Resolving Ambiguity of Species Limits and Concatenation in Multilocus Sequence Data for the Construction of Phylogenetic Supermatrices

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Abstract.—Public DNA databases are becoming too large and too complex for manual methods to generate phylogenetic supermatrices for multilocus sequence data. Delineating the terminals based on taxonomic labels is no longer practical because species identifications are frequently incomplete and gene trees are incongruent with Linnaean binomials, which results in uncertainty about how to combine species units among unlinked loci. We developed a procedure that minimizes the problem of forming multilocus species units in a large phylogenetic data set using algorithms from graph theory. An initial step established sequence clusters for each locus that broadly correspond to the species level. These clusters frequently include sequences labeled with various binomials and specimen identifiers that create multiple alternatives for concatenation. To choose among these possibilities, we minimize taxonomic conflict among the species units globally in the data set using a multipartite heuristic algorithm. The procedure was applied to all available GenBank data for Coleoptera (beetles) including > 10 500 taxon labels and > 23 500 sequences of 4 loci, which were grouped into 11 241 clusters or divergent singletons by the BlastClust software. Within each cluster, unidentified sequences could be assigned to a species name through the association with fully identified sequences, resulting in 510 new identifications (13.9%) of total unidentified sequences of which nearly half were “trans-locus” identifications by clustering of sequences at a secondary locus. The limits of DNA-based clusters were inconsistent with the Linnaean binomials for 1518 clusters (13.5%) that contained more than one binomial or split a single binomial among multiple clusters. By applying a scoring scheme for full and partial name matches in pairs of clusters, a maximum weight set of 7366 global species units was produced. Varying the match weights for partial matches had little effect on the number of units, although if partial matches were disallowed, the number increased greatly. Trees from the resulting supermatrices generally produced tree topologies in good agreement with the higher taxonomy of Coleoptera, with fewer terminals compared with trees generated according to standard filtering of sequences using species labels. The study illustrates a strategy for assembling the tree-of-life from an ever more complex primary database. [BlastClust; data mining; graph theory; incongruence; multipartite matching; species delimitation; supermatrix.]

Metadata from mining of public DNA databases are a rapidly growing resource for molecular phylogenetics (Driskell et al. 2004). Compilations of these data for construction of large phylogenetic trees and multiple gene sets are widely performed (Sanderson et al. 2008; Goloboff et al. 2009; Peters et al. 2011) and software for data manipulation is available (Jones et al. 2011). Whether produced based on existing gene annotations (Jones et al. 2011), or with similarity searches (Hunt and Vogler 2008; Sanderson et al. 2008), the initial products of these compilations are sets of presumed orthologous sequences partitioned by locus. Combined data analysis generally outperforms other methods of phylogenetic inference such as supertrees derived from individual gene fragments (McMahon and Sanderson 2006; de Queiroz and Gatesy 2007; Kupczok et al. 2010; Thomson and Shaffer 2010), and therefore sequences require concatenation across loci to generate a “supermatrix” or “superalignment.” There has been great interest in the behavior of supermatrices, for example, regarding the density of taxon sampling, the selection of loci and the number of loci and characters required, the effects of missing data and the optimal way in which to reduce them (Philippe et al. 2004; Delsuc et al. 2005; Gatesy et al. 2007; Simon et al. 2009; Meusemann et al. 2010).

However, little attention has been paid to the key step of defining the terminals for such analysis. Specifically, it is not straightforward how to delimit the species entities that represent the terminals at each locus, and how to combine sequence data from various loci. In other words, although methods have been developed for specifying the columns in a 2-dimensional data matrix (representing orthologous sequences), the rows of the matrix (representing the terminals) have not been addressed. The problem of defining species units is complicated by the recent trend of depositing sequences without full species identification (Ryberg et al. 2008), either labeled with incomplete taxon names or approximate species identifiers (“sp.”, “cf.”, “aff.”, “sp. near,” etc.), or with various alphanumerical specimen tags referring to individuals, rather than a taxon.

Inconsistent species identifications by various authors exacerbate the problem of name-based concatenation across loci. In addition, the phylogenetic history of various DNA markers may be incongruent, for example, due to hybridization (Funk and Omland 2003; Edwards 2009). This causes different taxonomic affiliations of gene copies depending on the locus under consideration and results in incongruent signal in a concatenated matrix.

This study sets out a procedure for generating a global (multilocus) species delination matrix by optimizing the concatenation process. This required that multiple exemplars of a species, from population
analyses, are grouped to provide an estimate of species entities independently of taxon labels or particular identifications (Tautz et al. 2003; Brock et al. 2009; Lee et al. 2012). These sequence clusters established for each locus have to be combined into a supermatrix. This is straightforward if all sequences in the clusters are labeled with the same species name (Fig. 1a), in which case any sequence can be picked to represent a given locus in the supermatrix with equal justification. However, often the sequence variation is not congruent with the taxon assignment, that is, a name may be associated with multiple clusters, or conversely a single taxon label may be distributed across multiple groups. In these cases, combining loci becomes ambiguous (Fig. 1b). As we will show, empirical data sets are greatly affected by complex pattern of ambiguity, causing great difficulties to join sequences in matrix construction.

