

1 Dense Sampling of Taxa and Genomes Untangles the Phylogenetic Backbone of a Non-  
2 model Plant Lineage Rife with Deep Hybridization and Allopolyploidy

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27        **Abstract:**

28        Phylogenetic networks, rather than purely bifurcating trees, more accurately depict the  
29        intricate evolutionary dynamics of most lineages, especially those characterized by  
30        extensive hybridization and allopolyploidization events. However, the challenges of  
31        achieving complete taxon sampling, and limited financial resources for studying non-  
32        model plant lineages, have hindered comprehensive and robust estimation of  
33        phylogenetic backbones with guidance from networks. The bellflower tribe,  
34        Campanuleae, characterized by a reticulate evolutionary history, serves as an ideal  
35        model to investigate how to diagnose nested ancient reticulation events. Here, by  
36        integrating multiple genomic data sources and a range of phylogenetic inference  
37        methods, we produced a robust phylogenetic backbone for the tribe Campanuleae. Our  
38        investigation of reticulate evolution indicates that hybridization and  
39        allopolyploidization were instrumental in shaping the diversity of the bellflower tribe,  
40        particularly during the initial diversification of the subtribe Phytematinae. Additionally,  
41        we ascertained that conflicting topologies resulting from distinct genomic datasets and  
42        inference methodologies significantly impact downstream estimates of divergence  
43        dating, ancestral area construction, and diversification rates. This study offers a  
44        universally relevant framework for deciphering how to use network-based phylogenetic  
45        structures using various genomic sources and inference methods. [Campanulaceae,  
46        Campanuleae, Cytonuclear discordance, paralog, phylogenomics, reticulate evolution]

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48 Building on the legacy of Darwin's Origin of Species (Darwin 1859), the Tree of  
49 Life (ToL) has been used as a model and a research tool to explore the evolution and  
50 relationships between living and extinct organisms with an assumption of bifurcating  
51 phylogeny (Mindell 2013). Given the prevalence of reticulation via incomplete lineage  
52 sorting (ILS), hybridization, polyploidization, and introgression, modeling the  
53 evolutionary connectivity of all life using a bifurcating phylogeny is problematic  
54 biologically and unrealistic (Rothfels 2021; Stull et al. 2023). A growing body of  
55 genomic and/or phylogenomic studies have provided mounting evidence supporting a  
56 network-like structure of life, such as the Bacteria and Archaea lineages (Dagan and  
57 Martin 2009; Gontier 2015), the vascular plant lineages (Leebens-Mack et al. 2019;  
58 Stull et al. 2021), birds (Jarvis et al. 2014), and mammals (Upham et al. 2019). Over the  
59 past decades, improved bioinformatic methods have been developed for teasing apart  
60 the various mechanisms underlying complex reticulate evolutionary histories, and  
61 multiple software programs have also been developed for resolving the same process,  
62 e.g., PhyloNet (Wen et al. 2018) and SNaQ (Solís-Lemus et al. 2017). However, the  
63 lack of a standardized procedure for untangling weblike relationships has impeded our  
64 understanding of evolutionary patterns and phylogenetic relationships.

65 In contrast to model plants such as *Arabidopsis thaliana* in Brassicaceae, rice  
66 (*Oryza sativa*) and maize (*Zea mays*) in Poaceae, and the tobacco plant (*Nicotiana*  
67 *tabacum*) in Solanaceae, non-model plants represent the vast majority of plant diversity  
68 on Earth, and most of them have significant ecological, agricultural, or medicinal

