SuperCRUNCH: A toolkit for creating and manipulating supermatrices and other large phylogenetic datasets

Daniel M. Portik[1,2*], John J. Wiens[1]

1. Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona

2. California Academy of Sciences, San Francisco, California

*Correspondence: daniel.portik@gmail.com

Running Title: SuperCRUNCH for phylogenetic data

Keywords: bioinformatics, GenBank, genomics, multiple sequence alignment, similarity filtering, phylogenetics, phylogeography, supermatrix

Abstract

1. Phylogenies with extensive taxon sampling have become indispensable for many types of ecological and evolutionary studies. Many large-scale trees are based on a "supermatrix" approach, which involves amalgamating thousands of published sequences for a group. Constructing up-to-date supermatrices can be challenging, especially as new sequences may become available almost constantly. However, few tools exist for assembling large-scale, high-quality supermatrices (and other large datasets) for phylogenetic analysis.

2. Here we present SuperCRUNCH, a Python toolkit for assembling large phylogenetic datasets. It can be applied to GenBank sequences, unpublished sequences, or combinations of GenBank and unpublished data. SuperCRUNCH constructs local databases and uses them to conduct rapid searches for user-specified sets of taxa and loci. Sequences are parsed into putative loci and passed through rigorous filtering steps. A post-filtering step allows for selection of one sequence per taxon (i.e. species-level supermatrix) or retention of all sequences per taxon (i.e. population-level dataset). Importantly, SuperCRUNCH can generate "vouchered" population-level datasets, in which voucher information is used to generate multi-locus phylogeographic datasets. Additionally, SuperCRUNCH offers many options for taxonomy resolution, similarity filtering, sequence selection, alignment, and file manipulation.

3. We demonstrate the range of features available in SuperCRUNCH by generating a variety of phylogenetic datasets. We provide examples using GenBank data, and combinations of GenBank and unpublished data. Output datasets include traditional species-level supermatrices, large-scale phylogenomic matrices, and phylogeographic datasets. Finally, we briefly compare the ability of SuperCRUNCH to construct species-level supermatrices to alternative approaches. SuperCRUNCH generated a large-scale supermatrix (1,400 taxa and 66 loci) from 16GB of

GenBank data in ~1.5 hours, and generated population-level datasets (<350 samples, <10 loci) in <1 minute. It also outperformed alternative methods for supermatrix construction in terms of taxa, loci, and sequences recovered.

4. SuperCRUNCH is a flexible bioinformatics toolkit that can be used to assemble datasets for any taxonomic group and scale (kingdoms to individuals). It allows rapid construction of supermatrices, greatly simplifying the process of updating large phylogenies with new data. It is also designed to produce population-level datasets. SuperCRUNCH streamlines the major tasks required to process phylogenetic data, including filtering, alignment, trimming, and formatting. SuperCRUNCH is open-source, documented, and freely available at https://github.com/dportik/SuperCRUNCH, with example analyses available at https://osf.io/bpt94/.

1 | INTRODUCTION

Large-scale phylogenies, including hundreds or thousands of species, have become essential for many studies in ecology and evolutionary biology. Many of these large-scale phylogenies are based on the supermatrix approach (e.g., de Queiroz & Gatesy, 2007), which typically involves amalgamating thousands of sequences from public databases (e.g., GenBank). Yet relatively few tools exist for automatically assembling these datasets. These include programs like PhyLoTA (Sanderson, Boss, Chen, Cranston, & Wehe, 2008), PHLAWD (Smith, Beaulieu, & Donoghue, 2009), phyloGenerator (Pearse & Purvis, 2013), SUMAC (Freyman, 2015), SUPERSMART (Antonelli et al., 2017), PhylotaR (Bennett et al., 2018) and PyPHLAWD (Smith & Walker, 2018). Each program has its own pros and cons for assembling molecular datasets. For example, several programs (e.g., PHLAWD, PyPHLAWD, PhyLoTA, PhylotaR, SUPERSMART) employ automated ("all-by-all") clustering of all sequences, which restricts the ability to target specific loci. In addition, the criteria for filtering steps and sequence selection are not always clear in these programs. However, their most severe limitation may be their reliance on GenBank databases to obtain starting sequences. This design generally prevents the inclusion of locally generated (e.g., unpublished) sequence data, thereby limiting analyses to published sequences. Furthermore, many methods were designed to create species-level datasets, in which a species is represented by one sequence per locus (e.g., a traditional supermatrix). It is often not possible to use these methods to intentionally generate phylogeographic (population-level) datasets, in which a species is represented by many individuals sequenced for anywhere from one gene to thousands of loci. The dramatic increase in the availability and size of phylogeographic datasets (McCormack et al., 2013; Garrick et al., 2015) has created a need for methods which can construct large-scale population-level datasets. Additionally, no current methods utilize voucher codes (e.g., a field series, museum number, or other identifier). These codes are critical for linking samples and building phylogeographic datasets. Thus, producing high-quality phylogenetic datasets is presently challenging using many of the available methods.

To address these challenges, we developed SuperCRUNCH, a semi-automated method for creating phylogenetic and phylogeographic datasets. SuperCRUNCH can be used to process sequences from GenBank, datasets containing only locally generated (unpublished) sequences, or a combination of sequence types. During initial steps, the sequence data are parsed into loci based on user-supplied lists of taxa and loci, offering fine-control for targeted searches. SuperCRUNCH allows any taxonomy to be used, and offers simple steps for identifying and resolving taxonomic conflicts. SuperCRUNCH also includes refined methods for similarity filtering, quality filtering, and sequence selection. By offering the option to select one representative sequence per species or retain all filtered sequences, SuperCRUNCH can be used to generate species-level datasets (one sequence per species per gene) and population-level datasets (multiple sequences per species per gene). SuperCRUNCH can also filter sequences using voucher codes, which can label and link sequences in phylogeographic datasets (e.g., a "vouchered" dataset). Analyses are highly scalable, and can range in size from small population-level datasets (one taxon, one gene) to large phylogenomic datasets (hundreds of taxa, thousands of loci). SuperCRUNCH is modular in design, offering flexibility across all major steps in constructing phylogenetic datasets, and analyses are transparent and highly reproducible. SuperCRUNCH is open-source, heavily documented, and freely available at https://github.com/dportik/SuperCRUNCH.

2 | INSTALLATION

SuperCRUNCH consists of a set of PYTHON modules that function as stand-alone command-line scripts. As of SuperCRUNCH v1.2, these modules can be run using Python 2.7 or 3.7. All modules can be downloaded and executed independently without the need to install SuperCRUNCH as a PYTHON package or library, making them easy to use and edit. Nevertheless, there are eight dependencies that should be installed that enable the use of all features in SuperCRUNCH. These include the BIOPYTHON package for PYTHON, and the following seven external dependencies: NCBI-BLAST+ (for BLASTN and MAKEBLASTDB; Altschul, Gish, Miller, Myers, & Lipman, 1990; Camacho et al., 2009), CD-HIT-EST (Li & Godzik, 2006), CLUSTAL-O (Sievers et al., 2011), MAFFT (Katoh, Misawa, Kuma, & Miyata, 2002; Katoh & Standley, 2013), MUSCLE (Edgar, 2004), MACSE (Ranwez, Douzery, Cambon, Chantret, & Delsuc, 2018), and TRIMAL (Capella-Gutiérrez, Silla-Martínez, & Gabaldón, 2009). Installation instructions for all dependencies is provided on the SuperCRUNCH github wiki (https://github.com/dportik/SuperCRUNCH/wiki).

3 | WORKFLOW

A comprehensive user-guide, including overviews for all major steps and detailed instructions for all modules, is available on the SuperCRUNCH github wiki. Several complete analyses are posted on the Open Science Framework SuperCRUNCH project page, available at: https://osf.io/bpt94. Here, we briefly outline the major steps in a typical analysis, including some technical details for key steps. However, we strongly encourage users to read the complete documentation available online.

3.1 | Overview

SuperCRUNCH is designed to work with fasta-formatted sequence data that have been previously downloaded (e.g., from GenBank) or are locally available (e.g., processed sequences from in-house projects). No connection to live databases (such as NCBI) is required. Three input files are needed to perform a typical analysis: a set of sequence records in fasta format, a list of taxonomic names, and a list of loci (or genes) and associated search terms. The contents of these input files are described in greater detail below. The general workflow involves assessing taxonomy, parsing loci, similarity filtering, sequence selection, sequence alignment, and various post-alignment tasks (Fig. 1). The taxonomy used is user-supplied (e.g., not explicitly linked to any online databases). Therefore, an important first step is to identify and resolve potential conflicts between the user-supplied taxon list and the taxon labels in the sequence records. Afterwards, searches are conducted to identify records that putatively belong to loci (based on the content of record labels). These records are then written to locus-specific files. The sequences in each locus are then subjected to more stringent filtering using similarity searching (via nucleotide BLAST). This step removes non-homologous sequences and trims homologous sequences to remove non-target regions. After similarity filtering, the sequence-selection step allows selection of one sequence per species per locus or including all sequences. For both options, several additional filters (e.g., requiring an error-free reading frame, minimum length, or voucher information) can be used to ensure only high-quality sequences are retained. Sequences can then be prepared for alignment (adjusting direction and/or reading frame) and subsequently aligned using several alignment methods. After alignment, sequences can be relabeled, and the alignments can be trimmed, converted to multiple formats, and concatenated. SuperCRUNCH analyses end with the production of fully formatted input files that are compatible with numerous phylogenetic and population-genetic programs. Below, we provide additional details for the major steps outlined here.

3.2 | Starting Sequences

SuperCRUNCH requires a single fasta file of nucleotide sequence records as the main input. The fasta file can contain records from GenBank, unpublished sequence records, or a combination. GenBank data can be obtained by searching for relevant taxonomy terms or organism identifier codes on that database, and downloading the records in fasta format. For clades with many species, downloading all records directly may not be possible. For these groups, results from multiple searches using key organism identifiers can be downloaded and combined into a single fasta file. Automated downloading of GenBank sequence data through SuperCRUNCH is currently not supported, but may be included in a future release. Locally generated data should be formatted similar to GenBank records. A typical record should contain an accession number (a unique identifier code), a taxon label (two-part or three-part name, genus/species or genus/species/subspecies), and locus information (gene abbreviation and/or full name). Voucher information is optional. Additional details and examples of how to label Sanger-sequenced and sequence-capture datasets are provided in the online documentation.

3.3 | Assessing Taxonomy

SuperCRUNCH allows any taxonomy to be used. Taxonomy is supplied as a simple text file with one taxon name per line. Two-part and three-part names can be used. SuperCRUNCH offers the option to include or exclude subspecies. If subspecies are excluded, the third component of any three-part name is ignored, thereby reducing it to a two-part name. A taxon list can therefore contain a mix of species and subspecies names, even if subspecies are not desired. Although SuperCRUNCH does not connect with any taxonomy databases, lists of taxon names for large clades can be obtained through such databases, including the NCBI Taxonomy Browser or Global Names Database (Patterson et al., 2016). Many groups also have taxonomic databases, such as the Reptile Database (Uetz, Freed, & Hošek, 2018) and AmphibiaWeb (2019). These usually contain up-to-date taxonomies in a downloadable format. Taxon names can also be extracted directly from fasta files using the *Fasta_Get_Taxa.py* module. This option is most useful for unpublished sequences and sequence sets with few species.

Ideally, the user-supplied taxonomy will match the taxon names in the sequence records. However, taxonomy can change rapidly and conflicts often arise. To pass initial filtering steps, a record must have a taxon label that matches a name in the user-supplied taxonomy. Before beginning any filtering steps, it is therefore important to understand how compatible the user-supplied taxonomy is with the sequence-record set. The *Taxa_Assessment.py* module will perform an initial search across records to identify all records with a taxon label contained in the provided taxonomy, and identify all records with an unmatched taxon label (which would fail initial filtering steps). A list of unmatched taxon names is provided as output. External tools such as organismal databases, TAXIZE/PYTAXIZE (Chamberlain & Szöcs, 2013; Chamberlain et al., 2017), or the resolver function in the Global Names Database (Patterson et al., 2016), can be used to identify a “correct” name for an unmatched name. If a set of updated names is supplied for a set of unmatched names, the *Rename_Merge.py* module can be used to relabel all relevant records with the updated names, thus allowing them to pass the initial filtering steps. The combination of these two taxonomy modules allows users to correct minor labeling errors (such as misspellings), reconcile synonymies, or completely update names to a newer taxonomy.

3.4 | Parsing Loci

The *Parse_Loci.py* module conducts searches for specific loci using a set of user-supplied search terms, including gene abbreviations and full gene names. All searches are conducted using SQL with a local database constructed from the input sequences, and the initial assignment of a sequence to a locus is based purely on matches to the record labeling. For a sequence to be written to a locus-specific file, it must match either the gene abbreviation or description for that locus, and it must have a taxon label present in the user-supplied taxonomy. This approach creates smaller locus-specific sequence sets from the initial sequence set, which are more tractable for downstream similarity searches (versus “all-by-all” clustering).

