantial improvement in phylogenetic resolution for each of the three methods when comparing the combined tree with the barcode tree, even though only 8% of the specimens, representing all genera, incorporated additional, non-barcode data. A similar trend of improvement was observed for relative branch length comparisons, where lower K scores and scale-factors closer to one indicate branch lengths that are more similar to each other between two trees (Supplementary Table S3 available on Dryad). According to this metric, the combined tree was also more similar to the backbone tree ($K = 1.57/0.006/0.10$; scale-factor = 1.05/0.92/0.70) than was the barcode tree ($K = 15.85/0.08/0.14$; scale-factor = 0.91/0.54/0.30).

At the species level, the percentage of nodes recovered in both the combined tree and the barcode tree was 42.33% in BEAST, 65.81% in IQtree and 57.52% in RAxML, indicating that there were meaningful differences between the two data sets in the phylogenetic relationships recovered within each genus. Differences between the three tree inference methods used to resolve topologies and relative branch lengths were appreciable, which may be related to the number of unresolved nodes. No supported changes in topology at the genus level could be detected in ML trees of the combined data set when we compared nonpartitioned analyses with analyses partitioned by codon position (Supplementary Figs. S2 and S3 available on Dryad). The only observed differences were associated with nodes that repeatedly showed low support across all methods used.

When testing for inconsistencies in resolving monophyletic genera using data from only the barcode tree, we determined that the barcode tree failed to cluster 5 of the 34 genera in the BEAST tree, 4 genera in the IQtree tree, and 3 genera in the RAxML tree, while the combined tree failed to cluster only one genus in the RAxML tree (Supplementary Table S4 available on Dryad).

In an initial exploratory step, the combined tree recovered four taxa that each had an unexpected placement or divergence that violated the criteria applied to delimit genera in Polyommata suggested by Talavera et al. (2013) (i.e., divergencies of <4–5 Myr). These four taxa were represented only by barcodes