The proposed procedure concatenates the representatives for each locus in a way that minimizes conflict of the taxon names (including specimen identifiers) attached to each group. Because multiple labels may be present in a single sequence cluster and a single label may be distributed across multiple clusters, there are many different ways in which to link 2 loci (Fig. 2). The challenge of optimally linking these entities is taken as a specific case of the general problem of maximizing matches in graph theory (Cherkassky et al. 1996). Here, the label similarities among species units from multiple loci are represented as a multipartite graph, where nodes in a partite set correspond to the labels of clusters generated in the initial clustering step, and edges link nodes from adjacent partite sets where species names are shared. Decomposing the collection of edges such that nodes have no greater than a single edge to adjacent node sets is by “graph matching” (Fig. 2), in which the algorithm aims to find the greatest possible number of edges or the greatest sum of weights. Thus, the preferred global solution is a combinatorial optimization problem on multipartite graphs. By applying an explicit weighting scheme for name matches and partial matches, the linking may either be very strict, avoiding mismatches of any kind (beyond those already contained within a single-locus cluster), or may be more permissive by allowing mixed clusters with multiple taxon labels. The latter will increase the number of loci that participate in a concatenate and so reduce the number of terminals in the resulting supermatrix, hence increase the gene occupancy, at the cost of greater conflict of names within the concatenates.

We applied this procedure to a large multilocus data set of many thousand taxa for an entire insect order, the Coleoptera (beetles). Starting from DNA-based clusters that were created with the rapid BlastClust procedure for establishing the primary entities, we first assessed which clusters are inconsistent with Linnaean names and therefore constitute difficulties for concatenation. The labels attached to these clusters were used to generate a maximum matching set with the greatest number of edges. This set links various loci in accordance with the taxonomy to the greatest degree possible given the inconsistencies of sequence labels in the clusters. We also compared the outcome of the analysis with a conventional name-based concatenation, to test the properties of the data matrices in regard to the number of terminals, the proportion of missing data, and potential improvement in tree topology.
FIGURE 2. Species-level clusters from multiple loci represented as a multipartite graph. Species names associated with hypothetical sequence clusters at 2 loci are shown. Edges link clusters of adjacent partite where taxonomic species are shared (thin and fat lines). a) Graph is decomposed to permit only single links (fat lines), but resulting in a suboptimal matching. No further edges may be matched due to taxonomic dissimilarity of clusters. Matching results in a matrix of 8 global species units, with a cell density of 0.75. b) Maximum matching (fat lines), forming a matrix of 6 global species units and cell density of 1.0. All cases shown here are for the bipartite matching of 2 loci only, which is repeated sequentially for the multilocus (multipartite) data set.

MATERIALS AND METHODS

Data Mining and Species Clustering at Each Locus

Sequences were obtained from the NCBI database by downloading the invertebrate release (DNA) flatfiles from ftp://ftp.ncbi.nih.gov/genbank/. In addition, we obtained the NCBI taxonomy database from ftp://ftp.ncbi.nih.gov/pub/taxonomy/, from which we built a unique name-based string for each taxon (the “taxon identifier” or “taxon ID”) corresponding to the taxonomic hierarchy (Hunt and Vogler 2008). Taxon IDs were generated for every node descended from the “Coleoptera” node (NCBI taxon number 7041), using the Linnaean binomials and 7 hierarchical levels of the Linnaean taxonomic system up to the species level currently applied in this database. The taxon IDs were assigned to DNA sequences from the flatfiles by matching the NCBI taxon numbers. The formatdb NCBI program (ftp://ftp.ncbi.nih.gov/toolbox/FAQ.html) was used to generate a Blast searchable database which was screened for partial gene sequences coding for Cytochrome Oxidase subunit I (COI, 3’-portion), 16S rRNA, 18S rRNA, and 28S rRNA, using multiple queries from a phylogenetically diverse range of Coleoptera for each locus. Sequences showing similarity above the e-value cutoff of 1e−5 were obtained using a BioPerl...
a single edge. A suboptimal decomposition is shown in

requires decomposition so that no vertex has more than

SpG. Delineation of the graph into global species units

operational units at different loci. For example, the lower

SpE and one for the shared

are composed of one or more species labels (SpA to

(loci), each containing 6 vertices (species units), which

in missing cells in the resulting global species units

are linked in the maximal cardinality matching shown

in Figure 2b, thus forming 6 global species units with a

matrix density of 100%. Finding a maximal matching in

general k-partite graphs is NP-hard (Garey and Johnson

1983), but a simple heuristic is to reduce the problem

to a series of bipartite matchings (Bandelt et al. 1994),

as maximal matching of a bipartite graph is solvable

in polynomial time (Papadimitriou and Steiglitz 1982).