The success of finding sequences using SuperCRUNCH depends on providing appropriate gene abbreviations and labels. We recommend searching on GenBank to identify common labeling or using gene databases such as GeneCards (Stelzer et al., 2016). There is no hard upper bound on how many loci can be searched for. Thus, SuperCRUNCH can be used to process large phylogenomic datasets (e.g., sequence capture experiments) including those with thousands of species and loci. Whole mitochondrial genomes can also be targeted for any search involving a particular mitochondrial gene (see below). Recommendations for optimizing locus searches for different data types are provided in the online documentation.

The choice of loci will be group-specific. Previous phylogenetic/phylogeographic papers can be used to identify appropriate loci. The best criteria for selecting loci remain unresolved. One relevant criterion is completeness (e.g., including only loci present in >20% of the species). For each search conducted with *Parse_Loci.py*, the number of sequences found for each locus will be output. Therefore, it can be used to survey the availability of sequences for each locus. A downstream step allows loci to be filtered based on a minimum number of required sequences, so decisions can be made after additional filtering.

The *Parse_Loci.py* module performs another important task: automatically detecting voucher information in those sequence record labels that containing a "voucher", "strain", or "isolate" field (see online documentation). This information is written into the records as a new tag that is discoverable in other downstream steps, allowing the creation of "vouchered" datasets.

3.5 | Similarity Filtering

SuperCRUNCH offers two parallel methods for filtering sequences based on similarity. Each method uses nucleotide BLAST to perform searches, but they differ in whether reference sequences are automatically selected (*Cluster_Blast_Extract.py*) or user-provided (*Reference_Blast_Extract.py*) (Fig. 2). The automatic selection of reference sequences is appropriate for loci consisting of "simple" sequence records (Fig. 2). We define "simple" record sets as those generally containing a single gene region with limited length variation, which results from use of the same primers (Sanger-sequencing) or probes (sequence capture) to generate sequences. The *Cluster_Blast_Extract.py* module can be used for these types of loci. These generally include nuclear markers and those from commercial probe sets (e.g., UCEs: ultraconserved elements). The *Cluster_Blast_Extract.py* module begins by clustering sequences based on similarity using CD-HIT-EST. It then identifies the largest sequence cluster, and designates that as the reference sequence set (Fig. 2). All starting sequences (including those in the reference cluster) are then blasted to this reference using BLASTn. This method is convenient for automating the process of similarity filtering for "simple" records and can be used to screen thousands of loci.

However, *Cluster_Blast_Extract.py* will fail for loci containing "complex" sequence records. "Complex" records include those containing the target region plus non-target sequence (e.g., other regions or genes). Common examples include long mtDNA fragments and whole mitogenomes (Fig. 2). Another type of "complex" record is a gene sequenced for different fragments that have little or no overlap. For these sequence sets, the *Reference_Blast_Extract.py* module should be used instead. Rather than identifying the reference set from the starting sequences via clustering, it requires a user-supplied reference sequence set to perform BLASTn searches (Fig. 2). An external reference set must be provided for each locus, and it ensures that only the desired regions are targeted and extracted. For example, a set of ND2 reference sequences can be used to extract only ND2 regions from a record set comprised of whole mitochondrial genomes, multi-gene mitochondrial sequences, and partial ND2 records.

For both modules, the BLASTn algorithm can be specified by the user (blastn, blastn-short, megablast, or dc-megablast), allowing searches to be tailored to inter- or intraspecific datasets. After BLASTn searches are conducted for a locus, sequences without significant matches are discarded. For all other sequences, the BLAST coordinates of all hits (excluding self-hits) are merged to identify the target region of the query sequence. Based on these coordinates, the entire sequence or a trimmed portion of the sequence is kept. The BLAST coordinate merging action often results in a single continuous interval (e.g., bases 1–800). However, non-overlapping coordinates can also be produced (e.g., bases 1–200, 450–800). Two common examples (sequences containing stretches of N's or gene duplications) are illustrated in Figure 3.

Multiple options are available for handling non-overlapping sequence intervals. The default option is "span", which bridges non-overlapping intervals <X base pairs apart, where X is

the default value (100 bp) or a user-supplied value. However, if the gap is >X bases, the longest interval is selected instead. The "nospan" method will simply select the longest interval of the coordinate set, and the "all" method will concatenate the sequence intervals together. Results from each option are shown in Figure 3.

An optional contamination-filtering step is available (*Contamination_Filter.py*). This step excludes all sequences scoring >95% identity for at least 100 continuous base pairs to the reference sequences. Here, the contamination reference sequences should correspond to the expected source of contamination (see documentation).

3.6 | Sequence Selection

SuperCRUNCH can construct two fundamentally different datasets: species-level supermatrices and population-level (phylogeographic) datasets. The *Filter_Seqs_and_Species.py* module is used to select the sequences necessary to construct either dataset (using the "oneseq" or "allseqs" options). For supermatrices, a single sequence is typically used to represent each species for each gene. If multiple sequences for a given gene exist for a given species (e.g., because multiple individuals were sampled), then an objective strategy must be used for sequence selection. *Filter_Seqs_and_Species.py* offers several options, including the simplest solution: sorting sequences by length and selecting the longest sequence ("length" method). An additional filter can be applied to protein-coding loci, termed "translate". This is an extension of the "length" method, which limits sequences to those containing a valid reading frame (determined by translation in all forward and reverse frames), thereby removing sequences with errors. However, if no sequences pass translation, the longest sequence is selected rather than excluding the taxon. The "randomize" feature can be used to select a sequence randomly from the set available for a taxon, which will generate supermatrix permutations. Finally, the "vouchered" option will only allow sequences with a voucher tag (generated by *Parse_Loci.py*). For all selection options, sequences must meet a minimum base-pair threshold set by the user. This will determine the smallest amount of data that can be included for a given marker for a given terminal taxon. However, the optimal minimum is another unresolved issue.

To build a population-level dataset, all sequences passing the minimum base pair threshold will be kept. The "translate" option can be used to only include sequences that pass translation, and the "vouchered" option will only include sequences with a voucher tag. The "vouchered" option should be selected to build a population-level dataset that allows samples to be linked by voucher information. Additional information on how various options affect supermatrix and population-level datasets is available online.

The *Filter_Seqs_and_Species.py* module provides key output files for reproducibility and transparency. For each locus, this includes a BatchEntrez-compatible list of all accession numbers from the input file, a per-species list of accession numbers, and a comprehensive summary of the sequence(s) selected for each species (accession number, length, translation test results, and number of alternative sequences available). The *Infer_Supermatrix_Combinations.py* module can be used to infer the total number of possible supermatrix combinations (based on the number of available alternative sequences per taxon per locus). Following the selection of representative sequences, the *Make_Acc_Table.py* module can be used to generate a table of GenBank accession numbers for all taxa and loci. This can be created for species-level supermatrices and "vouchered" population-level datasets.

3.7 | Multiple Sequence Alignment

SuperCRUNCH includes two pre-alignment steps and several options for multiple sequence alignment (Fig. 4). One pre-alignment module (*Adjust_Direction.py*) adjusts the direction of all sequences in each locus-specific fasta file in combination with MAFFT. This step produces unaligned fasta files with all sequences written in the correct orientation (thereby avoiding major pitfalls with aligners). Sequences for any locus can be aligned using the *Align.py* module with one of several popular aligners (MAFFT, MUSCLE, CLUSTAL-O) or with all aligners sequentially. For protein-coding loci, the MACSE translation aligner is also available, which is capable of aligning coding sequences with respect to their translation while allowing for multiple frameshifts or stop codons. To use this alignment method, the *Coding_Translation_Tests.py* module can be used to identify the correct reading frame of sequences, adjust them to the first codon position, and ensure completion of the final codon. Although MACSE can be run on a single set of reliable sequences (e.g., only those that passed translation), it has an additional feature allowing simultaneous alignment of a set of reliable sequences and a set of unreliable sequences (e.g., those that failed translation), using different parameters. The *Coding_Translation_Tests.py* module can be used to generate all the necessary input files to perform this type of simultaneous alignment using MACSE (see online documentation).

The alignment methods implemented in SuperCRUNCH are not intended to produce ultra-large alignments containing several thousand sequences. To create ultra-large alignments, we recommend using external alignment methods such as SATé-II (Liu et al., 2012), PASTA (Mirarab et al., 2015), or UPP (Nguyen et al., 2015). We also recommend using UPP to create alignments for loci containing a mix of full-length sequences and short sequence fragments, as these conditions are problematic for many alignment methods (Nguyen et al., 2015).

3.8 | Post-Alignment Tasks

After multiple sequence alignment, there are several tasks that can be help prepare datasets for downstream analyses. One important task involves relabeling sequences using the *Fasta_Relabel_Seqs.py* module, such that sequence labels are composed of taxon labels, accession numbers, voucher codes, or some combination. The relabeling strategy will depend on the type of dataset being produced (and whether concatenation is intended). Recommendations are provided in the online documentation. Regardless, this step is essential because full-length labels are incompatible with many downstream programs. Relabeled fasta files can be converted into other commonly used formats (nexus, phylip) using the *Fasta_Convert.py* module.

SuperCRUNCH offers two different approaches for automated alignment trimming, although the overall value of trimming remains debatable (Tan et al., 2015). The *Trim_Alignments_Trimal.py* module uses several implementations of TRIMAL (“gap-threshold”, “gappyout”, “noallgaps”) to trim alignments. The *Trim_Alignments_Custom.py* module is based on the custom trimming routine in PHYLUCE (Faircloth, 2016). This version allows edge trimming, row trimming, or both.

Relabeled alignment files can be concatenated using the *Concatenation.py* module. This module allows fasta or phylip input and output formats. The user can also select the symbol for missing data (-, N, ?). It produces a log file containing the number of loci for each terminal taxon and a data partitions file (containing the corresponding base pairs for each locus in the alignment). The *Concatenation.py* module can be used for any dataset in which labels are consistent across loci, including species-level supermatrices (with taxon labels) and “vouchered” population-level datasets (with taxon/voucher combination labels). See online documentation for more details.

4 | DEMONSTRATIONS AND COMPARISONS

To demonstrate the full range of features available in SuperCRUNCH, we constructed several types of datasets. These included small population-level datasets (<300 sequences, <10 loci), a "vouchered" phylogeographic dataset (~100 samples, 4 loci), traditional supermatrices (~1,500 species, ~70 loci), and phylogenomic supermatrices (~2,000 UCE loci, <20 samples). In addition, we demonstrate how SuperCRUNCH can be used to add published outgroup sequences to a supermatrix of locally generated sequences. Finally, we compared the ability of SuperCRUNCH to construct species-level supermatrices relative to the program PyPHLAWD (Smith & Walker, 2018), using two test clades (Iguania and Dipsacales). In addition to comparing supermatrix characteristics (taxa, loci, sequences), we also compared the resulting phylogenies (including the number genera and families recovered as monophyletic). Details are given in Supporting Information S1. All analyses are available as tutorials on the SuperCRUNCH project page on the Open Science Framework (https://osf.io/bpt94/). Analyses were run on an iMac with a 4.2 GHz quad-core Intel Core i7 with 32 GB RAM.

5 | RESULTS

Detailed results for all analyses are provided in Supporting Information S1, and are briefly summarized here. SuperCRUNCH produced a large supermatrix (~1,500 species, ~60 loci, ~13,000 sequences) in ~1.5 hours, but with more thorough settings ran up to 13 hours. This difference in runtimes is largely attributable to the alignment step, with MAFFT taking ~4 minutes and MACSE requiring 11 hours. SuperCRUNCH successfully reconstructed a published phylogeographic dataset (<1 min) and a published phylogenomic supermatrix (~25 min). It rapidly created new combinations of population-level datasets from multiple published sources (<1 min). It also added GenBank sequences for hundreds of outgroups to a local (unpublished) supermatrix project (<4 min).

SuperCRUNCH outperformed PyPHLAWD in all supermatrix comparisons, recovering more taxa and sequences in both test clades. Given the same starting sequences for the Iguania dataset, SuperCRUNCH found ~300 more taxa (1,359 vs. 1,069) and ~2,300 more sequences (12,676 vs. 10,397). PyPHLAWD experienced a severe performance drop for loci containing "complex" records (those with multiple loci or non-overlapping regions), and thereby lost 63% of the available mtDNA sequences (>2,000 sequences discarded). SuperCRUNCH supermatrices also generated higher quality phylogenies, recovering more genera as monophyletic in all comparisons. Additional results for these comparisons are discussed in Supporting Information S1, and all analyses are available on the Open Science Framework (https://osf.io/bpt94/).

6 | DISCUSSION

SuperCRUNCH is a versatile bioinformatics toolkit that can be used to create large phylogenetic datasets. It contains many novel features that distinguish it from other programs. Most importantly, SuperCRUNCH is not restricted to GenBank sequence data. It can be used to process unpublished sequences, and combinations of GenBank and unpublished data. Many programs rely on GenBank database releases (PhyLoTA, PyPHLAWD, SUMAC, SUPERSMART) to retrieve starting sequences and obtain metadata. In contrast, SuperCRUNCH infers metadata directly from user-supplied starting sequences, and constructs local databases to perform searches. This design explicitly allows for the inclusion of unpublished sequence data.

There are other programs designed to generate species-level supermatrices (phyloGenerator, PhylotaR, PyPHLAWD, SUPERSMART), but these workflows generally do not offer explicit options for creating population-level (phylogeographic) datasets. SuperCRUNCH includes a key step that allows for either selecting one sequence per species, or all sequences, generating either species-level supermatrices or population-level datasets. Furthermore, filtering options are available for both (passing translation, minimum length), ensuring only high-quality sequences are included in both types of datasets.