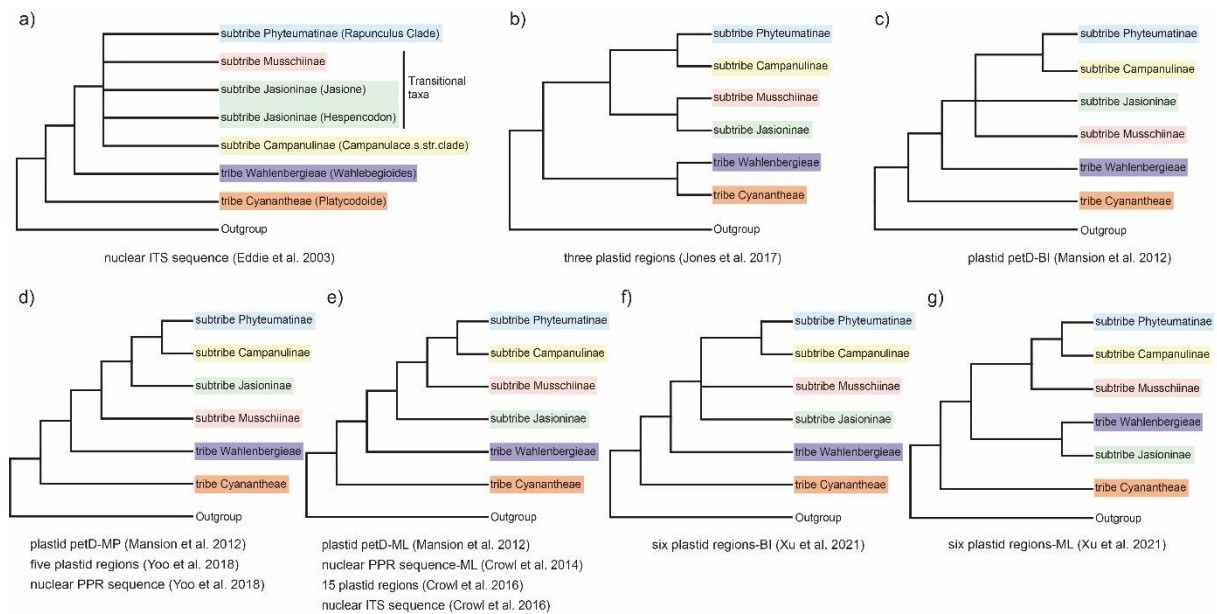


69 importance. Studying non-model plants is critical for gaining a broader understanding  
70 of plant biology, evolution, and adaptation, especially given the tremendous diversity of  
71 plants in nature. However, the understudied background knowledge, cost and resource  
72 constraints, and unavailable biological samplings greatly challenged the phylogenomic  
73 studies of non-model plants. Critically, when species diversity in non-model lineages is  
74 not thoroughly sampled using multiple sources of genomic data, it can be impossible to  
75 tease apart ancient evolutionarily significant events such as hybridization. The  
76 decreased High-Throughput Sequencing (HTS) cost, especially in China (Liu et al.  
77 2021), promoted the genome-level sequencing of non-model plants. It is important to  
78 investigate in depth a non-model plant lineage characterized by pervasive hybridization  
79 and allopolyploidy, and employ a multi-source genomic approach to explore its  
80 phylogenetic backbone and deep reticulation.

81 The Campanuleae tribe, the largest lineage in the Campanulaceae family with over  
82 620 species, has undergone extensive hybridization and polyploidy events, as noted in  
83 previous studies (e.g., Lammers 2007a, 2007b; Crowl et al. 2017). The tribe  
84 Campanuleae, along with other two tribes, Cyanantheae and Wahlenbergieae, forms the  
85 Campanuloideae subfamily (or Campanulaceae sensu stricto), which features radial  
86 floral symmetry and has a center of diversity in the Holarctic region (Hong and Wang  
87 2015). Since the description of *Campanula* L., numerous taxonomists have dedicated to  
88 proposing a “natural” infra-tribal classification based on morphological and  
89 karyological evidence, e.g., Candolle et al. (1830), Boissier (1875), Fedorov (1957),

90 and Damboldt (1976, 1978). While these investigations provided essential clues for  
91 understanding evolutionary relationships, convergent evolution, cryptic species, and  
92 frequent reticulate evolution events can hinder comprehensive and accurate taxonomic  
93 classification (Crowl et al. 2016, 2017). The genetic age enabled the clarification of  
94 some recalcitrant evolutionary relationships. For example, Eddie et al. (2003) estimated  
95 the phylogeny of Campanulaceae and diagnosed the polyphyly of *Campanula*, with  
96 *Edraianthus* and *Phyteuma* nested within *Campanula*, using nuclear ribosomal internal  
97 transcribed spacer (ITS) sequences (Fig. 1a). Subsequently, a series of phylogenetic  
98 studies inferred the maternally phylogenetic backbone of Campanulaceae using plastid  
99 regions (Fig. 1b-g; Mansion et al. 2012; Crowl et al. 2016; Jones et al. 2017; Yoo et al.  
100 2018; Xu and Hong 2021); these studies confirmed the polyphyly of *Campanula*.  
101 Recently, Xu and Hong (2021) generated a data matrix with extensive taxon sampling  
102 and six plastid regions, identifying four major clades (Fig. 1f, g; the Campanuleae I, II,  
103 III, and IV clades) and 24 subclades within the bellflower tribe, including 18 clades  
104 confirmed by Mansion et al. (2012) (further subdividing Cam 04 to two clades) and six  
105 separate genera: *Favratia*, *Feeria*, *Homocodon*, *Jasione*, *Peracarpa*, and *Trachelium*  
106 (Xu and Hong 2021). Because plastid markers have limited variability and maternal  
107 inheritance (Gitzendanner et al. 2018), these data painted an incomplete picture of the  
108 evolutionary relationships among the bellflower tribe. With the development of next-  
109 generation sequencing (NGS) technology and accompanying phylogenomic inference  
110 programs, we can now utilize hundreds or thousands of biparentally inherited nuclear

111 genes, whole plastomes, and mitochondrial genes for estimating phylogenies. Newly  
 112 developed approaches such as Deep Genome Skimming (DGS; Liu et al. 2021, 2022)  
 113 and target enrichment sequencing (Hyb-Seq; Weitemier et al. 2014; Baker et al. 2022)  
 114 are getting us closer to the goal of accurate phylogenies, which can be used to detect  
 115 underlying mechanisms for gene tree and cytonuclear discordance (Guo et al. 2021). It  
 116 is clear that multiple sources of genomic data can overcome some of the limitations and  
 117 idiosyncracieaes involved with using single markers types.



118 **Figure 1.** Phylogenetic hypotheses estimated in previous studies among the tribes  
 119 Campanuleae, Cyanantheae, and Wahlenbergieae, particularly emphasizing the  
 120 relationships among the four subtribes within the tribe Campanuleae, i.e.,  
 121 Campanulinae, Jasioninae, Musschiinae, and Phyteumatinae (referring to Fig. 2a). **a)** a  
 122 combined nuclear ITS1 and ITS2 sequences (MP tree; Eddie et al. 2003). **b)** three  
 123 plastid regions (*petD*, *rpl16*, and *trnK/matK*; MP, MO, and BI trees; Jones et al. 2017).  
 124 **c)** plastid *petD* sequence (BI tree; Mansion et al. 2012). **d)** plastid *petD* sequence (MP

125 tree; Mansion et al. 2012); five plastid regions (*atpB*, *matK*, *petD*, *rbcL*, and *trnL-F*)  
126 and nuclear PPR70 sequence (ML and BI trees; Yoo et al. 2018). **e)** plastid *petD*  
127 sequence (ML tree; Mansion et al. 2012); nuclear PPR70 sequence (ML tree; Crowl et  
128 al. 2014); 15 plastid regions (ML tree; Crowl et al. 2016); nuclear ITS sequence (ML  
129 tree; Crowl et al. 2016). **f)** six plastid regions (*atpB-rbcL*, *matK*, *petD*-intron, *rbcL*,  
130 *rpl16*, and *trnL-F*; BI tree; Xu and Hong 2021). **g)** six plastid regions (ML tree; Xu and  
131 Hong 2021).