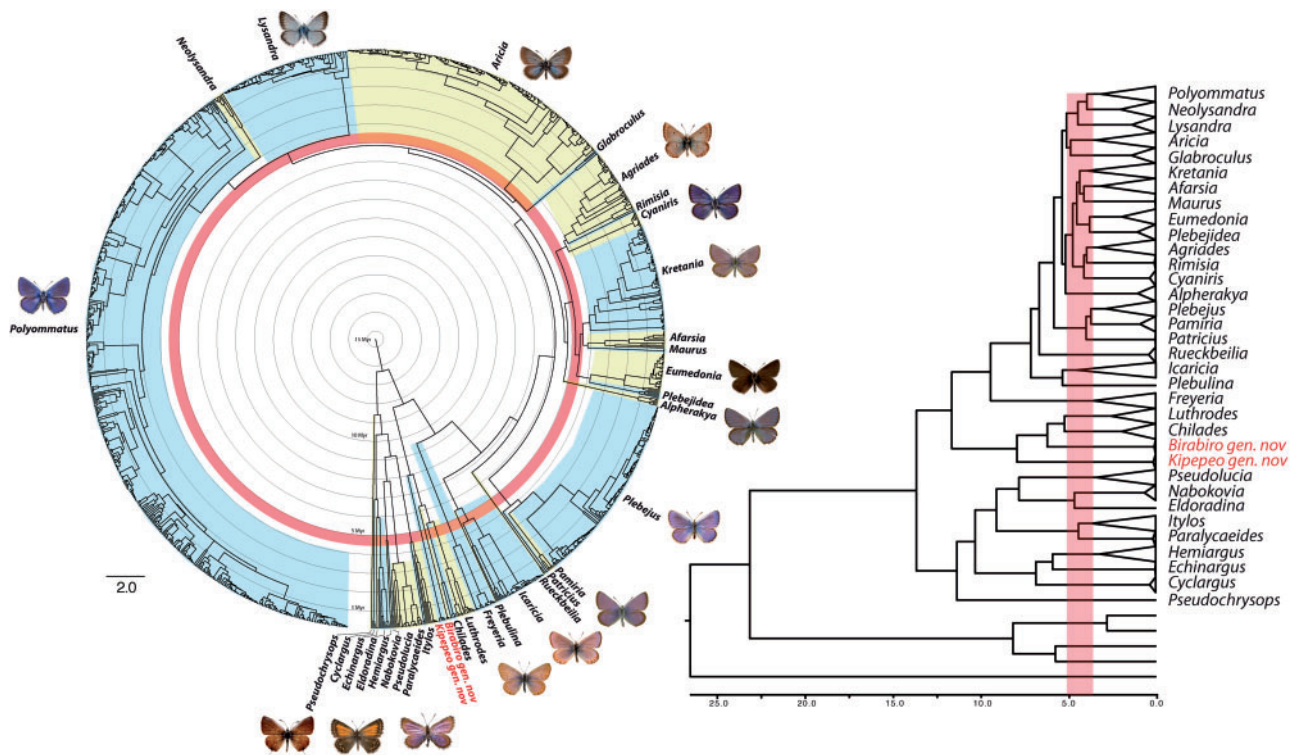


FIGURE 3. BEAST tree for the species-level data set of Polyommata butterflies (1365 specimens—ca. 80% of all taxa (left), and genus-level tree where nodes are collapsed into a single branch per genus (right), both showing the temporal banding used as a threshold for genus delimitation.

in the analysis. Since taxonomic changes might be required for these four taxa, we increased their molecular representation by sequencing the same additional genes for them that were included in the backbone database. Taxonomic decisions were then applied based on a tree incorporating these additional sequences (Fig. 3, Supplementary Fig. S1 available on Dryad).

Neolysandra corona was confirmed to be nested within *Polyommatus*, and thus, we transferred the taxon *corona* to *Polyommatus*.

Kretania psylorita's divergence (4.02 Myr) fell within the flexible temporal scheme of 4–5 Myr, and thus, we retained *psylorita* together with the rest of the taxa within *Kretania*, as defined here. However, the genus was not well supported and relationships shifted depending upon the method of phylogenetic reconstruction. Since *psylorita* is the type-species of the genus *Kretania*, this could have taxonomic consequences, but for now we have opted for the topology most frequently recovered, which is also in keeping with the morphology-based classification.

Divergences for *C. elicola* (6.68 [4.58–9.01] Myr) and *C. kedonga* (8.21 [5.65–10.77] Myr) were considerably older than 5 Myr, ages that in both cases indicated the need for a description of new, monotypic, genus. We describe these two new genera as *Birabiro gen. nov.* (type species *elicola*) and *Kipepeo gen. nov.* (type species *kedonga*) (see Appendix).

Finally, we use these results to propose a full division into subgenera of the large genus *Polyommatus*, including

the description of three new subgenera: *Escherilycaena subgen. nov.*, *Amandolycaena subgen. nov.*, and *Iranolysandra subgen. nov.* This new phylogenetic classification helps to resolve other debated cases such as that of *Chilades parrhasius*, which is transferred to *Luthrodes* (see Supplementary material available on Dryad for the full taxonomic description and discussion).

Simulations

The phylogenetic consequences of combining different sequencing strategies to infer higher-level systematics were further evaluated using simulated experiments. The proportion of nodes that were correctly resolved in genus-level trees increased with the percentage of fully sequenced tips in the matrices (Fig. 4a). For the phylogenetic data set, the proportion of nodes that were correctly resolved was 68.75% on average for the barcode data sets and reached a peak value of 94.06% for the combined data sets, whereas for the phylogenomic data set, these values ranged from 73.44% for the barcode data sets to 99.38% for the combined data sets.