A population-level (phylogeographic) dataset includes multiple sequences per species per locus. It is straightforward to collect all sequences available for a particular gene for a given species. However, there may be little overlap of sampling across loci. For example, different individuals may have been sequenced for different loci in different studies. Identifying sequences derived from the same sample can be difficult and requires integrating voucher information. Incorporating additional sequences (published or unpublished) into phylogeographic datasets can be challenging, given the difficulty of identifying and matching voucher information in sequence records. SuperCRUNCH automates these tasks, creating "vouchered" datasets. The "vouchered" feature of SuperCRUNCH only allows sequences with a voucher code to pass the filtering steps used to create a population-level dataset. The final sequences are relabeled using the voucher information (typically taxon name plus voucher code), such that sequences derived from the same sample share an identical label. Together, these features allow the rapid reconstruction of published phylogeographic datasets, merging of published and unpublished data to create new datasets, and construction of datasets from locally generated sequences (especially from sequence-capture experiments).

SuperCRUNCH differs from similar programs in that it initially identifies sequences using record labels, moves the relevant sequences to locus-specific files, and performs similarity searches on reduced-sequence sets. In contrast, many other programs attempt to cluster all starting sequences to produce putatively orthologous sequence clusters (PhyLoTA, PyPHLAWD, PhylotaR, and SUPERSMART). In general, these "all-by-all" clustering approaches do not allow target loci to be specified, require additional steps to identify the content of sequence clusters, and can result in the inclusion of paralogous sequences. Furthermore, clusters produced from a "complex" record set may be redundant, introducing biases into supermatrices (e.g., a single locus repeated multiple times). SuperCRUNCH putatively assigns sequences to a locus based on the presence of locus search terms in the record label (similar to phyloGenerator). This method allows specific loci to be targeted, establishes a clear identity for the sequences, and reduces the chance of including paralogous sequences (which should have a different gene label). Thus, SuperCRUNCH can accurately target and build datasets composed of thousands of loci, including UCEs and other sequence-capture loci. It is difficult to reliably perform this task using "all-by-all" clustering of starting sequences. Even the recently proposed "baited" clustering approach of PyPHLAWD, which requires a reference sequence set for each locus, is prohibitive for large genomic datasets (e.g., ~5,000 UCE loci). We acknowledge the success of the label-matching strategy relies on defining appropriate search terms. Unanticipated issues like gene name synonymies can inadvertently exclude relevant sequences (Supporting Information S1). Regardless, the label-matching method of SuperCRUNCH circumvents many issues outlined above, and outperformed the "baited" clustering methods of PyPHLAWD for all test cases (Supporting Information S1). Given that searches for loci are conducted using SQL, they are fast and can be executed using iteratively refined search terms to optimize results.

SuperCRUNCH also offers improved methods for similarity searches. These include the ability to specify BLASTn algorithms, improved BLAST coordinate merging and sequence trimming, and flexible choices for selecting reference sequences. Unless specified, the default algorithm used by nucleotide BLAST is megablast, which is best for finding highly similar sequences in intraspecific searches (e.g., population-level datasets). In contrast, discontiguous megablast performs substantially better for interspecific searches (Ma, Tromp, & Li, 2002; Camacho et al., 2009), and is preferable for species-level supermatrices. In many cases, merging the BLAST coordinates obtained from a query sequence is trivial and results in a single continuous target region. However, multiple non-overlapping target regions may also occur for a query sequence, and SuperCRUNCH offers several novel options to handle these cases (Fig. 3). Furthermore, SuperCRUNCH uses the resulting coordinates to automatically trim sequences to the target region, if necessary. This non-standard trimming action ensures that only sequence regions homologous to the reference-sequence set are kept. SuperCRUNCH also offers two options for designating reference sequences: reference sequences can be selected automatically from the sequence set, or can be supplied by the user (Fig. 2). Automatic selection of reference sequences is appropriate for "simple" sequence records (i.e., same gene regions), and can efficiently perform similarity searches for thousands of loci. User-supplied references are more appropriate for "complex" sequence records (multiple loci or non-overlapping regions), or whenever fine-control over the target region is desired. Although this latter option requires gathering reference sequences manually, it is powerful and can be used to extract a single mtDNA gene region from a record set containing a mix of whole mitochondrial genomes, long multi-gene mtDNA sequences, and shorter target sequences.

Despite many improvements implemented in SuperCRUNCH, an important and general issue is the accuracy of GenBank sequence data. This issue can affect SuperCRUNCH and all other programs that process GenBank data. For example, errors may arise through incorrect uploading of data, misidentified specimens, contamination, and other lab errors. Data errors can occur in record labels, and include incorrect gene, taxon, or voucher information. With regards to contamination, we identified two human mtDNA sequences labeled as lizards in our iguanian supermatrix analysis (HM040901.1, KP899454.1; Supplemental File 1). The contamination filter in SuperCRUNCH can detect and eliminate some problems of this kind, but it cannot readily identify cases of misidentified or mislabeled sequences within the focal group. Misidentified specimens are perhaps the most difficult problem to detect, particularly at a shallow taxonomic scale (e.g., a specimen assigned to the wrong species within the same genus or family). Although similarity filtering can generally be used to correctly establish gene identities, parallel approaches for identifying inaccurate taxon labeling within the focal group are generally lacking. Overall, data accuracy is a general problem for the supermatrix approach regardless of the methods used to process the data. Automatic identification of inaccurate sequence records would be a useful goal for future studies of supermatrix construction.

The initial motivation behind SuperCRUNCH was to increase transparency and reproducibility across all steps in dataset construction. We therefore encourage researchers running analyses with SuperCRUNCH to publish the information needed to reproduce their results. This includes accession numbers for the starting sequence set, the taxon list file, the locus search terms file, and the ancillary files and commands used to execute steps. We also emphasize that SuperCRUNCH is highly modular, and performing a SuperCRUNCH analysis does not require running the full pipeline. As such, SuperCRUNCH modules can be incorporated into any bioinformatics pipeline or used in conjunction with features of other currently available programs. Alternative programs offer important features that may serve different needs beyond those available in SuperCRUNCH (e.g., SUPERSMART performs phylogenetic analyses on the supermatrices that it generates). Given the rapid growth of sequence data on GenBank (NCBI, 2019), improved bioinformatics approaches to mine and manage phylogenetic datasets are needed.

ACKNOWLEDGMENTS

For financial support, we thank U.S. National Science Foundation Grant DEB 1655690. We thank early testers, including Itzue W. Caviedes-Solis, Cristian Román-Palacios, Benjamin R. Karin, and Pascal O. Title, and participants of the Trees in the Desert workshop (Tucson, AZ) for their beneficial feedback. We thank the Associate Editor and two anonymous reviewers for helpful comments that greatly improved the manuscript.

AUTHORS' CONTRIBUTIONS

DMP designed the methodology, wrote all code, and analyzed the data; DMP and JJW wrote the manuscript.

DATA ACCESSIBILITY

SuperCRUNCH is open-source and freely available at https://github.com/dportik/SuperCRUNCH. The complete materials (and instructions for replicating our analyses), including input and output files from each step, is available from the Open Science Framework (https://osf.io/bpt94/).

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FIGURE LEGENDS

FIGURE 1 A depiction of the general steps (and associated modules) involved in full SuperCRUNCH analyses. Each step is outlined in a corresponding entry of the same title in the Workflow section of the main text.

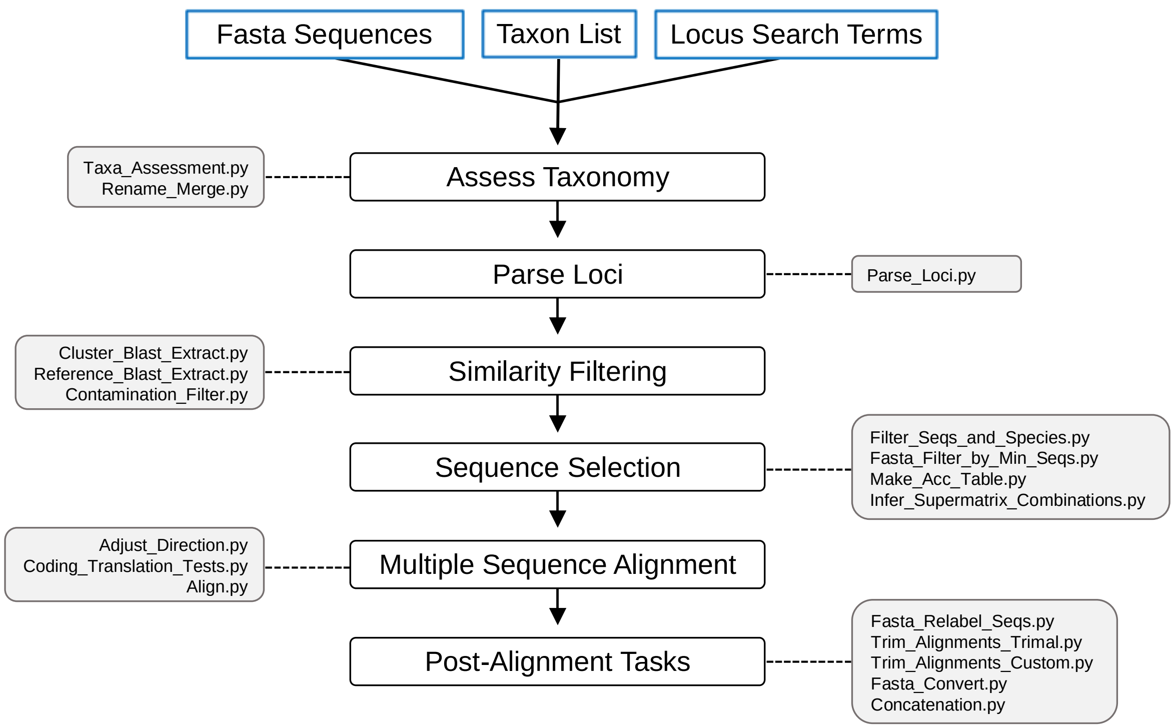


FIGURE 2 An illustration of the similarity searching workflows occurring in the *Cluster_Blast_Extract.py* and *Reference_Blast_Extract.py* modules. Green color represents target regions, and all other colors represent non-target regions.

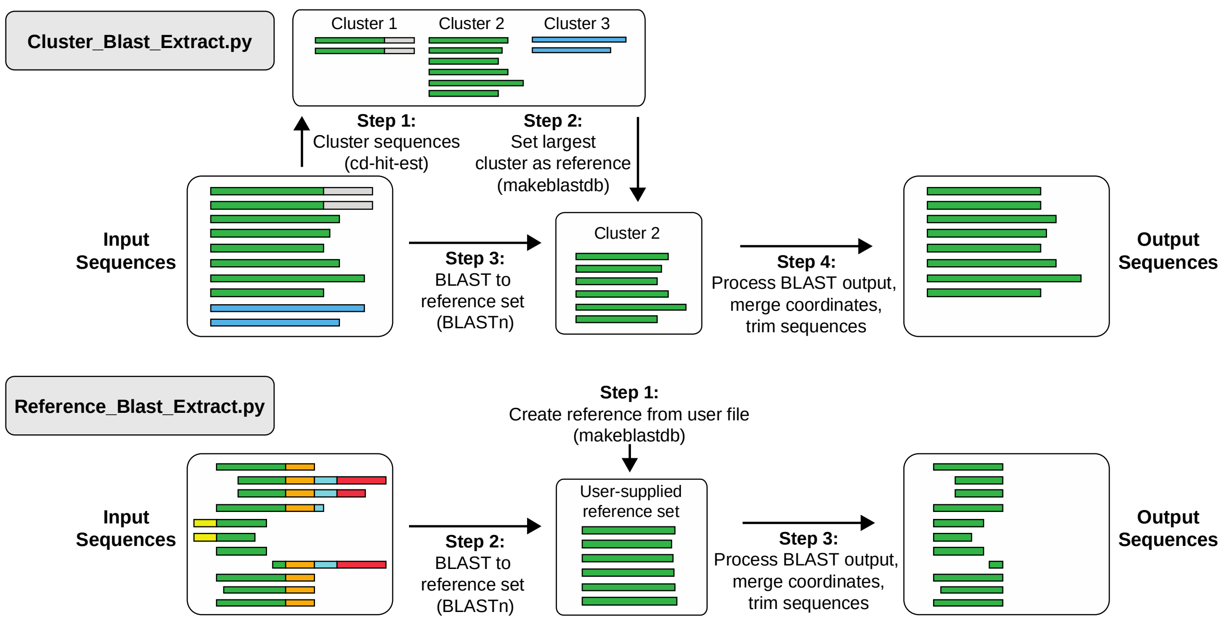
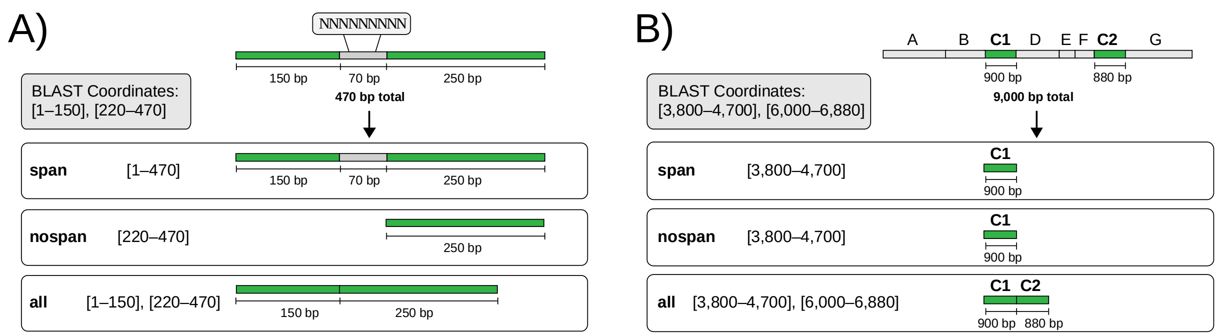


FIGURE 3 A demonstration of the options available for handling non-overlapping BLAST coordinates for query sequences with two common examples: (A) a sequence that contains a stretch of N's, and (B) a long sequence containing multiple genes (represented by letters) that also contains a gene duplication (indicated by C1 and C2), such as an organellar genome. In both sequences, green represents the target region and grey represents either missing data (A) or non-target regions (B). The resulting merged BLAST coordinates are shown for each sequence, along with which coordinates would be selected under the available options ("span", "nospan", and "all", see main text).