132 Complementary lines of evidence have shown that reticulate evolution played a  
133 significant role in the diversification of the bellflower tribe, especially via  
134 hybridization, polyploidization, and ILS (Lammers 2007b; Crowl et al. 2017).  
135 Integrating plastomes and 130 nuclear loci, Crowl et al. (2017) uncovered cryptic  
136 tetraploid and octoploid *Campanula*, a lineage with four species from the  
137 Mediterranean, and revealed that morphological traits failed to distinguish polyploid  
138 lineages because only one parental morphology is retained. Previous cytological  
139 evidence also showed that nearly 13% of the Campanuloideae are presumed polyploid  
140 derivatives (Lammers 2007a), indicating a substantial role of polyploidization in the  
141 evolutionary history of bellflowers and their relatives.

142 Increasing genomic data resources in public databases, including highly reusable  
143 data types (Guo et al. 2021), such as DGS, Whole-Genome Sequencing (WGS), and  
144 transcriptomic sequencing (RNA-Seq) enable using multiple lines of genomic evidence  
145 to investigate reticulation histories. In this study, we integrate multiple genomic data

146 sources into a phylogenomic study of Campanulaceae. In total, 659 single-copy nuclear  
147 (SCN) genes, derived from Hyb-Seq, RNA-Seq, DGS, as well as plastid protein-coding  
148 sequences (CDSs) will be used for phylogenomic analysis. Using this genomic dataset  
149 with different gene histories, we assessed cytonuclear discordance to identify potential  
150 ILS and hybridization events throughout the deep nodes along the phylogenetic  
151 backbone. Molecular dating and biogeographic analysis were used to infer when and  
152 where both recent and ancient hybridization and polyploidization occurred, promoting  
153 the diversification of Campanuleae. Explicitly, we aim to explore the utility of multi-  
154 source genomic data for 1) building a well-supported phylogenetic network backbone  
155 for a non-model plant lineage, the tribe Campanuleae, and 2) elucidating hybridization  
156 and polyploidization events deep in phylogeny based on phylogenomic and  
157 biogeographic analyses.

## 158 MATERIALS AND METHODS

### 159 *Taxon Sampling, DNA Extraction, and Sequencing*

160 In this study, we adopted the taxonomic system as described by Lammers (2007a,  
161 2007b), which offers a comprehensive synopsis of generic and species delimitation, and  
162 has gained extensive acceptance within the Campanulaceae community. Later, Mansion  
163 et al. (2012) subdivided *Campanula* sensu lato into 17 distinct clades, relying on  
164 chloroplast *petD* group II intron sequences. Our sampling was meticulously designed to  
165 encompass all 17 major clades of Campanuleae, to optimize our ability to obtain a well-

166 supported nuclear and plastid backbone for the bellflower tribe. Specifically, our  
167 samples comprised 134 accessions, which included 110 ingroup species (116  
168 individuals) spanning all 17 clades in Campanuleae and 18 outgroup species (Mansion  
169 et al. 2012; see Supplementary Table S1 for details).

170 Our approach integrated data from diverse sequencing strategies, to optimize the  
171 data available to guide phylogenomic inference, as demonstrated by Liu et al. (2021,  
172 2022). For the phylogenomic analyses of Campanuleae, we harnessed data from DGS,  
173 WGS, Hyb-Seq, and RNA-Seq (detailed in Supplementary Table S1). For DGS, WGS,  
174 and Hyb-Seq sequencing, we extracted total genomic DNAs from silica-gel dried  
175 leaves and, where applicable, herbarium/museum specimens. The extraction was  
176 performed using the modified CTAB (mCTAB) method (Li et al. 2013) and carried out  
177 at the Institute of Botany, Chinese Academy of Science (IBCAS). For DGS and WGS,  
178 we utilized the NEB Next Ultra DNA Library Prep Kit for Illumina (NEB, USA),  
179 following the manufacturer's guidelines. Sequencing was performed on the DNBSEQ-  
180 T7 and BGISEQ-500 Sequencing System (Novogene, Beijing), yielding paired-end  
181 reads of  $2 \times 150$  bp for 35 accessions and  $2 \times 100$  bp for five accessions, respectively  
182 (see Supplementary Table S1 for more information). For Hyb-Seq, library preparation  
183 was completed using the Fast Library Prep Kit (iGeneTech, Beijing). Subsequent  
184 solution-based hybridization and target enrichment were carried out with the TargetSeq  
185 One<sup>®</sup> Kit at the iGeneTech facility in Zhejiang, China. We used the Illumina NovaSeq  
186 6000 platform to generate paired-end reads ( $2 \times 150$  bp) for 82 accessions (refer to

187 Supplementary Table S1 for details). For RNA-Seq, we extracted total genomic RNAs  
188 from silica-gel dried leaves via the mCTAB method. The libraries were prepared using  
189 the NEBNext<sup>®</sup> Ultra RNA Library Prep Kit for Illumina (USA). This process resulted  
190 in paired-end reads of  $2 \times 150$  bp, which were sequenced on the Illumina NovaSeq  
191 6000 Sequencing System for a total of eight accessions (further details in  
192 Supplementary Table S1). All raw reads (130 accessions) sequenced for this study have  
193 been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject  
194 PRJNA895940.