The improvement curve was optimized when the selection of tips was guided to include one tip per genus (Fig. 4a). In these cases, combined trees having only 5% of fully sequenced tips (90.94% and 99.38% of correct nodes in the phylogenetic and phylogenomic data sets,

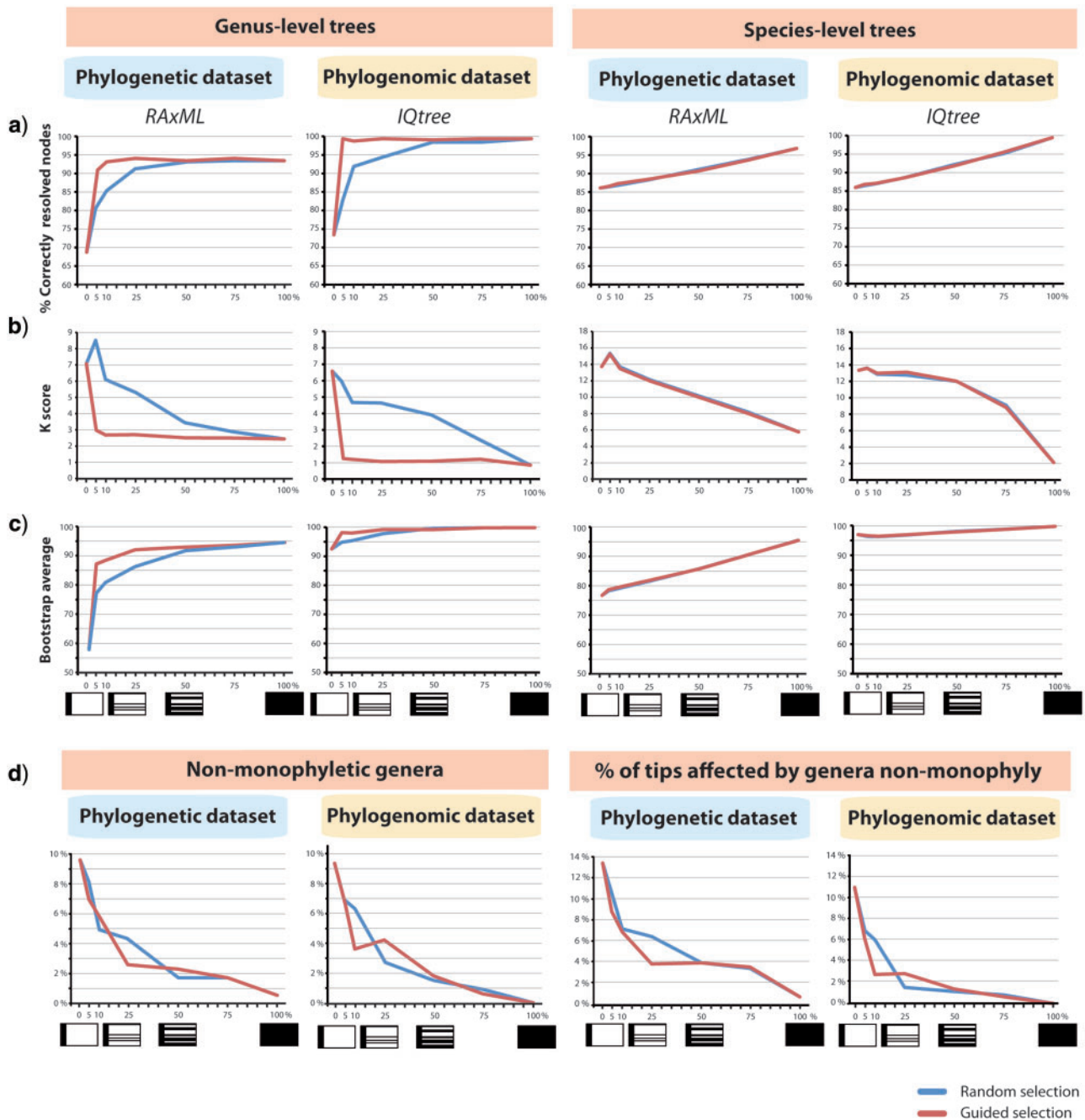


FIGURE 4. Phylogenetic performance evaluation from simulation experiments along a progressive percentage of fully sequenced representatives in combination with barcode data sets: a) Percentage of correctly resolved nodes, b) relative branch-length/divergence times (K score), c) average bootstrap values, and d) monophyly assessment for genera (number of nonmonophyletic genera and number of affected tips). Evaluation metrics are shown for both the species-level trees (1000 tips) and the genus-level trees (34 tips, after collapsing nodes defining genera into a single branch). Values from randomly selected representatives with all markers (in blue) and guided selection strategy using one representative per genus (in red) are compared. Results for a phylogenetic data set (7 markers + 1 barcode), and a phylogenomic data set (100 markers + 1 barcode) are shown. Trees were inferred using RAXML for the phylogenetic data set and IQtree for the phylogenomic data set.