SUPPORTING INFORMATION S1

for

SuperCRUNCH: A toolkit for creating and manipulating supermatrices and other large phylogenetic datasets

Daniel M. Portik[1,2*], John J. Wiens[1]

1. Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona

2. California Academy of Sciences, San Francisco, California

*Correspondence: daniel.portik@gmail.com

DEMONSTRATIONS, COMPARISONS, AND RESULTS

1 | *de novo* Supermatrix for Iguania

1.1 | Methods: Iguania-Fast and Iguania-Thorough Analyses

To demonstrate the full use of SuperCRUNCH we assembled a *de novo* supermatrix for Iguania, a clade of squamate reptiles that contains ~1900 species in 14 families (Uetz et al., 2018). This clade includes chameleons, dragons, flying lizards, iguanas, anoles, and other well-known lizards. For starting material, we downloaded all available iguanian sequence data from GenBank on November 30, 2018 using the organism identifier code for Iguania (txid8511[Organism:exp]). This produced a 13.2 GB fasta file that included 8,785,378 records. We obtained search terms for 69 loci (62 nuclear, 7 mitochondrial) that have been widely used in reptile phylogenetics or phylogeography (Townsend et al., 2008; Portik et al., 2012; Pyron et al., 2013). This includes 62 nuclear loci (*ADNP*, *AHR*, *AKAP9*, *AMEL*, *BACH1*, *BACH2*, *BDNF*, *BHLHB2*, *BMP2*, *CAND1*, *CARD4*, *CILP*, *CMOS*, *CXCR4*, *DLL1*, *DNAH3*, *ECEL1*, *ENC1*, *EXPH5*, *FSHR*, *FSTL5*, *GALR1*, *GHSR*, *GPR37*, *HLCS*, *INHIBA*, *KIAA1217*, *KIAA1549*, *KIAA2018*, *KIF24*, *LRRN1*, *LZTS1*, *MC1R*, *MKL1*, *MLL3*, *MSH6*, *MXRA5*, *MYH2*, *NGFB*, *NKTR*, *NOS1*, *NT3*, *PDC*, *PNN*, *PRLR*, *PTGER4*, *PTPN*, *R35*, *RAG1* (two fragments), *RAG2*, *REV3L*, *RHO*, *SLC30A1*, *SLC8A1*, *SLC8A3*, *SNCAIP*, *SOCS5*, *TRAF6*, *UBN1*, *VCPIP*, *ZEB2*, *ZFP36L1*) and 7 mitochondrial genes (*12S*, *16S*, *CO1*, *CYTB*, *ND1*, *ND2*, *ND4*). For the taxon names list, we used a modified version of the February 2018 release of the Reptile Database (which does not contain subspecies). The above starting materials were used to run two different analyses, including one high-quality analysis that used all modules and features (termed "Iguania-Thorough"; https://osf.io/9gs32/), and one analysis that used the fastest possible settings (termed "Iguania-Fast"; https://osf.io/x5hrm/).

For the Iguania-Thorough analysis, we performed an initial taxon assessment of the starting sequences (8,785,378 records). Using *Taxon_Assessment.py*, we found that 158,935 records had a name matching our taxonomy, and 8,626,443 records had a name that did not match a name in our taxonomy. After identifying all correctable unmatched names using the Reptile Database (Uetz et al., 2018), we successfully relabeled 1,860 records using corrected names. We merged the updated records with those that passed the initial taxon assessment, and the resulting sequence set contained 160,795 records. These records were used to search for the 69 loci using *Parse_Loci.py*, and we recovered two or more sequences for all but three loci (*KIAA1217*, *KIAA1549*, *MYH2*). We performed similarity filtering using *Cluster_Blast_Extract.py* for 58 nuclear loci (allowing automatic reference selection, intended for “simple” records). We performed similarity filtering using *Reference_Blast_Extract.py* for the 7 mitochondrial genes and for the nuclear protein-coding locus RAG1. This latter filtering module utilizes user-supplied references, and is intended for “complex” record sets (e.g., with little or no overlap for some sequences in some taxa). We included RAG1 in the user-supplied reference strategy because it has been sequenced in (depending on the species) either its entirety as well as for two non-overlapping fragments. We therefore targeted the two regions of RAG1 independently (labeled p1 and p2), which we considered as separate loci downstream. We used two reference sequence sets (*RAG1p1*, *RAG1p2*) created from the full RAG1 gene of seven tetrapod species (available at https://github.com/dportik/SuperCRUNCH/tree/master/data/reference-sequence-sets/vertebrate-RAG1). To target each of the 7 mitochondrial genes, we assembled a reference sequence set for each gene from 114 squamate mitochondrial genomes, which were downloaded from GenBank. We used the “extract annotation” feature of Geneious (https://www.geneious.com) to quickly obtain each gene region from all mitogenomes (in GenBank format). These reference sequence sets are available at: https://github.com/dportik/SuperCRUNCH/tree/master/data/reference-sequence-sets/squamate-mtdna. After similarity filtering, we selected representative sequences for each species per gene, using options specific to each type of marker (nuclear protein-coding, mtDNA protein-coding, and mtDNA rRNA genes). We used the "oneseq" and "translate" options of *Filter_Seqs_and_Species.py* for the 60 nuclear protein-coding loci (setting translation to standard code) and for the 5 mitochondrial protein-coding genes (setting translation to vertebrate mtDNA code), and the "oneseq" and "length" options for the two mitochondrial rRNA genes (12S, 16S). For each of these three runs, we enforced a minimum 200 bp length for all sequences. We used *Fasta_Filter_by_Min_Seqs.py* to remove alignments with fewer than 30 taxa, which eliminated 6 of the initial 67 loci. We performed sequence direction adjustments for the 61 loci using *Adjust_Direction.py*. We used the *Coding_Translation_Tests.py* module to prepare the 54 nuclear loci (setting translation to standard code) and the 5 mitochondrial protein-coding genes (setting translation to vertebrate mtDNA code) for translation alignment. We used the *Align.py* module to perform MACSE translation alignments, using the "pass_fail" option, for the 54 nuclear loci (setting translation to standard code) and the 5 mitochondrial protein-coding genes (setting translation to vertebrate mtDNA code). We performed sequence alignment for the two rRNA mitochondrial genes (12S, 16S) using Clustal-O using the defaults in *Align.py*. We constructed a table of GenBank accession numbers for all filtered sequences using the *Make_Acc_Table.py* module. We relabeled sequences in all alignment files using the "species" option in *Fasta_Relabel_Seqs.py*, and subsequently trimmed alignments using the gap-threshold option in trimAl with a threshold value of 0.1, as implemented in *Trim_Alignments_Trimal.py*. We converted fasta alignments to phylip and nexus format and concatenated alignments to produce the final supermatrix. In total, we ran 21 separate steps using 16 modules for the Iguania-Thorough analysis (Table S1). The input and output files for all steps, along with complete commands used to execute modules, are available are available on the Open Science Framework at: https://osf.io/9gs32/.

For the Iguania-Fast analysis, we skipped the taxonomy assessment step and began by searching for the 69 loci in the full set of 8,785,378 starting records using *Parse_Loci.py*. We performed similarity filtering using *Cluster_Blast_Extract.py* for 58 nuclear loci (allowing automatic reference selection), and performed similarity filtering using *Reference_Blast_Extract.py* for the 7 mitochondrial genes plus *RAG1* (with the same user-supplied references as above). Following similarity filtering, we selected sequences for all loci using the “length” option in *Filter_Seqs_and_Species.py*, requiring a minimum length of 200 bp. We used *Fasta_Filter_by_Min_Seqs.py* to remove alignments with fewer than 30 taxa, which eliminated 7 of the initial 67 genes. We performed sequence direction adjustments for the remaining 60 loci using *Adjust_Direction.py*, and performed multiple sequence alignment using MAFFT in *Align.py*. We constructed a table of GenBank accession numbers for all filtered sequences using the *Make_Acc_Table.py* module. We relabeled sequences in all alignment files using the “species” option in *Fasta_Relabel_Seqs.py*, and subsequently trimmed alignments using the gap-threshold option in trimAl with a threshold value of 0.1, as implemented in *Trim_Alignments_Trimal.py*. We skipped format conversion and concatenated the alignments to produce the final supermatrix. In total, we ran 11 separate steps using 11 modules for the Iguania-Fast analysis (Table S2). The input and output files for each step, along with complete instructions, are available at: https://osf.io/x5hrm/.

We investigated the quality of the phylogenetic trees resulting from each of the supermatrices (in terms of the number of monophyletic genera, subfamilies, and families). For each supermatrix we ran an unpartitioned RAxML analysis using the GTRCAT model and 100 rapid bootstraps to generate support values. We performed an assessment of these trees in conjunction with other trees of Iguania produced by PyPHLAWD and a constrained SuperCRUNCH analysis (see section 6 – Comparison to PyPHLAWD).

1.2 | Results: Iguania-Fast and Iguania-Thorough Analyses

The Iguania-Thorough analysis resulted in a supermatrix containing 61 loci, 1,426 species, and 13,307 total sequences. The analysis took 12 hours and 53 minutes to complete (not including user-time; Table S1). The initial record set contained over 8 million sequences. This set was narrowed down to 160,795 sequences corresponding to the 67 loci. This difference indicates that most sequences downloaded in our GenBank search were irrelevant for our purposes (shotgun genome sequences, mRNA, etc.). Of the 160,795 relevant sequences, 1,860 represent records that were "rescued" by updating an unmatched taxon label. The 160,795 sequences were narrowed down to 13,389 sequences during the sequence-selection step (in which one sequence per taxon per locus was selected). Although the analysis initially found 67 loci, the requirement of at least 30 species per locus eliminated six loci. Consequently, the final number of sequences in the supermatrix totaled 13,307. For the Iguania-Thorough analysis, most steps required only seconds to complete, and a majority of the analysis time is attributable to similarity filtering (~1 hour combined) and multiple sequence alignment (~11.5 hours combined; Table S1). The overall analysis time of ~13 hours was calculated based on running the modules/steps sequentially, but running the three alignment steps simultaneously (MACSE for mtDNA, MACSE for nucDNA, Clustal-O for noncoding mtDNA) would have reduced the total analysis time for Iguania-Thorough to ~7 hours.

The Iguania-Fast analysis resulted in a supermatrix containing 60 loci, 1,399 species, and 12,978 total sequences. The analysis took 1 hour and 28 minutes to complete (not including user-time; Table S2). This analysis did not include any taxonomy assessment, thereby losing the 1,860 records that were "rescued" in the Iguania-Thorough analysis. Rather, loci were parsed directly from the starting set of 8 million records. As a result, the Iguania-Fast analysis recovered less data (1,399 species, 12,978 sequences) than the Iguania-Thorough analysis (1,426 species, 13,307 sequences). This Iguania-Fast analysis also found 67 loci initially, but seven loci were discarded because they contained fewer than 30 species. The Iguania-Fast analysis also required ~1 hour for similarity filtering, but the multiple sequence alignment step using MAFFT took less than 5 minutes to complete.

Most steps of the Iguania-Fast and Iguania-Thorough analyses took similar amounts of time. However, alignment time (5 minutes vs. ~11.5 hours) appears to be the main driver of differences in total time for the Iguania-Fast analysis (~1.5 hours) and the Iguania-Thorough analysis (~13 hours). The Iguania-Thorough analysis resulted in more taxa and sequences, and this was entirely due to the taxonomy assessment step, which only required ~20 minutes to complete (excluding time to identify updated names). We therefore strongly recommend performing the taxonomy assessment step for SuperCRUNCH analyses, as it can result improve dataset quality with minimal computational time.

Trees produced from the Iguania-Thorough and Iguania-Fast datasets are discussed in Section 6 (Comparison to PyPHLAWD).

Table S1. Summary of all steps for the Iguania-Thorough analysis, which took ~13 hours to complete (not including user-time).