195 *Raw Reads Processing, and Nuclear SCN and Plastid Sequence Assembly*

196 In a recent series of studies, we have developed approaches to integrate multi-  
197 source genomic data for phylogenomic analyses (Liu et al. 2021, 2022; Jin et al. 2023).  
198 Here, the processing and assembly of raw reads follow the workflow established in  
199 previous studies. After sequencing, low-quality reads and base calls were trimmed, and  
200 adaptor sequences were removed using Trimmomatic v. 0.39 (Bolger et al. 2014). The  
201 quality of the results was subsequently checked using FastQC v. 0.11.9 (Andrews  
202 2018). Leveraging the transcriptome of *Adenophora polyantha* Nakai (SRA accession:  
203 SRX8528008), we screened putative single-copy genes with MarkerMiner v. 1.0  
204 (Chamala et al. 2015), which yielded 659 SCN genes. The clean reads were then used  
205 to assemble the SCN genes via the HybPiper v. 2.0 pipeline (Johnson et al. 2016).  
206 Specifically, the “hybpiper assemble” command was executed to assemble contigs and

207 extract sequences with the 659 SCN gene sequences as the references. We summarized  
208 gene recovery statistics using the “hybpiper stats” command. The gene recovery  
209 statistics were used to generate a visual representation of recovery efficiency through  
210 the “hybpiper recovery\_heatmap” command, enabling us to assess the assembly quality  
211 of each gene. Finally, the “hybpiper paralog\_retriever” command was run to obtain the  
212 sequences, potentially containing paralogs, for all recovered genes. This process  
213 generated an unaligned multi-FASTA file for each gene.

214         Given the history of gene rearrangements and diverse repeat sequences in the  
215 Campanulaceae plastome (Li et al. 2020), we focused on assembling only the protein-  
216 coding sequences (hereafter referred to as plastid CDSs) for plastid phylogenetic  
217 inference. We extracted 79 CDS sequences from three chloroplast genomes using  
218 Geneious Prime (Kearse et al. 2012); they are *Adenophora remotiflora* (GenBank  
219 accession: KP889213), *Campanula takesimana* (GenBank accession: KP006497), and  
220 *Trachelium caeruleum* (GenBank accession: EU090187). These 79 CDS sequences  
221 served as the reference for assembly. For the assembly of the plastid CDSs, we  
222 employed the HybPiper v. 2.0 pipeline (Johnson et al. 2016), mainly following the  
223 approach described in our earlier nuclear SCN genes assembly, except the final multi-  
224 FASTA file for each CDS sequence were retrieved using “hybpiper retrieve\_sequence”  
225 command.



226 *Orthology Inference and Data Matrices Generation for Nuclear SCN Genes*

227       Considering the prevalence of allopolyploid species documented in the Index to  
228 Plant Chromosome Numbers (IPCN), we applied several methods to identify paralogs  
229 and differentiate potential orthologs from homologs. We adopted the orthology  
230 inference methodology developed by the Ya Yang Group (Yang and Smith 2014;  
231 Morales-Briones et al. 2022). This strategy includes the Monophyletic Outgroups  
232 (MO), Rooted Ingroups (RT), and one-to-one orthologs (1to1) approaches. These  
233 methods are effective for minimizing the influence of paralogs in phylogenetic  
234 inference. The 1to1 method retains only homologs with no duplicated taxa, which avoid  
235 the introduction of potential paralogs. In the MO approach, paralogs were identified  
236 and pruned using homologs with monophyletic, non-repeating outgroups, and this  
237 process involved rerooting and pruning paralogs from root to tip. When the outgroup  
238 was absent, only those without duplicated taxa were used. Meanwhile, the RT method  
239 removed paralogs by extracting ingroup clades and cutting paralogs from root to tip.  
240 Similarly, when the outgroup was missing, only those sequences free from duplicated  
241 taxa were considered. For both methods, we set a minimum threshold of 25 ingroup  
242 taxa. The full details of paralog assessment and orthology inference are available online  
243 ([https://bitbucket.org/dfmoralesb/target\\_enrichment\\_orthology/src/master/](https://bitbucket.org/dfmoralesb/target_enrichment_orthology/src/master/)). We  
244 generated three datasets from these analyses to use as the basis for phylogenetic  
245 inference, i.e., the 1to1, MO, and RT datasets.

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*Cleaning of Nuclear and Plastid Sequences*

We implemented a series of processing steps to clean low-quality sequences, a method previously employed with success in our studies, such as Liu et al. (2021, 2022) and Jin et al. (2023). To achieve refined alignments despite sequences with inconsistent quality, we utilized MAFFT v. 7.505 (Nakamura et al. 2018) to align each SCN sequence, employing the Smith-Waterman algorithm and the “--maxiterate 1000” parameter. The resulting multiple sequence alignments were trimmed by trimAl v. 1.2 (Capella-Gutiérrez et al. 2009) to remove spurious sequences or poorly aligned regions. Specifically, columns with gaps in over 20% of the sequences or with a similarity score below 0.001 were removed using the parameters “-gt 0.8 -st 0.001”. Further cleaning of the sequences was performed using Spruceup (Borowiec 2019), which identified, visualized, and eliminated outlier sequences, with a window size of 50 and overlap of 25. Alignments produced before and after using Spruceup were concatenated and split, respectively, using AMAS v. 1.0 (Borowiec 2016). Recognizing that exceptionally short sequences could hinder accurate phylogenetic inference for each SCN gene, sequences shorter than 250 bp in each alignment were excluded using a Python script (`exclude_short_sequences.py`) from Liu et al. (2022). The cleaned sequences then served as inputs to infer gene trees through RAxML v. 8.2.12 (Stamatakis 2014), using the option “-f a” and 200 BS replicates for clade support evaluation. To ensure the accuracy of species tree inference, TreeShrink v. 1.3.9 (Mai and Mirarab 2018) was employed to identify and remove excessively long branches in each gene tree. After the

267 above processing steps were complete, the sequences were termed ‘clean nuclear  
268 genes’, with three separate data matrices: 1to1, MO, and RT approach.