Additionally, bipartite graphs are well studied, with

several algorithmic solutions. Here, we implement a

single hub heuristic (Bandelt et al. 1994), as follows: for k

loci, L1, L2, L3 ... Lk, compute edge weights between L1

and L2, and find maximal matching. The resulting linked

and unlinked operational units for L1 + L2 become the

vertices for a second round of graph matching, whereby

edge weights are calculated between L1 + L2 and L3,

to determine the maximal bipartite matching in this

second round. Rounds continue from n = 3...k−1, with

bipartite matching between L1 + L2 ... Lr and Lr+1.

Maximal weight matches are calculated according to

Galil (1986), as implemented in the Graph::Matching

Perl module written by Joris van Rantwijk and freely

available at http://jorisvr.nl/maximumpathmatching.html,

with the single hub multipartite heuristic implemented

in Perl (see Supplementary Material).

Weights were applied to the name matchings based

on unique taxon IDs (not individuals) matched between

units in 2 loci. As the default weights, we assigned an

arbitrary score of +2 if all members in an operational

unit (sequence cluster) obtained in one locus are the

same taxonomic species as all members in a unit from

another locus (full matches). This score is increased

by +1 for a match of a particular specimen that links

units in different loci via an alphanumeric species

name field, with strings containing only the

letters a–z and a single internal whitespace assumed to

be Linnaean, and specimen label otherwise. A score of

+1 is assigned to “partial” name matches, if a subset of

the sequences in a unit has at least one match to a species

name in a unit obtained for a second locus.

The global (multilocus) species units were labeled

according to the taxon ID most widely represented across

loci. This strategy would avoid that, for example, a

single mislabeled sequence would be used to label the

entire concatenate. The following algorithm was used

for assigning a taxon ID to a specific global species unit:

for each locus, obtain all unique taxon IDs, then for each

taxon ID, count the number of loci containing it. Where

sequences in a specific matching were assigned multiple

Linnaean binomials, the one with widest representation

across loci was selected, or where binomials were absent,

the most frequently non-binomial label was used. In

addition to assigning a species label to the concatenate,

this procedure was also used to choose the DNA

sequence that represents a locus in the concatenate.

Where multiple sequences for the preferred binomial

were available, the most complete sequence was used

(Stajich et al. 2002) wrapper script, in which the Fastacmd

tool (ftp://ftp.ncbi.nih.gov/toolbox/FAQ.html) was

used for trimming of sequences to the extent of the

query and obtaining the reverse complement sequence

where necessary.

The first step of the matrix building procedure was

the clustering of the DNA sequences into species-

level groups. We utilized a rapid Blast-based clustering

method implemented in NCBI’s BlastClust program
to define operational species-level units, as applied

previously to microbiological species discovery (Lee

et al. 2012). BlastClust performs all-against-all pairwise

alignment, followed by single linkage clustering

according to user-specified similarity thresholds. Rates

of sequence variation are highly locus specific (e.g.,

Rokas et al. 2002; Danforth et al. 2005), hence the degree

of sequence variation within a species is likely to differ

across the loci. We therefore assessed a range of single

linkage cutoffs for each of the 4 target loci for the highest

congruence with the Linnaean species names. For each

cutoff, we summed the number of correctly delimited

species, that is, cases in which multiple sequences

labeled as a particular taxonomic name are grouped

into a single cluster that are free of sequences identified

as belonging to other species. Unidentified sequences

are indicated by edges, where in the current instance,

relationships are established based on the sharing of

taxonomic matches (a concatenate), the graph is

multipartite

\[ \text{P} \text{artite } \text{Concatenation from Ambiguously Assigned } \text{Clusters} \]

The set of operational units, defined as BlastClust

clusters for a locus ki, can be represented as vertices Vi,i

in an undirected graph G. Relationships between vertices

are indicated by edges, where in the current instance,

relationships are established based on the sharing of

taxonomic names between units. Since units are grouped

into classes (loci), with the aim of creating a serial set of

matches (a concatenate), the graph is k-partite, where k

equals the number of loci. In a k-partite graph, edges

exist between vertex classes, but not within a vertex

class. Where 2 vertex classes are present, the graph is

bipartite. Figure 2 gives a toy example, with 2 partites

(loci), each containing 6 vertices (species units), which

are composed of one or more species labels (SpA to

SpG). Edges represent all taxonomic matches between

operational units at different loci. For example, the lower

left unit has 2 edges to the adjacent locus, one indicating

the shared presence of SpE and one for the shared

SpG. Delineation of the graph into global species units

requires decomposition so that no vertex has more than

a single edge. A suboptimal decomposition is shown in

Figure 2a, where 4 units in the first locus have been

matched to 4 units in the second locus, resulting in

8 global species units. There are no species matches

between partites in the remaining 4 units. This results

in missing cells in the resulting global species units

(a matrix density of 75%), corresponding to missing

character states in the supermatrix. In contrast, all units

are linked in the maximal cardinality matching shown

in Figure 2b, thus forming 6 global species units with a

matrix density of 100%. Finding a maximal matching in

general k-partite graphs is NP-hard (Garey and Johnson

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of sequence variation within a species is likely to differ

across the loci. We therefore assessed a range of single

linkage cutoffs for each of the 4 target loci for the highest

congruence with the Linnaean species names. For each

cutoff, we summed the number of correctly delimited
or a sequence was chosen at random if sequences were of equal length. A file with the matched clusters across the 4 loci and a key to the taxon identifiers are available as Supplementary Material (Dryad Digital Repository; doi:10.5061/dryad.qp567).