respectively), or 10% of fully sequenced tips (93.12% and 98.75% of correct nodes) already produced comparable topologies to the ones resulting from complete matrices with 100% of fully sequenced tips (93.44% and 99.38% of correct nodes) (Fig. 4a). This was not the case using a random selection strategy, where equivalent topologies

were achieved only when 50% of fully sequenced tips were included (93.12% and 98.44% of correct nodes), percentages that were likely to have included at least one representative per genus by chance (Fig. 4a). In species-level trees, a progressive improvement of phylogenetic accuracy was also observed, but only reached the

optimal when trees were reconstructed using 100% of the data (Fig. 4a). The percentage of correctly resolved nodes ranged from 86.1% in the barcode trees to 96.8% in the complete trees for the phylogenetic data set, and from 86% to 99.45% in the phylogenomic data set.

Tree shape as indicated by relative branch length assessments performed similarly to topological assessments (Fig. 4b). In these comparisons, higher K scores indicate more disparate branch lengths than lower K scores. For genus-level trees, an improvement (decrease) of K was generally observed when guided fully sequenced tips were progressively added: when none of these were present, $K = 7.07$ and $K = 6.58$ for phylogenetic and phylogenomic data sets respectively, whereas when only 5% of fully sequenced tips representing each genus were added, these values dropped to $K = 2.97$ and $K = 1.25$, respectively. The latter values were already quite close to those obtained when 100% of taxa were fully sequenced, with $K = 2.44$ and $K = 0.85$, respectively (Fig. 4b).

This rapid convergence in branch length differences did not occur for randomly selected, fully sequenced tips, where K only approached optimal values when 100% of fully sequenced tips were included ($K = 2.43$ and $K = 0.85$) (Fig. 4b).

When assessing lower-level phylogenetic relationships with species-level trees, K scores showed a similar pattern with a progressive improvement from 0% of fully sequenced tips where $K = 13.74$ and $K = 13.35$ for phylogenetic and phylogenomic data sets respectively, to 100% of fully sequenced tips where $K = 5.77$ and $K = 2.12$ (Fig. 4b). Random and guided selections did not show substantial differences in this case, suggesting that a guided selection of fully sequenced tips is mainly of benefit in resolving deeper level phylogenetic relationships.

Bootstrap values rapidly increased on average from the barcode data sets (57.88%) to the combined data sets, with 5% of fully sequenced tips (87.16% for guided selection and 77.16% for random selection) in the genus-level trees of the phylogenetic data set (Fig. 4c). Bootstrap values of the phylogenomic data sets increased from 92.51% to 98.12% (guided selection) and 94.83% (random selection) (Fig. 4c). Bootstrap values of the species-level trees showed a progressive improvement as fully sequenced tips were incorporated, independent of the sampling strategy (Fig. 4c). Although the average bootstrap does not provide information about which set of nodes contribute the most to the topological changes observed, the patterns are consistent between all of these indices, and give no indication that a few nodes might be strongly biasing the results.

The number of monophyletic genera in the phylogenetic data sets increased with the number of fully sequenced tips, starting with an average of 9.7% of nonmonophyletic genera out of 34 (involving on average 13.4% of affected tips) in barcode trees to 0.6% nonmonophyletic genera (involving 0.9% of affected tips) in complete (100% gene sampling) trees (Fig. 4d). Values in the phylogenomic data sets ranged

from an average of 9.1% of nonmonophyletic genera (involving 11.1% of affected tips) to none (Fig. 4d). No substantial differences were detected between randomly and guided selection strategies. Fewer genera were recovered as nonmonophyletic in the simulations than in the empirical data set, highlighting the simplicity of simulated evolution against the complexity of real evolutionary processes in nature. Nevertheless, the simulations show cases of tips that are hard to place into the right genera, possibly due to effects of short internode branching patterns or of “singletons,” genera represented by a single terminal species, either because of poor sampling or because monotypic lineages can be grouped together erroneously due to long-branch attraction.