269 *Accurate Phylogenetic Inference with Multiple Methods*

270 To obtain accurate phylogenies and identify the topological discordance between  
271 trees, we employed both concatenated and coalescent-based methods. The gene trees,  
272 refined by removing long branches via TreeShrink, served as the input trees to estimate  
273 the species tree using ASTRAL-III (Zhang et al. 2018), which is statistically  
274 consistency with the multi-species coalescent model. Notably, any input gene tree  
275 branches with low support ( $\leq 10$ ) were collapsed using phyx (Brown et al. 2017), as  
276 collapsing gene tree nodes with BS support below a threshold value can enhance  
277 accuracy (Zhang et al. 2018). Clean nuclear genes were used for both ML and BI tree  
278 inference. The most suitable partitioning schemes and molecular evolution models were  
279 identified through PartitionFinder2 (Stamatakis 2006; Lanfear et al. 2016), with default  
280 settings. The resulting schemes and models were then used in subsequent ML tree  
281 estimates via IQ-TREE2 v. 2.2.0.3 (Minh et al. 2020) — with 1000 SH-aLRT and  
282 ultrafast bootstrap replicates — and RAxML v. 8.2.12 (Stamatakis 2014) using the  
283 GTRGAMMA model for each partition and 200 rapid bootstrap (BS) replicates for  
284 clade support. BI analysis was conducted using MrBayes 3.2.7a (Ronquist et al. 2012),  
285 running Markov Chain Monte Carlo (MCMC) analyses for 50 million generations.  
286 Stationarity was achieved when the average standard deviation of split frequencies

287 remained under 0.01. Trees were analyzed every 1,000 generations, with the initial 25%  
288 of samples discarded as burn-in. Subsequent trees were used to generate a 50%  
289 majority-rule consensus tree.

290 We compiled a dataset comprising 79 plastid CDS sequences, hereafter referred to  
291 as the ‘plastid CDS dataset’, for phylogenetic analysis. While processing the plastid  
292 sequences and performing the phylogenetic inference, we largely follow the nuclear  
293 SCN methodology, excluding the steps of eliminating short sequences and long  
294 branches.

#### 295 *Gene Tree and Species Tree Discordance Analyses*

296 We used *phyparts* to calculate unique, conflicting, and concordant bipartitions  
297 within individual orthologs across the phylogeny (Smith et al. 2015). We conducted  
298 both quick concordance (-a 0) and full concordance (-a 1) analyses to address the issue  
299 arising from orthologs with missing taxa. The conflict analysis produces a pie chart for  
300 each node, segmented into five sections. These sections depict varying proportions of  
301 orthologs, such as those supporting the clade (in blue), those supporting the main  
302 alternative for that clade (in green), those supporting the remaining alternatives (in red),  
303 the uninformative (in dark grey), and the missing ones (in light grey). Additionally, we  
304 computed the ‘internode certainty all’ (ICA) scores on the input  
305 concatenated/coalescent-based tree based on the set of ortholog trees (Salichos et al.  
306 2014). The results from *phyparts* were illustrated using

307 `phypartspiecharts_missing_uninformative.py`, a Python script developed by Morales-  
308 Briones, which is available at:  
309 [https://bitbucket.org/dfmoralesb/target\\_enrichment\\_orthology/src/master/phypartspiech](https://bitbucket.org/dfmoralesb/target_enrichment_orthology/src/master/phypartspiecharts_missing_uninformative.py)  
310 [arts\\_missing\\_uninformative.py](https://bitbucket.org/dfmoralesb/target_enrichment_orthology/src/master/phypartspiecharts_missing_uninformative.py)

311 As a counterpart to *phyparts* in analyzing phylogenomic discordance, QS adeptly  
312 identifies discordance in large-sparse and genome-wide datasets. This method addresses  
313 challenges related to alignment sparsity and can differentiate between strong conflict  
314 and weak support (Pease et al. 2018). For each internal branch, QS produces three  
315 distinct scores: Quartet Concordance (QC), Quartet Differential (QD), and Quartet  
316 Informativeness (QI). Each approach for quantifying discord offers unique yet  
317 complementary insights. The results from QS are visually represented using the  
318 `plot_QC_ggtree.R`, an R package developed by Shui-Yin Liu, accessible at  
319 [https://github.com/ShuiyinLIU/QS\\_visualization](https://github.com/ShuiyinLIU/QS_visualization).