Step	Module	Input Details	Flag Information	Elapsed time
Assess Taxonomy	Taxa_Assessment.py	8,785,378 records	--no_subspecies	0:12:47
	Rename_Merge.py	Relabeled 1,860 records		0:06:42
Parse Loci	Parse_Loci.py	69 loci to search, 160,795 records	--no_subspecies	0:02:41
Similarity Filtering	Cluster_Blast_Extract.py	58 loci	-b dc-megablast, --max_hits 200, -m span, --threads 4	0:18:50
	Reference_Blast_Extract.py	9 loci	-b dc-megablast, --max_hits 200, -m span, --threads 4	0:48:39
	Contamination_Filter.py	7 mtDNA loci	-b megablast	0:00:06
Sequence Selection	Filter_Seqs_and_Species.py	60 nuclear coding loci	-s oneseq, -f translate, -m 200, --no_subspecies, --table standard	0:00:21
	Filter_Seqs_and_Species.py	5 mtDNA coding loci	-s oneseq, -f translate, -m 200, --no_subspecies, --table vertmtdna	0:00:34
	Filter_Seqs_and_Species.py	2 mtDNA noncoding loci	-s oneseq, -f length, -m 200, --no_subspecies	0:00:01
	Fasta_Filter_by_Min_Seqs.py	67 loci	--min_seqs 30	0:00:01
Sequence Alignment	Adjust_Direction.py	61 loci	--threads 8	0:00:51
	Coding_Translation_Tests.py	54 nuclear coding loci	--table standard	0:00:01
	Coding_Translation_Tests.py	5 mtDNA coding loci	--table vertmtdna	0:00:03
	Align.py	2 mtDNA noncoding loci	-a clustalo, --accurate, --threads 4	0:27:03
	Align.py	5 mtDNA coding loci	-a macse, --table vertmtdna, --mem 10, --pass_fail	5:30:37
	Align.py	54 nuclear coding loci	-a macse, --table standard, --mem 10, --pass_fail	5:24:27
Post-Alignment	Make_Acc_Table.py	61 loci		0:00:01
	Fasta_Relabel_Seqs.py	61 loci	-r species	0:00:01
	Trim_Alignments_Trimal.py	61 loci	-f fasta, -a gt, --gt 0.1	0:00:01
	Fasta_Convert.py	61 loci		0:00:01
	Concatenation.py	61 loci, 1,426 taxa, 13,307 seqs	--informat fasta, --outformat phylip, -s dash	0:00:01
			Total elapsed time	**12:53:49**

Table S2. Summary of all steps for the Iguania-Fast analysis, which took ~1.5 hours to complete (not including user-time).

Step	Module	Input details	Flag details	Elapsed time
Parse Loci	Parse_Loci.py	69 loci to search; 8,785,378 records	--no_subspecies	0:17:24
Similarity Filtering	Cluster_Blast_Extract.py	58 loci	-b dc-megablast, --max_hits 200, -m span, --threads 4	0:18:30
	Reference_Blast_Extract.py	9 loci	-b dc-megablast, --max_hits 200, -m span, --threads 4	0:47:07
Sequence Selection	Filter_Seqs_and_Species.py	67 loci	-s oneseq, -f length, -m 200, --no_subspecies	0:00:10
	Fasta_Filter_by_Min_Seqs.py	67 loci	--min_seqs 30	0:00:01
Sequence Alignment	Adjust_Direction.py	60 loci	--threads 8	0:00:50
	Align.py	60 loci	-a mafft, --threads 8	0:04:16
Post-Alignment	Make_Acc_Table.py	60 loci		0:00:01
	Fasta_Relabel_Seqs.py	60 loci	-r species	0:00:01
	Trim_Alignments_Trimal.py	60 loci	-f fasta, -a gt, --gt 0.1	0:00:01
	Concatenation.py	60 loci, 1,399 taxa, 12,978 seqs	--informat fasta, --outformat phylip, -s dash	0:00:01
			Total elapsed time	**1:28:22**

2 | UCE Supermatrix for the Genus *Kaloula*

2.1 | Methods: *Kaloula*-Vouchered and *Kaloula*-Species Analyses

To evaluate the ability of SuperCRUNCH to handle phylogenomic datasets, we attempted to reconstruct the UCE matrix published by Alexander et al. (2017). Their matrix was composed of 14 species in the frog genus *Kaloula* and included a maximum of 1,785 loci per sample. One species (*K. conjuncta*) contained four subspecies, and three taxa were represented by multiple vouchered samples. In total, their dataset included 24 samples, but 6 samples were not identified to species (denoted with "sp." or "cf.") and were excluded from our analyses. Therefore, the maximum number of samples we targeted was 18, which represented 14 species. Given the characteristics of this phylogenomic dataset, we performed two separate analyses. For our first analysis, we aimed to construct a "vouchered" UCE supermatrix that would include all 18 samples, which we termed the *Kaloula*-Vouchered analysis (https://osf.io/crzp5/). This analysis was intended to partially reconstruct the full "vouchered" matrix used by the authors for their study. For our second analysis, we aimed to construct a species-level UCE supermatrix that would only include a single representative for each of the 14 species, which we termed the *Kaloula*-Species analysis (https://osf.io/crzp5/). In this analysis, we expected the number of loci to increase for the 3 terminal taxa represented by multiple samples (*K. baleata, K. conjuncta conjuncta, K. conjuncta negrosensis*), because sequences would be drawn from multiple samples. We downloaded sequence data from GenBank on January 16, 2019 using the search terms "*Kaloula* ultra conserved element", which resulted in a 32MB fasta file containing 38,568 records. We generated a locus search terms file from the UCE 5k probe set file (available at https://github.com/faircloth-lab/uce-probe-sets), which targeted 5,041 distinct UCE loci. This general use UCE search terms file is freely available at:

https://github.com/dportik/SuperCRUNCH/tree/master/data/locus-search-terms. We created the taxon list directly from the starting sequence set using outputs from *Fasta_Get_Taxa.py*, which resulted in 10 species names and 4 subspecies names.

For the *Kaloula*-Vouchered and the *Kaloula*-Species datasets, we conducted the same general steps for both analyses. Given the taxon list was generated from the starting sequences, we skipped the taxonomy assessment and instead began the analyses by searching for the 5,041 UCE loci in the 38,568 starting records using *Parse_Loci.py*. This produced 1,785 UCE files that each contained more than two sequences. Given that all loci were previously identified and filtered in another pipeline (PHYLUCE; Faircloth, 2016), we did not perform similarity filtering. For the *Kaloula*-Vouchered dataset, we used *Filter_Seqs_and_Species.py* to select sequences with the "allseqs", "length", and "vouchered" options, requiring a minimum length of 150 bp. For the *Kaloula*-Species dataset, we used *Filter_Seqs_and_Species.py* to select sequences with the "oneseq" and "length" options, requiring a minimum length of 150 bp. In both datasets, one locus was dropped (for which all sequences were less than 150 bp in length). Accession tables were created using *Make_Acc_Table.py* (with or without the "voucherize" option), sequence directions were adjusted using *Adjust_Direction.py*, and all 1,784 loci were aligned using MAFFT. Sequences were relabeled with *Fasta_Relabel_Seqs.py* using the "species" option (*Kaloula*-Species) or the "species" and "voucherize" options (*Kaloula*-Vouchered), and alignments were concatenated to produce the final supermatrices. In total, we ran 8 separate steps using 8 modules for both the *Kaloula*-Vouchered analysis (Table S3) and the *Kaloula*-Species analysis (Table S4). The input and output files for each step, along with complete instructions, are available on the Open Science Framework for the *Kaloula*-Vouchered analysis (https://osf.io/zxnq8/) and the *Kaloula*-Species analysis (https://osf.io/crzp5/). We investigated whether the supermatrices we constructed resulted in phylogenies concordant with those presented by Alexander et al. (2017). For each supermatrix we ran an unpartitioned RAxML analysis (Stamatakis, 2014), and used the GTRCAT model and 100 rapid bootstraps to generate support values. We compared the resulting topologies to those obtained by Alexander et al. (2017).

2.2 | Results: *Kaloula*-Vouchered and *Kaloula*-Species Analyses

The *Kaloula*-Vouchered analysis resulted in a phylogenomic supermatrix containing 1,784 loci, 18 samples, and 28,790 total sequences. The analysis took ~25 minutes to complete (not including user-time; Table S3). The number of UCE loci recovered per individual ranged from 1,276–1,664. The *Kaloula*-Species analysis resulted in a phylogenomic supermatrix containing 1,784 loci, 14 species, and 22,717 total sequences. The analysis took ~20 minutes to complete (not including user-time; Table S4). The number of UCE loci recovered per sample ranged from 1,276–1,777. As expected, the terminal taxa represented by multiple samples displayed an increase in the number of sequences recovered (*K. baleata*: from 1,649 to 1,765; *K. c. conjucta*: from 1,649 to 1,756; *K. c. negrosensis*: from 1,664 to 1,777). The *Kaloula*-Vouchered and *Kaloula*-Species analyses successfully found all 1,785 UCE loci reported by Alexander et al. (2017), but one locus was dropped due to short sequence lengths (all <150 bp). The phylogenies produced from both supermatrices are congruent with results obtained by Alexander et al. (2017). The tree files are available online for the *Kaloula*-Vouchered analysis (https://osf.io/zxnq8/) and the *Kaloula*-Species analysis (https://osf.io/crzp5/)

Table S3. Summary of all steps for the *Kaloula*-Vouchered analysis, which took ~25 minutes to complete (not including user-time).

Step	Module	Input details	Flag details	Elapsed time
Parse Loci	Parse_Loci.py	5,041 loci to search; 38,568 records		0:02:06
Sequence Selection	Filter_Seqs_and_Species.py	1,785 loci	-s allseqs, -f length, -m 150, --vouchered	0:01:13
	Make_Acc_Table.py	1,784 loci	--voucherize	0:00:02
Sequence Alignment	Adjust_Direction.py	1,784 loci	--threads 8	0:07:22
	Align.py	1,784 loci, mafft	-a mafft, --threads 8	0:14:23
Post-Alignment	Fasta_Relabel_Seqs.py	1,784 loci	-r species, -s, --voucherize	0:00:03
	Concatenation.py	1,784 loci, 18 taxa, 28,790 seqs	--informat fasta, --outformat phylip, -s dash	0:00:01
			Total elapsed time	**0:25:10**

Table S4. Summary of all steps for the *Kaloula*-Species analysis, which took ~20 minutes to complete (not including user-time).

Step	Module	Input details	Flag details	Elapsed time
Parse Loci	Parse_Loci.py	5,041 loci to search; 38,568 records		0:02:05
Sequence Selection	Filter_Seqs_and_Species.py	1,785 loci	-s oneseq, -f length, -m 150	0:00:13
	Make_Acc_Table.py	1,784 loci		0:00:02
Sequence Alignment	Adjust_Direction.py	1,784 loci	--threads 8	0:07:03
	Align.py	1,784 loci, mafft	-a mafft, --threads 8	0:10:25
Post-Alignment	Fasta_Relabel_Seqs.py	1,784 loci	-r species, -s	0:00:03
	Concatenation.py	1,784 loci, 14 taxa, 22,717 seqs	--informat fasta, --outformat phylip, -s dash	0:00:01
			Total elapsed time	**0:19:52**

3 | Phylogeographic Dataset for *Trachylepis sulcata*

3.1 | Methods: *Trachylepis*-Phylogeography and *Trachylepis*-Species Analyses

To evaluate the ability of SuperCRUNCH to reconstruct phylogeographic datasets from GenBank data, we attempted to reconstruct the dataset of Portik et al. (2011) using their published GenBank sequences. This dataset, which was partially published in Portik et al. (2010), consists of four loci sequenced for 88 samples of the lizard species complex *Trachylepis sulcata*, and several outgroups. We downloaded sequence data from GenBank on July 20, 2019 using the search term "*Trachylepis sulcata*", which resulted in 442 records (<1 MB in size). We created a locus search terms file specific to the four loci included in Portik et al. (2011): *EXPH5, KIF24, RAG1,* and *ND2*. These are a subset of the loci included in the Iguania supermatrix analyses. We obtained taxon names directly from the starting sequence set using *Fasta_Get_Taxa.py*, and used the outputs to create a taxon list that targeted the focal species (*T. sulcata*) and six outgroup species (*T. aurata*, *T. punctulata, T. varia*, *T. variegata*, *T. vittata,* and *T. wahlbergii*). To reconstruct the phylogeographic dataset of Portik et al. (2011) we ran a "vouchered" analysis (termed *Trachylepis*-Phylogeography), which would include all vouchered samples in the final alignments. For comparison, we also created a species-level supermatrix which would be composed of the seven species (termed *Trachylepis*-Species).