DISCUSSION

Robustness of the Combined Approach

All tree evaluation methods assessed, both for empirical and simulated data, show important improvements in phylogenetic accuracy when progressively increasing fully sequenced tips (Fig. 4, [Supplementary Table S3](#) available on Dryad). Topology, bootstrap support, and concordance in relative branch lengths are particularly strengthened when fully sequenced tips are not added randomly but are selected with the goal of representing at least one tip per genus (Fig. 4). Taxonomically balanced, multigene phylogenetic information seems efficient at counteracting the leading signal of the single-gene COI history in the combined phylogenies. Trees with 5–10% fully sequenced tips are comparable to those with 100% fully sequenced tips, but not to trees inferred from only barcodes. Interestingly, this effect mostly applies to deeper level phylogenetic relationships (i.e., genus-level trees) (Fig. 4).

The K score can be interpreted as a proxy for divergence time estimates. Missing data have previously been estimated to have little influence in the accuracy of divergence dating in BEAST ([Zheng and Wiens 2015](#)). Our empirical results also show little difference in divergence times when comparing the backbone and the taxonomy-guided combined data sets. This is also reflected in the simulations, which achieve near optimal values at 10% sampling provided fully sequenced tips are selected to be representative of each genus. However, data sets where fully sequenced tips are added randomly do not achieve optimal values until sampling is 100% complete (Fig. 4).

The placement of species into genera with which they are traditionally associated is reflected by the number of monophyletic genera recovered by an analysis. Our empirical data show that taxa are likely to be misplaced into genera with which they are not normally associated in phylogenies based exclusively on data from COI-based barcodes, with up to five genera recovered as nonmonophyletic (affecting 768 of the tips of the tree)

(Supplementary Table S4 available on Dryad). However, inaccurate placements are reduced in phylogenies based on the combined data sets. The same result is obtained with simulations, where the number of monophyletic genera improves progressively with the addition of fully sequenced tips (Fig. 4d).

Studies carried out by Cho et al. (2011) and Kawahara et al. (2011) show at the order and family level respectively that increased gene sampling improves estimates of deep relationships as indicated by higher support values. Our simulated findings are generally compatible with these results (Fig. 4), which have also been observed in multiple other phylogenies when increasing the number of characters (Rokas et al. 2003; Baptiste et al. 2002; Dunn et al. 2008; Zwick et al. 2011; Wilson 2011; Wilson et al. 2011; Kuntner et al. 2019).

The simulated phylogenomic analyses (Fig. 4) show that data sets with large barcode representation can be successfully combined with modern genomic data sets where taxa have been sampled for a large number of genes. The overall performance of the simulated combined phylogenomic data set (100 genes + barcode) is better than that of the phylogenetic combined data set (7 genes + barcode), as expected by the much greater number of characters. Again, in order to produce the best possible trees, it is key that taxa with genomic data represent a diversity of higher-level taxonomic categories. Thus, phylogeneticists are encouraged—and many do so instinctively—to strategically design their sampling to include 1) taxonomically distributed and representative species characterized with genomic data as well as 2) well-sampled barcode data from individuals representing as many species as possible in order to recover large-scale phylogenetic relationships.

DNA Barcodes as a Tool for Higher-Level Systematics: A New Value

A great many DNA barcodes representing a wide array of organisms have been generated and deposited in public repositories in recent years. Several markers function as DNA barcodes, with mitochondrial COI typically representing animals, and others such as ITS2 representing fungi, *rbcL* or *matK* representing plants, and 16S rRNA representing bacteria. To date, nearly 9.2 million barcoded specimens are available on the BOLD database, and nearly 4 million can be extracted from GenBank for COI.