### 320 *Coalescence Simulation for Testing the Effect of ILS*

321 Gene tree discordance can arise from single evolutionary events, including ILS,  
322 hybridization, and allopolyploidization, or some combination of these factors. To  
323 distinguish among these, we implemented a series of analyses. We utilized coalescence  
324 simulation to test if ILS could explain gene tree conflicts; this method has proven  
325 effective in recent research (Moralis-Briones et al. 2021; He et al. 2022; Liu et al.  
326 2022). First, we employed the previously described method (in the phylogenetic

327 inference section) to estimate the species tree using ASTRAL-III (Zhang et al. 2018).  
328 Using this ASTRAL ultrametric species tree, we simulated 10,000 gene trees under the  
329 multi-species coalescent (MSC) model with the “sim.coaltree.sp” function in the R  
330 package Phybase v. 1.5 (Liu and Yu 2010). We subsequently compared the distribution  
331 of tree-to-tree distances between simulated and empirical gene trees using the  
332 DendroPy v. 4.5.2 Python package (Sukumaran and Holder 2010). The result was  
333 visualized in a column chart where the extent of overlap between simulated and  
334 empirical gene tree bars represents the goodness-of-fit of the coalescent model,  
335 indicating whether ILS is a plausible explanation for gene tree discordance.

### 336 *Inference of Global Split Networks*

337 The split network is a valuable tool for visualizing inconsistencies within a dataset.  
338 In such a network, ancestral species are not designated by specific nodes. Instead,  
339 parallel edges denote the splits that derive from the dataset, and their length indicates  
340 the importance of these splits. For the tribe Campanuleae, we constructed a split  
341 network using SplitsTree v. 4.19.0 (Huson and Bryant 2006), drawing on aligned SCN  
342 sequences from the MO dataset. This construction involved applying split  
343 decomposition to the uncorrected\_P distances. Given the large divergence in this  
344 dataset, we presented the split network produced by the NeighborNet method for  
345 achieving higher resolution, and we then employed the EqualAngle network  
346 construction algorithm to infer the split network.

347 *Phylogenetic Network Estimation*

348 In this study, we used network approaches investigate the early diversification of  
349 major clades within the tribe Campanuleae, encompassing three tribes and four  
350 subtribes. To reduce the computational burden of the network analysis, we selected a  
351 subset of 11 species. This dataset is specifically tailored to test the potential ancient  
352 reticulate evolution events that occurred among the three tribes and four subtribes.

353 Given the comprehensive taxon sampling within the subtribe Phyteumatinae, we  
354 grouped all 78 samples (73 species) into six monophyletic clusters, i.e., six subclades,  
355 across 12 nuclear and four plastid trees. Additionally, we generated another dataset,  
356 comprising seven species, to assess the impacts of hybridization and  
357 allopolyploidization during the initial diversification of the subtribe Phyteumatinae.

358 We used Species Networks applying Quartets (SNaQ) approach, as detailed by  
359 Solís-Lemus and Ané (2016), for the Maximum pseudolikelihood estimation of species  
360 networks. This method is integrated within the PhyloNetworks package (Solís-Lemus et  
361 al. 2017) in Julia. Notably, it accounts for ILS via the coalescent model while  
362 addressing horizontal gene inheritance through reticulation nodes in the network. The  
363 methodology leverages pseudolikelihood, avoiding the intensive computation  
364 associated with full likelihood and facilitating estimations at the quartet level. This  
365 enhances computational efficiency due to its easy parallelization (Solís-Lemus and Ané  
366 2016). For phylogenetic network analysis, we followed the comprehensive protocol  
367 provided by Solís-Lemus, available at <https://github.com/crs14/PhyloNetworks.jl/wiki>.

368 We used  $h$  values ranging between 0 and 6, undertaking 50 runs for the network  
369 inference.

370 *Allopolyploidy Analysis*

371 Gene-tree Reconciliation Algorithm with Multi-labeled trees (MUL-trees) for  
372 Polyploid Analysis (GRAMPA) adapted an algorithm for topology-based gene-tree  
373 reconciliation to work with MUL-trees (Thomas et al. 2017), and this program,  
374 GRAMPA, can identify the parental lineages that hybridized to form  
375 auto/allopolyploids. Given the similarity between processes such as hybridization and  
376 allopolyploidization, we also tested potential auto/allopolyploidy events among the  
377 major clades in whole tree (including tribe Wahlenbergieae, tribe Cyanantheae, and four  
378 clades in tribe Campanuleae) and among the six subtribes in clade I of the tribe  
379 Campanuleae. Because GRAMPA can only infer one WGD at a time, we employed the  
380 method proposed by Morales-Briones et al. (2022) to successively explore WGD events  
381 along the backbone of a phylogeny. We applied this approach to the backbone of the  
382 tribe Campanuleae. Briefly, we specified the clades mentioned above as -h1 and -h2 and  
383 perform reconciliation search using all MO ortholog trees against the MO species tree  
384 estimated with ASTRAL-III (Zhang et al. 2018). The MO ortholog trees, and species  
385 tree after removing the clade identified as polyploid in former GRAMPA analysis, were  
386 used to run the next round of analysis on the remaining clades with the same settings  
387 until no polyploid clade were detected.



388 *Dating Analysis and Ancestral Area Reconstruction*

389 The earliest known macrofossils of the family Campanulaceae are seeds from  
390 *Campanula paleopyramidalis*, discovered in Miocene deposits ca. 17-16 million years  
391 ago (Mya), of Nowy Sacz in the Carpathians, Poland (Oszast and Stuchlik 1977;  
392 Łańcucka-Środoniowa 1979; Nemcok et al. 1998). These fossilized seeds have been a  
393 pivotal calibration point in several prior studies, including Cellinese et al. (2009),  
394 Mansion et al. (2012), Olesen et al. (2012), Crowl et al. (2014, 2016), and Jones et al.  
395 (2017). This species is closely related to the extant *Campanula pyramidalis*. We utilized  
396 this fossil as the MRCA of a combined clade comprising *Campanula carpatica*, *C.*  
397 *pulla*, *C. rainerii*, and *Favratia zoysii*. Furthermore, we incorporated two secondary  
398 calibration points to constrain the deeper nodes in Campanulaceae: the crown clade of  
399 the family Campanulaceae is dated between 72.24 to 52.66 Mya, and the crown clade  
400 of the subfamily Campanuloideae is dated between 63.52 to 44.58 Mya (Li et al. 2019).