Building Phylogenetic Trees from Concatenates

The above process of selecting taxon labels and concatenating the labels across loci was followed to build data matrices accordingly. In an initial step, all available sequences from each locus were aligned using BlastAlign (Belshaw and Katzungakis 2005), a program based on the Blast algorithm successfully used to align large numbers of rRNA sequences in Coleoptera (Hunt et al. 2007; Hunt and Vogler 2008). Alignments were produced for each of the 4 loci (COI, 16S, 18S, and 28S) and representatives for each operational unit were selected. (Note that the above step of clustering did not require multiple sequence alignment because the BlastClust algorithm produces clusters according to pairwise similarity.)

The quality of data sets generated under different edge weighting regimes was assessed on the phylogenetic trees inferred from the concatenated supermatrices. Direct comparisons of trees under likelihood require that the supermatrices obtained under the various weighting regimes have the same number and composition of concatenates (= terminals in the phylogenetic analysis), whereas the heuristic matching of loci under different weights results in matrices of variously formed concatenates. We therefore searched the supermatrices for concatenates which are identical between regimes. This set of equivalent concatenates were retained both in the trees and supermatrices, while all others were pruned (see fig. 1 of Poe (1998) for pruning method).

Hence, the tree topology was assessed only for a core set of concatenates recovered in all weighting schemes, whose relationships change due to differences in the variable concatenates (whose relationships were not assessed). Tree searches were performed on each of the resulting supermatrices under ML and the GTRCAT model with RAxML v. 7.2.8 (Stamatakis 2006), and with the NJ method implemented in Paup*4b (Swofford 2002), using ML distances and gamma distributed rates. To test for significance in the likelihood differences across weighting regimes, the branch lengths of the pruned topology were optimized first, and then the likelihood was calculated for each site. Site likelihoods were then bootstrapped using Consel (Shimodaira and Hasegawa 2001) to calculate approximately unbiased (AU) test statistics. The taxonomic retention index (RI; Hunt and Vogler 2008) was used to assess the fit of a tree to the Linnaean taxonomy. The RI was obtained by turning the taxon IDs created from the NCBI taxonomy database (see above) for each terminal into a set of binary pseudocharacters for various levels of the taxonomic hierarchy. State changes in each of these characters were scored for the trees using Paup to calculate the RI for each character, and the ensemble tRI for all characters

in the matrix was based on taxonomic state changes of 1376 taxonomic groups (898 genera, 161 tribes, 175 subfamilies, 120 families, and 22 superfamilies).

RESULTS

Clustering GenBank Entries and Grouping of Unidentified Sequences

The database contained 23,555 sequences and 10,503 taxon labels, including 7712 Linnaean binomials and 2791 alphanumeric name codes (referred to as unidentified in the following). The latter also contained 180 partial identifications (“sp.” “cf.” “nr.” or “aff.”). It is not known how many species these unidentified sequences equate to, but an estimate was made for each locus based on the level of intraspecific versus interspecific sequence divergence in a clustering with the BlastClust algorithm. Using all sequences with Linnaean taxon labels present at least twice in the database, similarity cutoffs were varied for the range of 90–100% in steps of 0.25%, and for each value, the proportion of correctly recognized species was determined (Fig. 3). Several hundred of these clusters matched the Linnaean names in the case of the mitochondrial markers and just over one hundred for the nuclear markers (Table 1). The slowly evolving loci (18S and 28S) returned a peak of correctly defined clusters at very high sequence similarity (linkage cutoff 99.75%), that is, intraspecific variation was very low, while the peak for the faster evolving COI and 16S was around 96 and 98.5%, respectively, with a very broad maximum. However, even under these optimal cutoff values, congruence of the clusters with the established taxonomy was low, with at most 52% (COI), 46% (16S), 37% (18S), and 69% (28S) of clusters unambiguously associated with a single Linnaean binomial (Table 1).

We then applied these optimal values to the total database, which returned 4760, 3260, 2092, and 1129 clusters for COI, 16S, 18S, and 28S (total 11,241 clusters; Table 2), including those sequences that were not grouped with any others (singletons). These clusters generated under the optimal cutoff were considered as operational units to represent species-level entities. These entities also contained many unidentified sequences. For example, in the COI locus, 1262 unidentified sequences were members of 1023 units, which included 935 units (including singletons) with all members unidentified, and 88 “mixed” units of unidentified and identified sequences (Table 2). Association with these units resulted in unambiguous species identification where the identified sequences in the cluster belong to a single species. For COI, 119 unidentified sequences were assigned species names in this way. The proportions were similar for other loci, with a total of 295 sequences newly identified in this manner (Table 2). This species assignment was improved by assignment to a name at a secondary locus if sequences for a given unidentified specimen were part of
a mixed cluster at another locus (Fig. 4). This trans-locus assignment increased the number of newly identified sequences by a further 215, for a total of 13.9% of the 3658 unidentified sequences in the data set (Table 2).