Potential applications of DNA barcodes are varied. First, they have been used as references for species-level identification since their conception (Hebert et al. 2003), and their impact on taxonomy is undeniable (Miller 2007; Hubert and Hanner 2015; Dincă et al. 2015; Miller et al. 2016). Conceptual variations of the initial DNA barcode idea such as metabarcoding have expanded into many other fields of molecular ecology and community ecology (Creer et al. 2016). DNA barcodes are also widely applied in phylogeography and surveys of intraspecific variability. After much initial

debate, it is now well established that DNA barcoding (and any other single-marker approach) can be a useful tool to identify potential cryptic species, although an integrative approach is necessary for confirmation (e.g., nuclear markers, morphology, and ecology) (Will et al. 2005; DeSalle et al. 2005; Talavera et al. 2013b; Dincă et al. 2015; Hernández-Roldán et al. 2016; Lukhtanov et al. 2016; Gaunet et al. 2019).

Few studies have assessed whether DNA barcodes can be helpful at placing unidentified species into higher-level taxonomic categories (Wilson et al. 2011; Coddington et al. 2016). Here, we show that DNA barcoding can potentially be applied to assign taxa to genera (or higher categories) provided a solid and representative higher-level backbone phylogeny exists. Our results indicate that large data sets of barcodes can be used to identify cases where taxa have been wrongly assigned to higher-level taxonomic categories, a frequent problem in diverse groups with complex taxonomy, where synapomorphies helping to delineate genera have been difficult to find.

In the case where potential higher-level cryptic taxa are indicated by the results, these can be the focus of further taxonomic assessments following standard principles of phylogenetic systematics, such as the addition of molecular characters that aid in phylogenetic placement. Our proposed workflow for phylogenetic systematic assessments (Fig. 5, Supplementary material available on Dryad) takes advantage of the huge number of sequenced specimens available in public databases with the aim of accelerating taxonomic resolution at higher-taxonomic levels. It may facilitate molecular-based taxonomy in research labs where phylogenomic techniques are not yet easily available and, ultimately, benefit the common goal of taxonomic stability.

CONCLUSIONS

Phylogenetic inference based exclusively on DNA barcodes has been shown, both here and elsewhere, to perform poorly. However, we show how in combination with a backbone of carefully sampled, representative taxa for which a large number of additional markers have been sequenced, these short barcode sequences can nevertheless be used effectively to produce reliable phylogenies and improve higher-level systematics in large data sets. Our simulation tests show that a multigene sampling for as few as 5–10% of the specimens in the total data set can produce high-quality phylogenies, comparable to those resulting from 100% multigene sampling, provided a strategic selection has been made of higher-level representatives for multigene sequencing (approximately one per genus). These results are found at both a phylogenetic and phylogenomic scale, thus accounting for a wide range of imbalance in the number of characters between the combined barcode and backbone matrices. Thus, as long as backbone matrices are taxonomically representative, data coming from probe capture, transcriptomic or