We conducted the same general steps for the *Trachylepis*-Phylogeography and the *Trachylepis*-Species analyses. Given the taxon list was generated from the starting sequences, we skipped the taxonomy assessment and instead began the analyses by searching for the four loci in the 442 starting records using *Parse_Loci.py*. We performed similarity filtering using *Cluster_Blast_Extract.py* for all loci. For the *Trachylepis*-Phylogeography dataset, we used *Filter_Seqs_and_Species.py* to select sequences using the "oneseq", "length", and "vouchered" options, requiring a minimum length of 200 bp. For the *Trachylepis*-Species dataset, we used *Filter_Seqs_and_Species.py* to select sequences using the "oneseq" and "length" options, requiring a minimum length of 200 bp. Accession tables were created using *Make_Acc_Table.py* (with or without the "voucherize" option), sequence directions were adjusted using *Adjust_Direction.py*, and all loci were aligned using MAFFT with *Align.py*. Sequences were relabeled with *Fasta_Relabel_Seqs.py* using the "species" option (*Trachylepis*-Species) or the "species" and "voucherize" options (*Trachylepis*-Phylogeography), file formats were converted using *Fasta_Convert.py*, and concatenation was performed using *Concatenation.py*. In total, we ran 10 separate steps using 10 modules for the *Trachylepis*-Phylogeography analysis (Table S5) and the *Trachylepis*-Species analysis (Table S6). The input and output files for each step, along with complete instructions, are available on the Open Science Framework for the *Trachylepis*-Phylogeography analysis (https://osf.io/bgc5z/) and the *Trachylepis*-Species analysis (https://osf.io/umswn/). We investigated if the phylogenies produced from each supermatrix were concordant with results presented by Portik et al. (2011). For each supermatrix we ran an unpartitioned RAxML analysis using the GTRCAT model and 100 rapid bootstraps to generate support values. We compared the resulting topologies to those obtained by Portik et al. (2011).

3.2 | Results: *Trachylepis*-Phylogeography and *Trachylepis*-Species Analyses

The *Trachylepis*-Phylogeography analysis successfully reconstructed the phylogeographic dataset of Portik et al. (2011). The analysis found sequences of the four loci (*EXPH5, KIF24, RAG1, ND2*) for all 88 vouchered samples of *Trachylepis sulcata*, which resulted in a total of 326 sequences. In addition, vouchered samples representing several outgroups from Portik et al. (2010) and Portik et al. (2011) were also recovered, including *T. aurata* (2 individuals), *T.*

punctulata (3), *T. varia* (6), *T. variegata* (5), *T. vittata* (2), and *T. wahlbergii* (2). This resulted in an additional 74 sequences, and the final concatenated alignment of all four loci (e.g., the phylogeographic supermatrix) contained a total of 108 vouchered samples and 400 sequences. The *Trachylepis*-Phylogeography analysis took 37 seconds to complete (not including user-time; Table S5). The *Trachylepis*-Species analysis was run as a comparison to the *Trachylepis*-Phylogeography analysis. It was used to select one representative sequence per species per locus, for the purpose of creating a species-level matrix from these population-level data. The *Trachylepis*-Species analysis resulted in a matrix containing 7 species (*T. aurata, T. punctulata*, *T. sulcata*, *T. varia*, *T. variegata*, *T. vittata*, and *T. wahlbergii*), four loci, and a total of 26 sequences. The *Trachylepis*-Species analysis took 21 seconds to complete (not including user-time; Table S6). The phylogenies produced from the phylogeographic matrix and the species-level matrix were congruent with results presented by Portik et al. (2010) and Portik et al. (2011). The tree files are available online for the *Trachylepis*-Phylogeography analysis (https://osf.io/bgc5z/) and the *Trachylepis*-Species analysis (https://osf.io/umswn/).

Table S5. Summary of all steps for the *Trachylepis*-Phylogeography analysis, which took <1 minute to complete (not including user-time).

Step	Module	Input details	Flag details	Elapsed time
Parse Loci	Parse_Loci.py	4 loci to search; 442 records		0:00:01
Similarity Filtering	Cluster_Blast_Extract.py	4 loci	-b dc-megablast, -m span, --threads 4	0:00:13
Sequence Selection	Filter_Seqs_and_Species.py	4 loci	-s allseqs, -f length, -m 200, --vouchered	0:00:01
	Make_Acc_Table.py	4 loci	--voucherize	0:00:01
Sequence Alignment	Adjust_Direction.py	4 loci	--threads 8	0:00:02
	Align.py	4 loci	-a mafft, --threads 8	0:00:16
Post-Alignment	Fasta_Relabel_Seqs.py	4 loci	-r species, --voucherize	0:00:01
	Fasta_Convert.py	4 loci		0:00:01
	Concatenation.py	4 loci, 108 taxa, 400 seqs	--informat fasta, --outformat phylip, -s dash	0:00:01
			Total elapsed time	**0:00:37**

Table S6. Summary of all steps for the *Trachylepis*-Species analysis, which took <30 seconds to complete (not including user-time).

Step	Module	Input details	Flag details	Elapsed time
Parse Loci	Parse_Loci.py	4 loci to search; 442 records		0:00:01
Similarity Filtering	Cluster_Blast_Extract.py	4 loci	-b dc-megablast, -m span, --threads 4	0:00:13
Sequence Selection	Filter_Seqs_and_Species.py	4 loci	-s oneseq, -f length, -m 200	0:00:01
	Make_Acc_Table.py	4 loci		0:00:01
Sequence Alignment	Adjust_Direction.py	4 loci	--threads 8	0:00:01
	Align.py	4 loci	-a mafft, --threads 8	0:00:01
Post-Alignment	Fasta_Relabel_Seqs.py	4 loci	-r species	0:00:01
	Fasta_Convert.py	4 loci		0:00:01
	Concatenation.py	4 loci, 7 taxa, 26 seqs	--informat fasta, --outformat phylip, -s dash	0:00:01
			Total elapsed time	**0:00:21**

4 | Population Datasets for *Callisaurus* and *Uma*

4.1 | Methods: *Callisaurus*-Population and *Uma*-Population Analyses

We used SuperCRUNCH to generate new combinations of population-level datasets from published sequences. We created independent population-level datasets for the lizard genera *Callisaurus* and *Uma* (family Phrynosomatidae). These were chosen because we knew in advance that some phylogeographic data were present across multiple studies (including Lindell et al., 2005; Schulte & de Queiroz, 2008; Gottscho et al., 2017). However, we did not know which loci would be most strongly represented. We therefore used SuperCRUNCH to survey the availability of sequences. We downloaded sequence data from GenBank on January 25, 2019 using the search term "Phrynosomatidae", which resulted in a 52MB fasta file containing 82,557 records. We obtained a taxon list directly from the fasta file, which was pruned to contents of each respective genus for separate searches. For *Callisaurus*, this included 11 taxon names (*Callisaurus draconoides*, *Callisaurus d. bogerti*, *Callisaurus d. brevipes, Callisaurus d. carmenensis, Callisaurus d. crinitus, Callisaurus d. draconoides, Callisaurus d. inusitanus, Callisaurus d. myurus, Callisaurus d. rhodostictus, Callisaurus d. splendidus, Callisaurus d. ventralis*). For *Uma*, this included 10 taxon names (*Uma exsul, Uma inornata*, *Uma notata, Uma n. cowlesi, Uma n. notata, Uma n. rufopunctata, Uma paraphygas, Uma rufopunctata, Uma scoparia, Uma s. scoparia*). We recognize that some of the taxon names are synonyms, but we included all names (without corrections) so as to find all available sequences and to obtain counts of sequences for each respective name. We used the same set of 69 locus search terms from our Iguania analysis to perform searches.

We conducted the same general steps for the *Callisaurus*-Population and *Uma*-Population analyses. Given the taxon list was generated from the starting sequences, we skipped the taxonomy assessment and instead began each analysis by searching for the 69 loci in the 82,557 starting records using *Parse_Loci.py*. This resulted in the recovery of 50 loci for *Callisaurus* and 52 loci for *Uma*. We performed similarity filtering using *Cluster_Blast_Extract.py* for all loci. We used *Filter_Seqs_and_Species.py* to select sequences using the "allseqs" and "length" options (but importantly not the "voucher" option), requiring a minimum length of 200 bp. We used *Fasta_Filter_by_Min_Seqs.py* to remove alignments with fewer than 8 sequences, which resulted in the retention of 7 loci for *Callisaurus* and 5 loci for *Uma*. Sequence directions were adjusted using *Adjust_Direction.py*, and all loci were aligned using MAFFT in *Align.py*. Sequences were relabeled with *Fasta_Relabel_Seqs.py* using the "species_acc" option (which is a combination of the taxon name and accession number), and file formats were converted using *Fasta_Convert.py*. In total, we ran 8 separate steps using 8 modules for both the *Callisaurus*-Population analysis (Table S7) and the *Uma*-Population analysis (Table S8). The input and output files for each step, along with complete instructions, are available for the *Callisaurus*-Population analysis at https://osf.io/7gujb/, and for the *Uma*-Population analysis at https://osf.io/e28tu/. For all genes, we ran separate unpartitioned RAxML analyses. We used the GTRCAT model, and performed 100 rapid bootstraps to generate support values for the gene trees.

4.2 | Results: *Callisaurus*-Population and *Uma*-Population Analyses

We used SuperCRUNCH to generate new combinations of published sequences, with the intention of finding loci suitable for population-level analyses. We created a population-level dataset for the lizard genus *Callisaurus* and a separate one for the genus *Uma*.

For the *Callisaurus*-Population analysis, we found 7 loci that contained eight or more sequences, including five mitochondrial genes (*12S*, *n*=8; *CO1*, *n*=8; *CYTB*, *n*=93; *ND1*, *n*=8; *ND2*, *n*=8) and two nuclear loci (*MC1R*, *n*=70; *RAG1*, *n*=70). Among these seven genes, three contained the most sequences (*CYTB*, *MC1R*, *RAG1*), each with 70 or more. Across all genes, there were 10 taxa represented (*C. draconoides, C. d. bogerti, C. d. brevipes, C. d. carmenensis, C. d. crinitus, C. d. draconoides, C. d. inusitanus, C. d. myurus, C. d. rhodostictus, C. d. splendidud, C. d. ventralis*). The *Callisaurus* analysis took 35 seconds to complete (not including user-time; Table S7).

For the *Uma*-Population analysis, we found 5 genes that contained eight or more sequences, which were all mitochondrial genes (*12S*, *n*=8; *CO1*, *n*=19; *CYTB*, *n*=191; *ND1*, *n*=8; *ND2*, *n*=8). Among these genes, *CYTB* contained the greatest number of sequences (*n*=191) by a considerable margin. Across all loci, there were 10 taxa found (*U. exsul, U. inornata, U. notata, U. n. cowlesi, U. n. notata, U. n. rufopunctata, U. paraphygas, U. rufopunctata, U. scoparia, U. s. scoparia*). The *Uma* analysis took 31 seconds to complete (not including user-time; Table S8).

Because sequences in these datasets were not necessarily from vouchered samples, the sequences were labeled using the species name and GenBank accession number. This allowed every sequence within an alignment to have a unique name, but as a result concatenation was not possible. For these datasets, we created a gene tree from each alignment. The individual gene trees produced from the 7 loci for *Callisaurus* and the 5 loci for *Uma* were congruent with phylogenetic results presented by Schulte & de Queiroz (2008), Lindell, Méndez-de la Cruz, and Murphy (2005), and Gottscho et al. (2017). All gene tree files are available online for the *Callisaurus*-Population analysis (https://osf.io/7gujb/) and the *Uma*-Population analysis (https://osf.io/e28tu/).

Table S7. Summary of all steps for the *Callisaurus*-Population analysis, which took under a minute to complete (not including user-time).

Step	Module	Input details	Flag details	Elapsed time
Parse Loci	Parse_Loci.py	69 loci to search; 82,557 records		0:00:05
Similarity Filtering	Cluster_Blast_Extract.py	50 loci	-b dc-megablast, -m span, --threads 4	0:00:09
Sequence Selection	Fasta_Filter_by_Min_Seqs.py	46 loci	--min_seqs 8	0:00:01
	Filter_Seqs_and_Species.py	7 loci	-s allseqs, -f length -m 200	0:00:01
Sequence Alignment	Adjust_Direction.py	7 loci	--threads 8	0:00:02
	Align.py	7 loci, mafft	-a mafft, --threads 8	0:00:14
Post-Alignment	Fasta_Relabel_Seqs.py	7 loci	-r species_acc, -s	0:00:01
	Fasta_Convert.py	7 loci		0:00:01
			Total elapsed time	**0:00:35**

Table S8. Summary of all steps for the *Uma*-Population analysis, which took under a minute to complete (not including user-time).