Incongruence of Clustering across Loci

Given the incongruence between sequence clusters and taxonomic species, we tested whether some of these novel groupings were corroborated by independent genetic loci. This test was performed on a subset of clusters that contained sequences labeled with 2 or more different species labels exclusively in that cluster, where these names were also present in other loci. These labels at a secondary locus may be consistent

FIGURE 3. Blastclust cutoff values plotted against the number of correctly delimited species, that is, the proportion of clusters that include all sequences associated with a particular Linnaean name. The results are shown for 4 loci and all Coleoptera sequences with multiple entries per species name. The panels are scaled to the maximum possible value of correctly defined species for each gene.

### Table 1. Optimal BlastClust cutoffs for the 4 genetic markers and number of species correctly delimited

<table>
<thead>
<tr>
<th>Locus</th>
<th>COI</th>
<th>16S</th>
<th>18S</th>
<th>28S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal BlastClust cutoff (%)</td>
<td>96.75</td>
<td>98.5</td>
<td>99.75</td>
<td>99.75</td>
</tr>
<tr>
<td>Number of species tested</td>
<td>474</td>
<td>5260</td>
<td>128</td>
<td>109</td>
</tr>
<tr>
<td>Clusters congruent at optimal cutoff</td>
<td>249</td>
<td>195</td>
<td>47</td>
<td>75</td>
</tr>
</tbody>
</table>

### Table 2. Species assignment to unidentified sequences via clustering at optimal clustering cutoff

<table>
<thead>
<tr>
<th>Locus</th>
<th>COI</th>
<th>16S</th>
<th>18S</th>
<th>28S</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clustering</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequences</td>
<td>13557</td>
<td>5338</td>
<td>2486</td>
<td>2174</td>
<td>23103</td>
</tr>
<tr>
<td>Clusters (including singletons)</td>
<td>4760</td>
<td>5260</td>
<td>128</td>
<td>109</td>
<td>11241</td>
</tr>
<tr>
<td>Clusters all members identified</td>
<td>3737</td>
<td>2517</td>
<td>1366</td>
<td>712</td>
<td>8322</td>
</tr>
<tr>
<td>Clusters all members</td>
<td>935</td>
<td>688</td>
<td>674</td>
<td>399</td>
<td>2696</td>
</tr>
<tr>
<td>Clusters mixed identified and unidentified</td>
<td>88</td>
<td>55</td>
<td>52</td>
<td>28</td>
<td>223</td>
</tr>
<tr>
<td>Clusters with multiple binomials</td>
<td>243</td>
<td>178</td>
<td>95</td>
<td>57</td>
<td>573</td>
</tr>
<tr>
<td>Name assignments</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total binomials</td>
<td>3577</td>
<td>2695</td>
<td>1482</td>
<td>824</td>
<td>8576</td>
</tr>
<tr>
<td>Total unidentified sequences</td>
<td>1282</td>
<td>1023</td>
<td>800</td>
<td>553</td>
<td>3658</td>
</tr>
<tr>
<td>Non-assignable sequences</td>
<td>1188</td>
<td>987</td>
<td>727</td>
<td>499</td>
<td>3401</td>
</tr>
<tr>
<td>Sequences assigned to binomials</td>
<td>119</td>
<td>82</td>
<td>46</td>
<td>48</td>
<td>295</td>
</tr>
<tr>
<td>Trans-locus assignments</td>
<td>70</td>
<td>89</td>
<td>16</td>
<td>40</td>
<td>215</td>
</tr>
<tr>
<td>Concatenation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unambiguous by string matches</td>
<td>4090</td>
<td>2783</td>
<td>1671</td>
<td>979</td>
<td>9723</td>
</tr>
<tr>
<td>Names dispersed among clusters</td>
<td>670</td>
<td>477</td>
<td>221</td>
<td>150</td>
<td>1518</td>
</tr>
</tbody>
</table>

Notes: The “clusters” refer to all separate entities produced in a BlastClust analysis at the optimal value (Table 1), including a large number of sequences not grouped with any others (singletons).
with the first, that is, grouping the same labels into a single cluster, or they may be inconsistent, being distributed among several clusters. All members of these mixed clusters were then assessed for “consistent” or “inconsistent” trans-locus clustering. Likewise, we assessed the placement of unidentified sequences (alphnumerical specimen tags) and their linkage with clusters labeled with more than one binomial. The number of trans-locus inconsistencies of clusters affected between half and one-third of all sequences representing taxa with membership in multiple clusters (Table 3). The proportion was particularly high in the slowly varying 18S gene, compared with the faster evolving mitochondrial genes. For example, 125 of the 251 (49.8%) 18S clusters grouped species that were ungrouped at other loci. This was also evident from the (much larger) class of unidentified sequences, where trans-locus clustering was also inconsistent (Table 3).