401 To investigate the impact of conflicting topologies on dating analyses, we used the  
402 MCMCTree package in PAML v. 4.9j to estimate divergence times of Campanulaceae  
403 based on multiple distinct topologies (concatenated and coalescent-based trees) and  
404 datasets (plastome and nuclear). We started by determining the nucleotide substitution  
405 rate and Hessian Matrix through the MCMCTree program, with the independent rates  
406 clock model and the GTR substitution model. For each dataset, we performed two  
407 separate runs of the MCMCTree analysis, every time initiated with different seeds. The  
408 initial 1,000,000 generations of each Markov Chain Monte Carlo (MCMC) were

409 discarded as burn-in. Subsequently, we collected samples every ten generations,  
410 amounting to a total of 500,000 samples. These samples were assessed using Tracer v.  
411 1.7.1 (Rambaut et al. 2018) to confirm convergence and ensure that the effective  
412 sample size (ESS) of all parameters exceeded 200. Finally, we visualized the results  
413 from both runs using FigTree v. 1.4.4 and cross-validated them to ascertain  
414 convergence.

415 We utilized the software BioGeoBEARS v. 1.1.2 (Matzke 2018) implemented in  
416 RASP v. 4.2 to reconstruct the ancestral area of the bellflower tribe. Based on the  
417 current distribution of Campanulaceae, we classified its geographic range into six  
418 distinct regions: (A) Europe, (B) Northern Asia, (C) Africa, (D) North America, (E)  
419 Southern East Asia combined with Australasia, and (F) South West Asia. Three distinct  
420 estimated trees were used as input to test the effect of topological discordance on  
421 ancestral area reconstruction, in which a maximum of four areas were specified. Six  
422 models provided by BioGeoBEARS v.1.1.2 were utilized to estimate the biogeographic  
423 history, and the AICc values from these models were then compared to identify the  
424 optimal model for reconstructing the ancestral area of Campanuleae.

#### 425 *Chromosome Number Reconstructions*

426 We collected haploid chromosome numbers (n) from both the Chromosome Count  
427 Database (<http://ccdb.tau.ac.il>) and the Campanulaceae monograph by Lammers  
428 (2007a). Using the PAML ultrametric tree inferred from the nuclear concatenation-

429 based tree as input, we employed ChromEvol v. 2.2 to infer the ancestral haploid  
430 chromosome number through a likelihood-based approach. Notably, species lacking  
431 chromosomal data are denoted with the symbol ‘X’. When a single species exhibited  
432 multiple chromosome counts, we selected the count observed most frequently for  
433 subsequent analysis. Our analysis was conducted under the parameter “\_mainType =  
434 All\_Models”, encompassing ten evolutionary models, and we utilized  
435 “\_simulationsNum 1000000” to enhance the precision of our analysis. The best-fit  
436 model was estimated using the Akaike Information Criterion (AIC) score.

#### 437 *Diversification Analyses*

438 We used Bayesian Analysis of Macroevolutionary Mixtures (BAMM; Rabosky et  
439 al. 2014a) to estimate the diversification rate of Campanulaceae. The dated tree,  
440 constructed from the aforementioned concatenated orthologs tree, served as the input  
441 tree. We adopted a non-random incomplete taxon sampling strategy to avoid error  
442 introduced by incomplete sampling species and computed the sampling fraction for  
443 each clade within Campanulaceae. MCMC simulation with four chains was performed  
444 for 10,000,000 generations under the “species-extinction” model and sampling every  
445 1,000 generations. The first 1,000,000 generations were discarded as burn-in, and the  
446 remaining samples were then analyzed using the R package BAMMTOOLS (Rabosky  
447 et al. 2014b) to assess whether the effective sample size (ESS) exceeded 200 and to  
448 generate plots.

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## RESULTS

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### *SCN and Plastid Genes Assembly and Nuclear Orthology Inference*

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This study integrated multiple sources of genomic data, including 82 Hyb-Seq, eight RNA-Seq, and 48 DGS data, for phylogenomic analyses. The nuclear assembly from HybPiper resulted in a variable number of SCN genes, ranging from 519 to 654 (Supplementary Fig. S1). Non-chimeric sequences recovered from HybPiper have been utilized to perform orthology inference using three different methods, and this process resulted in three distinct datasets: 1to1 containing 445 genes, MO with 645 genes, and RT with 660 genes. The final alignment of these three datasets, each with 134 taxa, contained 598,993, 864,819, and 887,333 characters, respectively. We employed HybPiper for the assembly of 79 plastome protein-coding sequences (CDSs), and the recovery efficiency was visualized as a heatmap (Supplementary Fig. S2).