Step	Module	Input details	Flag details	Elapsed time
Parse Loci	Parse_Loci.py	69 loci to search; 82,557 records		0:00:06
Similarity Filtering	Cluster_Blast_Extract.py	52 loci	-b dc-megablast, -m span, --threads 4	0:00:17
Sequence Selection	Fasta_Filter_by_Min_Seqs.py	45 loci	--min_seqs 8	0:00:01
	Filter_Seqs_and_Species.py	5 loci	-s allseqs, -f length -m 200	0:00:01
Sequence Alignment	Adjust_Direction.py	5 loci	--threads 8	0:00:01
	Align.py	5 loci, mafft	-a mafft, --threads 8	0:00:03
Post-Alignment	Fasta_Relabel_Seqs.py	5 loci	-r species_acc, -s	0:00:01
	Fasta_Convert.py	5 loci		0:00:01
			Total elapsed time	**0:00:31**

5 | Adding Outgroups to an Unpublished Supermatrix Project: Family Hyperoliidae

5.1 | Methods: Hyperoliid-Outgroup Analysis

We used SuperCRUNCH to perform a common but sometimes exceedingly difficult task in phylogenetics: adding published outgroup sequences to an unpublished sequencing project. The unpublished sequences were generated as part of Portik et al. (2019), which focused on the systematics of hyperoliid frogs (family Hyperoliidae). These sequences are now available on GenBank (MK497946–MK499204; MK509481–MK509743), but for this demonstration we used the unpublished version of these sequences. This local dataset consisted of six loci sequenced for ~128 species, but many species were represented by multiple vouchered samples. It contains a total of 266 samples. The fasta file for the unpublished dataset contained 1,522 records (1.3MB), and the records were labeled according to the conventions described in the online documentation (https://github.com/dportik/SuperCRUNCH/wiki/2:-Starting-Sequences#ULS). For this analysis, we wanted to add all available GenBank sequence data for the family Arthroleptidae, which is the sister family of Hyperoliidae (the ingroup). We used the search term “Arthroleptidae” to download all available data from GenBank on October 25, 2019, which resulted in a fasta file containing 2,977 records (3MB). For the local sequences, we wanted to treat all samples as distinct (equivalent to a vouchered analysis), whereas for the outgroups we simply wanted to include all possible data for a given species (equivalent to a species-level analysis, in which sequences for a species most likely come from different samples). Within the local sequences, we intentionally labeled the records such that the species names was followed immediately by a museum/field identifier, which allowed us to take advantage of the flexible “subspecies” option in SuperCRUNCH. The subspecies option allows any three-part name to be used, and the third part of a name can contain either a subspecies label or any alphanumerical identifier. We therefore used *Fasta_Get_Taxa.py* with the "numerical" option to obtain a list of "subspecies" from the local sequences that actually contained the museum/field codes instead of a subspecies label (e.g., *Genus species identifier* as opposed to *Genus species subspecies*), and treated these as "subspecies" throughout the analysis. We independently used *Fasta_Get_Taxa.py* to obtain all taxon names in the arthroleptid outgroup sequences, and created a taxon list composed only of species labels (e.g., no subspecies included). The species labels of the arthroleptids (the outgroup) were combined with the "subspecies" labels of the hyperoliids (the ingroup) to create a combined taxon list. We merged the fasta files of the hyperoliid sequences and the arthroleptid sequences to create a single fasta file of sequences. We constructed locus search terms for the 6 loci, which included one mitochondrial gene (*16S*) and five nuclear loci (*FICD*, *KIAA2013*, *POMC*, *RAG1*, *TYR*). We used these materials to create a custom supermatrix with SuperCRUNCH, which we termed the Hyperoliid-Outgroup analysis.

We wanted to ensure that the sequences obtained for the outgroups closely matched the regions for each gene present in our hyperoliid (local) sequence data. To accomplish this, we used our local sequences as references during similarity filtering. In order to create the six necessary reference files (a locus-specific fasta file composed of only hyperoliid sequences), we ran *Parse_Loci.py* using the hyperoliid fasta file. We then ran *Parse_Loci.py* on the combined hyperoliid and arthroleptid fasta file (containing GenBank and local sequences) to obtain all sequences for each locus, and subsequently ran *Reference_Blast_Extract.py* for each of the six loci using the corresponding hyperoliid reference set. We used *Filter_Seqs_and_Species.py* to select sequences with the "oneseq" and "length" options, requiring a minimum length of 200 bp. Sequence directions were adjusted using *Adjust_Direction.py*, and all loci were aligned with

MAFFT using *Align.py*. Sequences were relabeled with *Fasta_Relabel_Seqs.py* using the "species" option with the "subspecies" feature (allowing the hyperoliid sequences to be labeled as *Genus species identifier*). After relabeling, concatenation was performed using *Concatenation.py*. In total, we ran 8 separate steps using 7 modules for the Hyperoliid-Outgroup analysis (Table S9). The input and output files for each step, along with complete instructions, are available at: https://osf.io/q9nyx/. We ran an unpartitioned RAxML analysis on the final supermatrix using the GTRCAT model and 100 rapid bootstraps.

5.2 | Results: Hyperoliid-Outgroup Analysis

The Hyperoliid-Outgroup analysis successfully incorporated GenBank sequences (Arthroleptidae) and locally generated sequences (Hyperoliidae) to produce a supermatrix that contained 6 loci, 365 terminals, and 1,724 sequences. The hyperoliid sequences included multiple vouchered samples for many taxa, and the subspecies feature in SuperCRUNCH was used to include museum/field identifiers as the "subspecies" component in their taxon names. This strategy allowed us to successfully include all 266 samples (rather than selecting representative sequences for each of the 128 species). In contrast, for the arthroleptids we simply wanted to obtain the most complete data possible per species (linking vouchers was not relevant). SuperCRUNCH allowed us to target species-level sampling for the outgroup, but population-level sampling for the ingroup. We produced a dataset containing 1,509 sequences and 266 samples for hyperoliids (local), and containing 225 sequences and 99 species for arthroleptids (GenBank). The Hyperoliid-Outgroup analysis took 3 minutes and 45 seconds to complete (not including user-time; Table S9). The phylogeny produced from the Hyperoliid-

Outgroup supermatrix is congruent with that estimated by Portik et al. (2019). The tree file is available online (https://osf.io/q9nyx/).

Table S9. Summary of all steps for the Hyperoliid-Outgroup analysis, which took ~4 minutes to complete (not including user-time).

Step	Module	Input Details	Flag Information	Elapsed time
Parse Loci	Parse_Loci.py	6 loci, 4,499 records	(for combined dataset)	0:00:01
	Parse_Loci.py	6 loci, 1,522 records	(for hyperoliid sequences only)	0:00:01
Similarity Filtering	Reference_Blast_Extract.py	6 loci	-b dc-megablast, --max_hits 200, -m span, --threads 4	0:03:38
Sequence Selection	Filter_Seqs_and_Species.py	6 loci	-s oneseq, -f length, -m 200	0:00:01
Sequence Alignment	Adjust_Direction.py	6 loci	--threads 8	0:00:01
	Align.py	6 loci	-a mafft, --threads 8	0:00:01
Post-Alignment	Fasta_Relabel_Seqs.py	6 loci	-r species, -s	0:00:01
	Concatenation.py	6 loci	--informat fasta, --outformat phylip, -s dash	0:00:01
			Total elapsed time	**0:03:45**

6 | SuperCRUNCH Comparison to PyPHLAWD

6.1 | Methods – PyPHLAWD Comparison

We compared the ability of SuperCRUNCH to construct species-level supermatrices, relative to PyPHLAWD (Smith & Walker, 2018), using two test clades (Iguania and Dipsacales). Importantly, we focused on PyPHLAWD given that Smith & Walker (2018) showed that PyPHLAWD outperformed other methods for supermatrix construction (specifically phyloGenerator, PhyLoTA, and SUPERSMART). Therefore, if our method outperforms PyPHLAWD, then it should represent the state-of-the-art for supermatrix construction. The performance criteria used by Smith & Walker (2018) was the number of taxa and sequences retrieved by each method. We used these criteria as well, but we also considered whether the clades recovered by each method were consistent with current taxonomy. This latter criterion should help detect whether the supermatrices generated by each method tend to yield problematic phylogenetic results. These problematic results will not be apparent simply from the number of taxa and sequences retrieved by each method.

PyPHLAWD retrieves sequences by interfacing directly with a GenBank database release. It was initially designed to produce orthologous sequence clusters using "all-by-all" clustering methods, but it also offers an option to target specific loci by incorporating sets of user-supplied reference sequences (referred to as a "baited" analysis). To search for the same set of 69 loci for Iguania, we performed a "baited" analysis in PyPHLAWD ("v1.0", Aug 20, 2018 release) with baits consisting of sequences used for the Iguania-Thorough analysis (e.g., mtDNA references) or obtained from them (e.g., the nuclear loci recovered). This PyPHLAWD analysis (termed Iguania-PyPHLAWD) was run using the default settings in the configuration file. All output files for the Iguania-PyPHLAWD analysis are provided at: https://osf.io/vyxj4/.

PyPHLAWD does not offer an option to exclude subspecies, and the taxonomy used relies on the NCBI Taxonomy database. As a result, the taxonomy for the PyPHLAWD analysis was incompatible with the taxonomy we obtained from the Reptile Database (Uetz et al., 2018). Therefore, after the analysis we “corrected” the taxonomy by updating names and removing all subspecies in the alignments. As the steps for performing concatenation in PyPHLAWD were unclear, we concatenated the updated alignments using the *Concatenation.py* module in SuperCRUNCH to produce a final supermatrix.

Although we had already run two species-level supermatrix analyses for Iguania using SuperCRUNCH (Iguania-Thorough, Iguania-Fast), these analyses used sequences downloaded from GenBank directly. For these analyses, it would not be possible to determine if differences in the resulting supermatrices (relative to PyPHLAWD) were due to differences in methodology or differences in the starting sequences used. To allow a direct comparison to PyPHLAWD, we used the Iguania sequence set fetched directly by PyPHLAWD (134,028 records) to perform an additional SuperCRUNCH analysis (termed Iguania-Constrained). For this analysis, we performed an initial taxon assessment of the starting sequences and relabeled records using updated names. Locus parsing, similarity filtering, sequence selection, direction adjustment, and alignment used the same options and settings as the Iguania-Fast analysis. We chose the Iguania-Fast settings because several of these steps resembled options in PyPHLAWD, including sequence selection by length, and alignment with MAFFT. We did not remove any loci based on a minimum requirement for the number of sequences (<30) to allow better comparison to PyPHLAWD. Alignments were relabeled, trimmed, and concatenated following the steps outlined in the Iguania-Thorough analysis. In total, we ran 12 separate steps using 12 modules for the Iguania-Constrained analysis (Table S10). The input and output files for each step, along with complete instructions, are available at: https://osf.io/za2ug/.

We further compared the ability of SuperCRUNCH and PyPHLAWD to construct supermatrices by performing additional analyses using the plant clade Dipsacales. This was the example dataset used by Smith and Walker (2018). Following Smith and Walker (2018), we searched for the same four loci (ITS, matK, rbcl, trnL-trnF) using a "baited" analysis in PyPHLAWD ("v1.0", Aug 20, 2018 release) using their provided bait sets. This PyPHLAWD analysis (termed Dipsacales-PyPHLAWD) was run using the default settings in the configuration file. All output files for the Dipsacales-PyPHLAWD analysis are provided at: https://osf.io/7jqe4/.

We performed a SuperCRUNCH analysis using the Dipsacales sequence set fetched by PyPHLAWD using the GenBank release database (12,348 records), which we termed Dipsacales-Constrained. The taxonomy table produced by PyPHLAWD was used to create a taxon list for this analysis, which included subspecies. The taxonomy table also contained the original description lines for the downloaded sequences, which we examined to create locus search terms for the four loci. We searched for sequences using *Parse_Loci.py*. We performed similarity filtering for each of the four loci using *Reference_Blast_Extract.py*, using the corresponding bait set as the reference sequences. We selected representative sequences per taxon for all loci using the "onseq" and "length" options in *Filter_Seqs_and_Species.py*, requiring a minimum length of 200 bp. We performed sequence direction adjustments for the remaining 60 loci using *Adjust_Direction.py*, and performed multiple sequence alignment using MAFFT in *Align.py*. We constructed a table of GenBank accession numbers for all filtered sequences using the *Make_Acc_Table.py* module. We relabeled sequences in all alignment files using the "species" option and "subspecies" feature in *Fasta_Relabel_Seqs.py*, and subsequently trimmed alignments using the gap-threshold option in trimAl with a threshold value of 0.1, as implemented in *Trim_Alignments_Trimal.py*. We skipped format conversion and concatenated the alignments to produce the final supermatrix. In total, we ran 9 separate steps using 9 modules for the Dipsacales-Constrained analysis (Table S11). The input and output files for each step, along with complete instructions, are available at: https://osf.io/937yu/.

We constructed a total of four supermatrices for Iguania (Iguania-Thorough, Iguania-Fast, Iguania-Constrained, Iguania-PyPHLAWD), and two supermatrices for Dipsacales (Dipsacales-PyPHLAWD, Dipsacales-Comparison). We summarized differences in the content of the supermatrices, including the total number of taxa, loci, and sequences. In addition, we evaluated differences in the resulting phylogenetic trees from these supermatrices. For each supermatrix we ran an unpartitioned RAxML analysis, and used the GTRCAT model and 100 rapid bootstraps to generate a phylogeny with support values. We evaluated the number of genera, subfamilies, and families recovered as monophyletic in the four different Iguania trees, and the number of monophyletic genera in the two different Dipsacales trees. All tree files are available at: https://osf.io/vgwu3/.

6.2 | Results – PyPHLAWD Comparison

6.2.1 | Supermatrix Results

SuperCRUNCH generally outperformed PyPHLAWD in all comparisons and resulted in higher numbers of sequences and taxa for the Iguania and Dipsacales datasets (Tables S12–S14). The analysis of Dipsacales was smaller in scope. SuperCRUNCH obtained better results, but the supermatrices generated by each method were generally similar (versus iguanians, see below).

For Dipsacales, we successfully replicated the results of Smith and Walker (2018) in terms of the number of species recovered. The Dipsacales-PyPHLAWD analysis resulted in a supermatrix containing 4 loci, 641 taxa, and 1,510 total sequences (Table S14), and took 1 minute and 16 seconds to complete. The Dipsacales-Constrained analysis in SuperCRUNCH resulted in a supermatrix containing 4 loci, 651 taxa, and 1,589 total sequences, and took 1 minute and 14 seconds to complete (not including user-time; Table S11). The Dipsacales-Constrained analysis recovered more sequences for 3 out of the 4 loci, and both analyses recovered an equal number of sequences for 1 locus (Table S14).