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The most straightforward category for concatenation across loci were “singleton” sequences that did not group with any others and therefore were unique in representing this binomial. Although a large proportion of entities in a given locus were singletons (e.g. 3655 sequences in COI, against 1105 true clusters composed of more than one sequence), a binomial that is a singleton in one locus was frequently linked with a cluster in another one. Only 1104 of minimally 7366 terminals (see below) in the final matrix were “singleton concatenates” composed of singletons in each locus available for the taxon. The remaining included many clusters with multiple taxon labels that were dispersed among several clusters in at least one locus. This problem affected 47.5% of all binomials represented by more than one sequence in COI (225 of 474 binomials), 53.6% for 16S, 63.3% for 18S, and 31.2% for 28S. Conversely, a cluster may contain multiple binomials that were dispersed across multiple clusters in at least one other locus. In total, there were 1518 clusters affected by these inconsistencies, corresponding to 13.5% of altogether 11 241 clusters. This leaves 9723 clusters that could be concatenated unequivocally using basic species name string matching (see Table 2 for breakdown by locus).

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### Search for Optimal Concatenates

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### Varying Edge Weighting Regimes according to Matches in Linnaean Taxonomy

Applying the sequential bipartite matching algorithm to the 11 241 operational units from the 4 loci, maximal matchings were generated under a series of edge weighting regimes. For the 2:1:1 weighting regime (for full, partial, and specimen match; see “Material and Methods” section), the procedure returned 7366 terminals, and this value was essentially unchanged under different weights for partial matches (Table 4). Likewise, the number of chimerical concatenates was similar across weighting regimes, as was the proportion of missing data (62%). However, if the “partial” match score was set to 0 (weighting scheme 2:0:0), that is, the algorithm does not consider any solution that implements an edge between units with multiple names, the number of terminals increased, and consequently the resulting data matrix showed an increase in the proportion of missing data (64.8%; Table 4, final column). This increase in the number of terminals affected in particular the mixed-name units, which increased to 565 over 520 units obtained with the 2:1:1 concatenation scheme. When partial matching is permitted, these
mixed-name clusters are mostly concatenated with mixed-name clusters in other loci, resulting in overall fewer terminals.

The concatenated were used for phylogenetic tree searches by retrieving the corresponding sequence information, retaining a single DNA sequence for each cluster for each locus, and concatenating these across loci. For each tree, we recorded the fit to the taxonomy (using the tRI, calculated from 1376 taxonomic groups of 5 ranks) and the likelihood of trees, after pruning all concatenates that are not universal to all weighting schemes to ensure comparability (see “Materials and Methods” section). The correspondence between lnL and tRI was low across weighing regimes, for the ML trees. For example, the tree attaining the highest tRI (2:1:0) was rejected (AU < 0.05) according to bootstrap analysis of site likelihoods. The quality of NJ trees was much lower than those obtained under ML, as indicated by the 2 site likelihoods. The quality of NJ trees was much lower than those obtained under ML, as indicated by the 2 site likelihoods. The quality of NJ trees was much lower than those obtained under ML, as indicated by the 2 site likelihoods. The quality of NJ trees was much lower than those obtained under ML, as indicated by the 2 site likelihoods.

Comparing Supermatrices from Sequence-based and Name-based Data Sets

We also generated a concatenated supermatrix of the 4 loci based on Linnaean taxon labels (species names and unidentified specimen labels). The original sequences were filtered to leave a single sequence per species label, using the most complete sequence or, where sequences were of the same length, randomly choosing one individual. This “name-based” supermatrix included

<table>
<thead>
<tr>
<th>Match</th>
<th>2</th>
<th>2</th>
<th>2</th>
<th>2</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial match</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4. Comparing the impact of different edge weighting regimes on the resulting matrices and trees

Table 5. Number of matched species units with data present/absent for the 4 target loci, separate for the name-based and cluster-based supermatrices

<table>
<thead>
<tr>
<th>Number of Loci</th>
<th>COI</th>
<th>16S</th>
<th>18S</th>
<th>28S</th>
<th>Cluster based</th>
<th>Name based</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Loci</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>276</td>
<td>404</td>
<td>31.2</td>
</tr>
<tr>
<td>3 Loci</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>233</td>
<td>241</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>327</td>
<td>363</td>
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</tr>
<tr>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>31</td>
<td>40</td>
<td>22.5</td>
</tr>
<tr>
<td>2 Loci</td>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
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<td>57</td>
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<td></td>
<td>✓</td>
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<td>✓</td>
<td>✓</td>
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<td>1360</td>
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<td>✓</td>
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<td>112</td>
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<td>✓</td>
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<td>158</td>
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<td>10.7</td>
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<td></td>
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<td>✓</td>
<td>✓</td>
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<tr>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>64</td>
<td>89</td>
<td>28.1</td>
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<tr>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>82</td>
<td>91</td>
<td>9.9</td>
</tr>
<tr>
<td>1 Locus</td>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
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<td>2714</td>
<td>−13.9</td>
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<tr>
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<td>993</td>
<td>1053</td>
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<tr>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>144</td>
<td>155</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Note: The last column gives the percentage by which the number of cluster-based concatenates is lower than name-based concatenates (negative values if the number of cluster-based concatenates is greater).