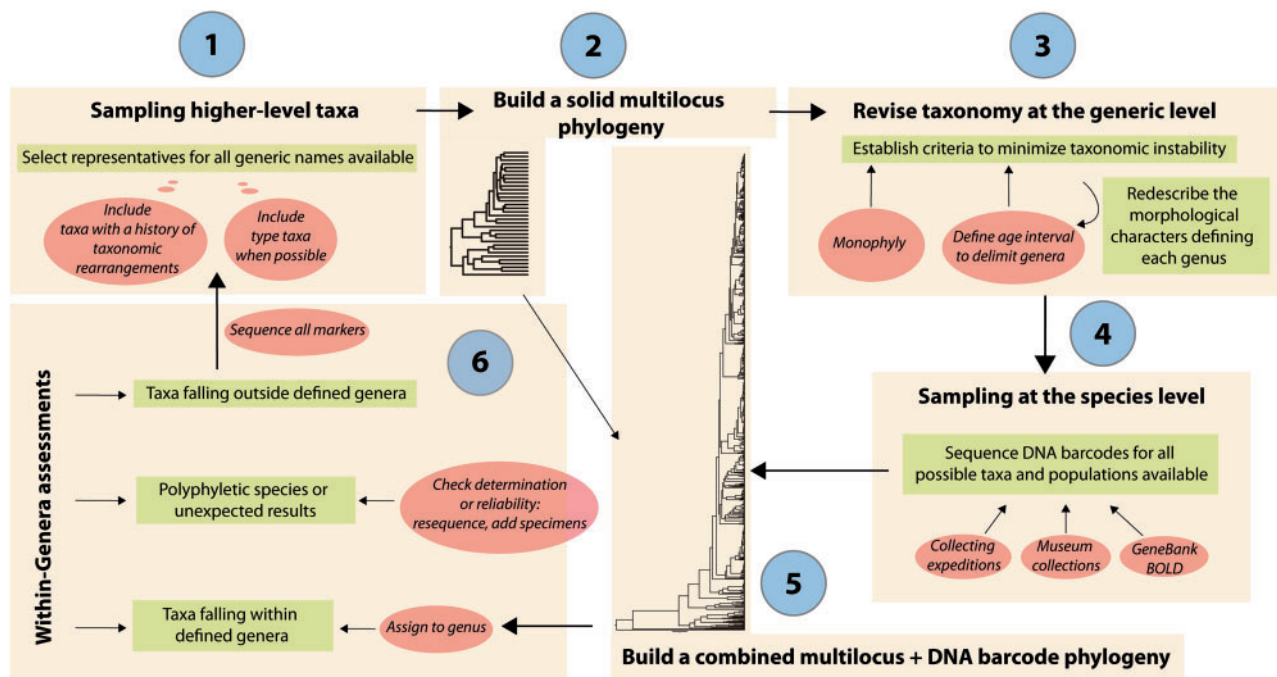


FIGURE 5. Diagram of the proposed workflow for higher-level systematic assessments.

genomic techniques can be effectively combined with barcodes to generate phylogenetically accurate, large-scale molecular characterizations of biodiversity.

SUPPLEMENTARY MATERIAL

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.m0cfxpp0d>.

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APPENDIX 1

Taxonomic Descriptions

Kipepeo Lukhtanov, Talavera, Pierce & Vila **gen. nov**
 urn:lsid:zoobank.org:act:03C66BDD-56AF-47D1-94F5-FABD45094262

Type species: *Everes kedonga* Grose-Smith, 1898
 The name is masculine in gender.

Diagnosis. The genus *Kipepeo* differs in male genitalia (Supplementary Fig. S3a and b available on Dryad) from the representatives of the closest genera *Luthrodes*, *Chilades* and *Birabiro* by relatively short and broad valves with angular, tooth-similar lower process; in wing pattern it differs by wing underside with distinct enlarged roundish ocelli and by hind wing underside with a row of large orange submarginal spots (Supplementary Figs. S1 and S2a available on Dryad). It can also be distinguished from other genera by unique molecular characters from COI, COII, Wg, ITS2, CAD, and H3 (see Supplementary material available on Dryad for the full taxonomic description and discussion).

Etymology. The name refers to the word “butterfly” in the Swahili language, specific to East Africa.

Birabiro Lukhtanov, Talavera, Pierce & Vila **gen. nov.**

urn:lsid:zoobank.org:act:015918DC-EE7A-477D-9902-2D0D0175EFBB

Type species: *Cupido elicola* Strand, 1911

The name is masculine in gender.

Diagnosis. The genus *Birabiro* differs from the closest genera *Luthrodes* and *Chilades* by the trapezoidal (not fusiform) shape of the valve in male genitalia (Supplementary Fig. S4 available on Dryad). The only representative of this genus (*Birabiro elicola*) has a plesiomorphic pattern on the wing underside, with the presence of all the basic elements typical of the non-Neotropical *Polyommatus* (Supplementary Fig. S2b available on Dryad). Thus, this pattern has no diagnostic value to distinguish the genus *Birabiro*. However, *Birabiro* represents a distinct monophyletic entity on the basis of molecular characters. It can be distinguished from other genera by unique molecular characters from COI, COII, ITS2, and H3 (see Supplementary material available on Dryad). The genus *Birabiro* differs from the genus *Kipepeo* by the wing pattern and the structure of the male genitalia, as well as by above mentioned molecular characters (see Supplementary material available on Dryad for the full taxonomic description and discussion).