The Iguania-PyPHLAWD analysis resulted in a supermatrix containing 66 loci, 1,069 species, and 10,397 total sequences (Table S13), and took 18 minutes to complete. The Iguania-Constrained analysis in SuperCRUNCH generated a supermatrix containing 67 loci, 1,359 taxa, and 12,676 total sequences (Table S13). The analysis took 1 hour and 8 minutes to complete (not including user-time; Table S10). Among the 66 loci shared between the two analyses, the Iguania-PyPHLAWD analysis recovered more sequences for 5 loci (7%), the Iguania-Constrained analysis recovered more sequences for 19 loci (29%), and both analyses recovered equal numbers of sequences for 43 loci (64%; Table S12). These two analyses relied on starting sequences obtained through a GenBank database release. They were outperformed by all other SuperCRUNCH analyses of Iguania that used data downloaded from GenBank directly by us (including Iguania-Thorough and Iguania-Fast; Table S13). In particular, the Iguania-Thorough analysis in SuperCRUNCH vastly outperformed the Iguania-PyPHLAWD analysis. It recovered more species (1,426 vs. 1,069) and total sequences (13,307 vs. 10,397) despite having fewer loci in the final supermatrix (it initially found 67 loci but discarded 6 because of the minimum sequence filter).

A per-locus comparison for Iguania revealed the largest difference in performance between the PyPHLAWD analysis and any of the three SuperCRUNCH analyses was for loci containing "complex" records (those consisting of multiple loci or non-overlapping regions). These included the mitochondrial genes, as well as *RAG1* (Table S12). Given the same set of starting sequences, PyPHLAWD found only 37% of the total mtDNA sequences recovered by SuperCRUNCH in the Iguania-Constrained analysis, and recovered only four of the seven mitochondrial genes with reasonable success (>50 sequences).

The likely source of this issue is the close match between baits and sequences required by PyPHLAWD, with close matches required in both length and divergence (S. Smith, personal communication). PyPHLAWD does not automatically trim query sequences after similarity searches. Rather, it passes or fails an entire sequence based on set threshold values (such as percent identity and minimum or maximum length). In the case of "complex" mtDNA records, the entire multigene sequence would pass or fail, rather than being trimmed to the target region. It may have been possible for us to obtain better results by changing these default settings. However, given this design and the sequence length heterogeneity present in "complex" records, it is unclear if there is an optimal setting that would allow all target sequences to be found for these types of loci. Inspection of the similarity searching outputs from SuperCRUNCH confirms this idea (i.e., outputs containing the starting lengths, BLAST coordinates, and trimmed lengths of input sequences). In the case of *CO1* (for which no sequences were recovered with PyPHLAWD), we used several reference sequences spanning the entire length of the *CO1* gene (~1,500 bp) as the "baits". All input sequences containing *CO1* fell into two categories: (1) they were purely on target but <700 bp in length, or (2) they were "complex" records in which *CO1* represented less than 50% of the length of the total sequence. Both of these scenarios appeared to cause severe problems for the similarity searches in PyPHLAWD (particularly the matched length requirement between bait and sequence), and the default settings caused both categories of sequences to fail this filter. Although we might have obtained better results for one category of sequences for *CO1* by using different settings, this would have driven losses for the other category of sequences. Given that the characteristics of the input sequences are generally unknown (e.g., "simple" vs. "complex", length heterogeneity), this makes finding appropriate "baits" and defining appropriate settings extremely challenging. Thus, significant losses of data for some loci are expected to occur with PyPHLAWD, as we observe here.

The results for the nuclear loci (e.g., "simple" records) were more similar between methods, and both methods obtained the same number of sequences for 43 loci. In some cases, PyPHLAWD obtained more sequences for nuclear loci. Two of these cases revealed a limitation of the label-searching method of SuperCRUNCH, as synonomous gene names for the two loci (R35 vs. GPR14, ZEB2 vs. ZHFX1b) resulted in the loss of actual homologous sequences by SuperCRUNCH. However, we also identified at least one case in which PyPHLAWD included paralogous sequence data (ENC1 contained ENC6 sequences), which artificially inflated the total number of sequences for that locus. We did not observe these paralogous sequences (of ENC6) in the ENC1 alignment created using SuperCRUNCH. Instead, these paralogous sequences were eliminated because they did not contain the gene abbreviation or description terms for ENC1.

6.2.2 | Phylogenetic Results

We compared phylogenetic trees estimated from the supermatrices for the four analyses of Iguania, and the two analyses of Dipsacales. For each iguanian phylogeny, we evaluated the number of genera, subfamilies, and families recovered as monophyletic. The Iguania-Thorough, Iguania-Fast, and Iguania-Constrained analyses recovered all 14 families as monophyletic with high support, whereas Iguania-PyPHLAWD only recovered 13 of 14 families as monophyletic. The PyPHLAWD analysis failed to support the monophyly of Agamidae (i.e., Chamaeleonidae is nested inside Agamidae), even though agamid monophyly has been strongly supported in previous supermatrix analyses (Pyron et al. 2013; Zheng & Wiens, 2016) and phylogenomic analyses (Streicher, Schulte, & Wiens 2016). All four Iguania analyses recovered all 10 subfamilies as monophyletic with high support (all >95%). Comparisons of genera were more complex, as the trees each contained a different number of species and genera. The Iguania-Thorough phylogeny contained 111 genera, including 68 monophyletic genera, 16 non-monophyletic genera, and 27 genera represented by a single species. The Iguania-Fast phylogeny contained 108 genera, including 65 monophyletic genera, 15 non-monophyletic genera, and 28 genera represented by a single species. The Iguania-Constrained phylogeny contained 106 genera, including 64 monophyletic genera, 13 non-monophyletic genera, and 29 genera represented by a single species. The Iguania-PyPHLAWD phylogeny contained 94 genera, including 58 monophyletic genera, 12 non-monophyletic genera, and 24 genera represented by a single species. Although PyPHLAWD and SuperCRUNCH produced somewhat similar scores for these metrics, the Iguania-Thorough phylogeny contained an additional 357 taxa and 17 genera not present in the PyPHLAWD phylogeny. The phylogenies and corresponding assessments of the groupings present are provided at: https://osf.io/zxhby/.

For the plant family Dipsacales, we only compared the monophyly of genera. In this regard, the Dipascales phylogeny produced from the SuperCRUNCH supermatrix was also higher quality than the phylogeny from the PyPHLAWD supermatrix. These trees contained different numbers of species, but the same number of genera. The Dipsacales-Constrained phylogeny from SuperCRUNCH contained 42 genera, including 24 monophyletic genera, 9 non-monophyletic genera, and 9 genera represented by a single species. The Dipsacales-PyPHLAWD phylogeny contained 42 genera, including 20 monophyletic genera, 11 non-monophyletic genera, and 11 genera represented by a single species. The phylogenies and corresponding assessments of the groupings present are provided at: https://osf.io/zxhby/.

Table S10. Summary of all steps for the Iguania-Constrained analysis, which took ~1 hour to complete (not including user-time).

Step	Module	Input Details	Flag Information	Elapsed time
Assess Taxonomy	Taxa_Assessment.py	134,028 records	--no_subspecies	0:00:10
	Rename_Merge.py	Relabeled 430 records		0:00:01
Parse Loci	Parse_Loci.py	69 loci to search, 133,962 records	--no_subspecies	0:00:25
Similarity Filtering	Cluster_Blast_Extract.py	58 loci	-b dc-megablast, --max_hits 200, -m span, --threads 4	0:18:04
	Reference_Blast_Extract.py	9 loci	-b dc-megablast, --max_hits 200, -m span, --threads 4	0:44:41
Sequence Selection	Filter_Seqs_and_Species.py	67 loci	-s oneseq, -f length, -m 200, --no_subspecies	0:00:10
Sequence Alignment	Adjust_Direction.py	67 loci	--threads 8	0:00:56
	Align.py	67 loci	-a mafft, --threads 4	0:04:27
Post-Alignment	Make_Acc_Table.py	67 loci		0:00:01
	Fasta_Relabel_Seqs.py	67 loci	-r species	0:00:01
	Trim_Alignments_Trimal.py	67 loci	-f fasta, -a gt	0:00:01
	Concatenation.py	67 loci, 1,359 taxa, 12,676 seqs	--informat fasta, --outformat phylip, -s dash	0:00:01
			Total elapsed time	**1:08:58**

Table S11. Summary of all steps for the Dipsacales-Constrained analysis, which took ~1 minute to complete (not including user-time).

Step	Module	Input Details	Flag Information	Elapsed time
Parse Loci	Parse_Loci.py	4 loci to search, 12,348 records		0:00:01
Similarity Filtering	Reference_Blast_Extract.py	4 loci	-b dc-megablast, -m span, --threads 4	0:00:26
Sequence Selection	Filter_Seqs_and_Species.py	4 loci	-s oneseq, -f length, -m 200	0:00:01
Sequence Alignment	Adjust_Direction.py	4 loci	--threads 8	0:00:07
	Align.py	4 loci	-a mafft, --accurate, --threads 8	0:00:35
Post-Alignment	Make_Acc_Table.py	4 loci	-s	0:00:01
	Fasta_Relabel_Seqs.py	4 loci	-r species, -s	0:00:01
	Trim_Alignments_Trimal.py	4 loci	-f fasta, -a gt, --gt 0.1	0:00:01
	Concatenation.py	4 loci, 651 taxa, 1,589 seqs	--informat fasta, --outformat phylip, -s dash	0:00:01
			Total elapsed time	**0:01:14**

Table S12. The total number of sequences for each locus in the supermatrix produced by each analysis for the clade Iguania.

Locus Type	Locus Name	Iguania-PyPHLAWD	Iguania-Constrained	Iguania-Fast	Iguania-Thorough
mtDNA	12S	325	489	499	530
	16S	9	544	560	580
	CO1	-	353	482	481
	CYTB	348	494	508	516
	ND1	109	203	208	209
	ND2	523	928	945	1006
	ND4	1	504	540	552
Nuclear	ADNP	142	142	142	145
	AHR	133	133	133	137
	AKAP9	177	180	180	184
	AMEL	10	13	<30	<30
	BACH1	183	183	183	187
	BACH2	29	30	30	31
	BDNF	375	379	380	394
	BHLHB2	146	146	146	149
	BMP2	140	140	140	143
	CAND1	145	145	145	149
	CARD4	139	139	139	141
	CILP	143	143	143	145
	CMOS	443	439	447	463
	CXCR4	143	143	143	145
	DLL1	140	140	140	140
	DNAH3	137	137	137	141
	ECEL1	150	150	150	150
	ENC1	94	41	41	45
	EXPH5	148	148	148	149
	FSHR	145	145	145	148
	FSTL5	141	141	141	144
	GALR1	137	137	137	138
	GHSR	132	132	132	132
	GPR37	140	140	140	144
	HLCS	137	137	137	139
	INHIBA	140	140	141	145
	KIAA1217	-	-	-	-
	KIAA1549	-	-	-	-
	KIAA2018	15	15	<30	<30
	KIF24	139	139	139	139
	LRRN1	132	132	132	132
	LZTS1	122	122	122	122

MC1R	40	40	40	40
MKL1	114	114	151	153
MLL3	123	123	123	123
MSH6	163	164	164	168
MXRA5	104	104	107	107
MYH2	-	-	-	-
NGFB	169	169	169	173
NKTR	215	221	221	227
NOS1	9	9	<30	<30
NT3	350	355	365	368
PDC	19	19	<30	<30
PNN	269	269	274	277
PRLR	420	420	438	440
PTGER4	147	147	147	149
PTPN	114	114	115	115
R35	300	288	299	303
RAG1p1	344	429	439	455
RAG1p2	290	361	390	397
RAG2	-	2	<30	<30
REV3L	28	29	<30	30
RHO	38	13	58	58
SLC30A1	144	144	144	147
SLC8A1	144	144	144	148
SLC8A3	143	143	143	146
SNCAIP	249	249	249	252
SOCS5	17	17	<30	152
TRAF6	149	149	149	151
UBN1	147	148	148	151
VCPIP	131	131	131	133
ZEB2	164	154	154	157
ZFP36L1	141	141	141	143

Table S13. The total number of loci, taxa, sequences, and the concatenated alignment length produced by each analysis for the clade Iguania.

Analysis	Loci	Species	Genera	Sequences	Alignment length
Iguania-PyPHLAWD	66	1,069	94	10,397	66,100 bp
Iguania-Constrained	67	1,359	106	12,676	58,315 bp
Iguania-Fast	60*	1,399	108	12,978	52,827 bp
Iguania-Thorough	61*	1,426	111	13,307	53,319 bp

*Note that an additional filter that required a minimum of 30 taxa per locus removed several loci for Iguania-Fast (*n*=7) and Iguania-Thorough (*n*=6).

Table S14. The total number of sequences for each locus in the supermatrix produced by each analysis for the clade Dipsacales.

Locus	Dipsacales-PyPHLAWD	Dipsacales-Constrained
ITS	556	583
matK	322	344
rbcl	299	299
trnL-trnF	333	363

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