7605 terminals with greater intra-genus representation compared with the cluster-based matrix, including a few hypervariable genera with > 200 differently named terminals possibly derived from population studies. The proportion of missing data at 59.7% was lower than the cluster-based analysis (62.0%). The sequence-based concatenation resulted in a general reduction in concatenates, and in particular in the multilocus concatenates (Table 5). Contrary to this trend, the number of COI clusters was greatly increased in the sequence-based clusters, suggesting that this fastest diverging gene frequently split Linnaean entities, unlike the other loci. This increase was not observed in the multilocus concatenates of the COI gene, which suggests that the concatenation step reduces the number of entities, that is, clusters that split Linnaean species are concatenated based on alternative names in mixed clusters present at other loci.

For comparisons of the resulting tree topologies, we pruned all terminals that were not identical in both trees, leaving a core set of 5485 terminals. As expected for a matrix of greater size, the search time differed significantly for the taxon-based matrix compared with the sequence-based matrix. Based on the 1000 bootstrap replicates, a mean search time of 16.4 ± 1 h was required for the name filtered, against 12.8 ± 1 h for the cluster-based data matrix (P = 0.012, W = 758.579, unpaired Wilcoxon rank sum test). After extensive searches, the tRI was 0.890 and 0.889 for the name-based and name-based data sets, respectively. To obtain an indication of significance for these values, the ensemble tRI was repeated for each of 1000 bootstrap trees for both data sets, resulting in a tRI of 0.890 and 0.889 for the name-based analysis, which was a highly significant difference (P < 0.001, W = 262741, unpaired Wilcoxon rank sum test). The tree from sequence-based
concatenation showed a small but significantly increased bootstrap support (0.604 ± 0.014, vs. 0.602 ± 0.014; P = 0.019, V = 762.162, paired Wilcoxon signed rank test), whereby searches were performed only on a set of shared sequences to find equivalent nodes. Finally, the likelihoods of the 2 topologies were assessed after a round of thorough likelihood optimization. While not significant, the tree from the cluster-based matrix ranked as showing higher likelihood (AU = 0.859, PP = 1.0, SH = 0.864).

**DISCUSSION**

It is widely recognized that gene annotations are insufficient and data mining therefore requires careful partitioning of orthologous sequences (Sanderson et al. 2008; Smith et al. 2009; Peters et al. 2011), as the first step in building a supermatrix. However, the literature has not addressed the allied problem of defining the terminals. Instead, virtually all recent studies define the species axis of the matrix by using the Linnaean names, which seems no longer adequate given the increasing proportion of un-named sequences and inconsistent identifications. The magnitude of the problem is apparent from the Coleoptera database that contains nearly 1000 “unidentified” species-level clusters (including singleton sequences) without assignment of Linnaean names and 1200 uncurated orthologous sequences for the COI gene alone. Altogether some 15.5% out of 23555 sequences are “unidentified” (Table 2). More importantly, the names in databases do not coincide with species-level clusters obtained from the sequences themselves. Blast clustering produced species-level entities containing multiple names in some 23% of clusters with > 1 sequence, even after careful selection of the best performing cutoff values. In as many as 50% of the binomials represented by > 1 sequence, a given taxon ID was split among different clusters, that is, the annotations contradict the sequence-based entities, and to make matters worse, these splits frequently are “inconsistent” between loci (Table 3). Choosing representatives for each cluster and deciding what annotation to use in a supermatrix therefore become very complicated. We here developed a procedure for consolidating these single locus species clusters into a supermatrix. The approach maximizes the number of matches of taxon IDs among loci globally over the entire database, using established procedures from graph theory. The key feature of the method is that it matches each set of names (each cluster) with no more than one other set, until no more matching sets are available, to produce the maximum matching set. This process of bipartite matching is iterated for multiple loci to provide a heuristic solution for the NP-hard problem of multipartite matching.