Etymology. The name refers to the word “butterfly” in the Amharic language, specific to Ethiopia.

Polyommatus (Iranolysandra) Lukhtanov, Talavera, Pierce & Vila **subgen. nov.**

urn:lsid:zoobank.org:act:BAF45FDC-0398-4103-BF2F-B569BB014336

Type species: *Lysandra corona* Verity, 1936

Diagnosis. The wing pattern of *Iranolysandra* (Supplementary Fig. S5a available on Dryad) is most similar to those found in two other genera: *Neolysandra* (the sister genus to *Polyommatus*) and *Glaucopsyche* (very distant genus with completely different structure of genitalia). All these taxa share a similar wing pattern that seems to have evolved independently three times. However, *Iranolysandra* represents a distinct monophyletic entity on the basis of molecular characters. It can be distinguished from other subgenera of the genus *Polyommatus* by using molecular markers from COI, COII, ITS2, and CAD (see Supplementary material available on Dryad for the full taxonomic description and discussion).

The subgenus *Polyommatus (Iranolysandra)* includes the species: *P. (I.) corona*, *P. (I.) fatima*, *P. (I.) stempfferi* and *P. (I.) fereiduna*.

Etymology. The name *Iranolysandra* reflects the distribution area of the subgenus (distributed mostly in Iran) and its phenotypic similarity to the species of the genus *Neolysandra*.

Polyommatus (Amandolycaena) Lukhtanov, Talavera, Pierce & Vila **subgen. nov.**

urn:lsid:zoobank.org:act:7363C141-F71A-4844-9728-0676F4FD43E0

Type species: *Papilio amandus* Schneider, 1792

Diagnosis. The wing pattern of *Amandolycaena* (Supplementary Fig. S5b available on Dryad) seems to represent a plesiomorphic character found in the genus *Polyommatus*. It is most similar to those found in other subgenera of *Polyommatus*: *Polyommatus sensu stricto*, *Plebicula*, *Thersitesia*, *Sublysaandra*, and *Escherilycaena*. However, *Amandolycaena* represents a distinct monophyletic entity on the basis of molecular characters. It can be distinguished from other subgenera of the genus *Polyommatus* by using molecular markers from COI, COII, and Wg (see Supplementary material available on Dryad for the full taxonomic description and discussion).

The subgenus includes a single species *Polyommatus (Amandolycaena) amandus* (Schneider, 1792).

Etymology. The name *Amandolycaena* reflects the name of the type-species (*Papilio amandus*) and includes the word *Lycaena* that has been used in the past as a genus name for blue butterflies.

Polyommatus (Escherilycaena) Lukhtanov, Talavera, Pierce & Vila **subgen. nov.**

urn:lsid:zoobank.org:act:2FBDDBBB-1073-43CE-845C-1D3491544390

Type species: *Papilio escheri* Hübner, [1823]

Diagnosis. The wing pattern of *Escherilycaena* (Supplementary Fig. S8 available on Dryad) seems to represent a plesiomorphic character found in the genus *Polyommatus*. It is most similar to those found in other subgenera of *Polyommatus*: *Polyommatus sensu stricto*, *Plebicula*, *Thersitesia*, *Sublysaandra*, and *Amandolycaena*. However, *Escherilycaena* represents a distinct monophyletic entity on the basis of molecular characters. It can be distinguished from other subgenera of the genus *Polyommatus* by using molecular markers from COI, COII, EF-1a, Wg, CAD, H3, and 28S (see Supplementary material available on Dryad for the full taxonomic description and discussion).

The subgenus includes a single species *Polyommatus (Escherilycaena) escheri* Hübner, [1823].

Etymology. The name *Escherilycaena* reflects the name of the type-species (*Papilio escheri*) and includes the word *Lycaena* that has been used in past as a genus name for blue butterflies.

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