The procedure is flexible in that it can incorporate a weighting function to reflect the greater certainty about the link of some vertices than others. This is particularly relevant if clusters can be linked across loci via sequences obtained from a single individual: in these cases, the link is unambiguous, and this gives confidence that these clusters should be concatenated beyond the taxon ID. We implemented this by adding extra weight to the matching function, which was set arbitrarily to add 50% greater weight above that for a “specimen match” (= identical taxon ID in clusters in 2 loci). Conversely, the certainty of a match may be reduced if certain taxon IDs differ in one or both of the clusters (= a “partial match”), which can be given lower weight than the match of all taxon IDs (= a “full match”). It may also be desirable to avoid partial matches altogether, which we implemented with the “partial match = 0” setting. In the Coleoptera data, only this latter approach had a substantial effect on the supermatrix, showing the greater number of terminals and more missing data expected if partial matches are disallowed. Other weighting schemes are conceivable. For example, the current approach does not take into account the number of sequences in each locus to be used in the weighting, which may lead to an over-proportional impact of a small number of aberrant sequences. This could be addressed by different weighting schemes that take into account the number of sequences supporting any one taxon ID. Likewise, additional information associated to particular sequences could be considered, such as geographic provenance of sequences, to preferentially match up clusters based on sequences from the same collecting locality or habitat. The most illustrative example of species membership. The “specimen match” could also be implemented by using the specimen voucher information if available on GenBank, instead of using alphanumerical name codes to insure that unique specimens are used, rather than unidentified entities. It also remains to be seen to what degree other parts of GenBank are affected by incongruence in species-level clusters. Currently, the Coleoptera database is well curated and consists of a majority of sequences assigned to Linnaean binomials, for example, 3577 binomials in the database for 4760 clusters in COI (75.1%) and a similar proportion of binomials to clusters in the other loci (Table 2). However, the proportion of unidentified sequences is likely to increase steeply in the future, for example, through the rapidly growing DNA barcodes (COI sequences) labeled by a “barcode index number” (Ratnasingham and Hebert 2007), an arbitrary alphanumerical code referring to DNA-based clusters similar to the clusters created here, and the adoption of high-throughput sequencing technologies, which places greater demand on taxonomic identifications via clustering.

Perhaps the greatest effect on the composition of the supermatrix is exerted by the clustering step preceding the concatenation. We used BlastClust as a pragmatic approach based on similarity cutoffs in a 2-step procedure whereby we first established the cutoff level that is most appropriate to the specific database by comparing clusters and Linnaean names to mirror traditional species circumscriptions, and then applied the preferred value to the wider database including...
“unidentified” or partially identified sequences. The approach is simplistic, by using a similarity criterion and universal cutoff values for species delimitation, and assuming correct taxonomic identifications and taxon concepts in GenBank submissions. Database entries are notorious for a low fit of sequence clusters and Linnaean names (Meier et al. 2006), unlike studies based on dedicated sequencing efforts, which estimated this discrepancy to affect at most 5% of all clusters (Hajibabaei et al. 2006). This suggests inconsistent naming as well as a focus on problematic groups in the primary studies from which the database is built. The discrepancies also differ among loci. For example, in the slowly evolving 18S and 28S loci, we found that multiple binomials were commonly collapsed into a single entity, indicating over-clustering that was not observed in fast-changing mitochondrial loci (Table 2). The specifics of the clustering procedure may have contributed to the high incongruence with the Linnaean binomials, while other clustering procedures have recently been found to perform better (Lee et al. 2012), and tree-based procedures (Pons et al. 2006) may be preferable on theoretical grounds.

Once loci are partitioned and species clusters are delineated, the matrix building step has to draw on the units as a set of fixed entities that are to be linked in the most appropriate way. The linking parameters affect the relative proportion of missing data and indirectly conflicting terminals. Assuming correct taxonomic identifications and taxon concepts, the outcomes of these tree searches are somewhat inconclusive, the proposed procedure will improve the matrix by the more efficient use of the rapidly growing sequences from incompletely identified individuals, objective selection of exemplars for inclusion in the supermatrix, and formal resolution of inconsistencies in public sequence repositories. In addition, the clustering step that precedes the concatenation also results in a substantially greater proportion of data that bear on the phylogeny of a focal group. Compared with conventional name-based analyses, the use of sequence clusters reduces the number of terminals with little phylogenetic information content from population studies. In the case of the Coleoptera database, this resulted in ~300 fewer terminals, a ~20% faster search time and a slightly, but significantly improved topology as measured by the tRI, LnI, and bootstrap support.

It remains to be studied what these mixed concatenates mean biologically. In our analysis, there are 2 layers contributing to mixed species units, at the level of the primary clusters and when matching clusters across loci. Mixed sequence clusters concern tip-level incongruence within genera, although a few clusters also included among-genus, among-subfamily and even higher taxa inconsistencies. The latter probably result from errors in gene annotations or identification, while at the tip-level true gene tree incongruence may be responsible for the clustering of multiple names (in addition to inappropriate clustering methods and parameter settings).

The second layer responsible for mixed terminals results from the focus matching step itself, which may aggravate the problems from mixed primary clusters. However, in the Coleoptera data set, this potentially negative effect is offset by the overall reduced proportion of mixed terminals, which is evident in single-locus terminals, a ~20% faster search time and a slightly, but significantly improved topology as measured by the tRI, LnI, and bootstrap support.

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ACKNOWLEDGMENTS

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