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### *Nuclear Phylogenetic Inference and Gene Tree Discordance Analyses*

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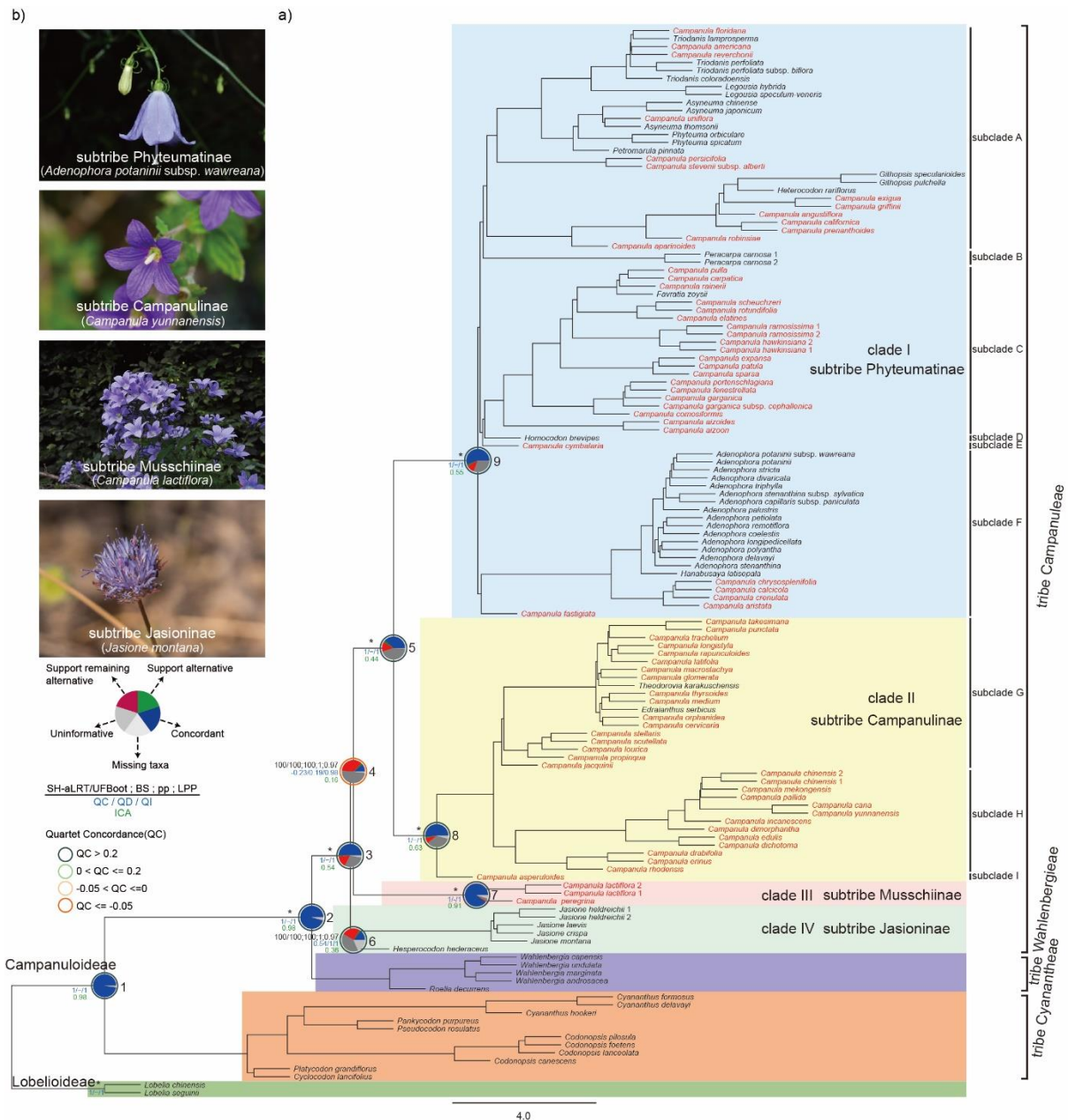
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Integrating concatenation and coalescent-based methods, we generated four trees for each dataset based on various phylogenetic inference methods: two trees from ML (RAxML and IQ-TREE2), one from Bayesian Inference (MrBayes), and one species tree estimated based on coalescent theory (ASTRAL-III). These 12 nuclear trees confirmed the monophyly of the subfamily Campanuloideae and the three-tribe classification within Campanuloideae: tribe Campanuleae, tribe Cyanantheae (orange),

468 and tribe Wahlenbergieae (purple) (Supplementary Figs. S3-S14), almost all the  
 469 informative gene trees were concordant with these nodes (node 1: 521 out of 522, ICA  
 470 = 0.98; node 2: 510 out of 512; ICA = 0.98), and full QS support (1/-/1) (Fig. 2a;  
 471 Supplementary Figs. S15-S22).



472 **Figure 2.** A represented tree-like phylogenetic backbone of the subfamily  
 473 Campanuloideae inferred from SCN genes, emphasizing the major clades in the tribe

474 Campanuleae. **a)** Species tree of the tribe Campanuleae in the framework of the  
475 subfamily Campanuloideae inferred from ASTRAL-III of the nuclear Monophyletic  
476 Outgroup (MO) orthologs. Summarized phylogenetic supports of the focal nine nodes  
477 from four trees based on the nuclear MO dataset were presented above the branch.  
478 From left to right (labeled in black above branch), the SH-aLRT support and Ultrafast  
479 Bootstrap (UFBoot) estimated from IQ-TREE2 (details referring to Supplementary Fig.  
480 S8); the bootstrap support (BS) values from RAxML analysis (details referring to  
481 Supplementary Fig. S7); Bayesian posterior probability values (pp) from MrBayes  
482 (details referring to Supplementary Fig. S9); the local posterior probability (LPP) from  
483 ASTRAL-III (details referring to Supplementary Fig. S10) (e.g., 100/100; 100; 1; 0.97);  
484 asterisks (\*) indicated full support (100/100; 100; 1; 1). Values for Quartet  
485 Concordance/ Quartet Differential/ Quartet Informativeness estimated from Quartet  
486 Sampling analysis were provided below branches (e.g., 1/-/1, labeled in blue) (details  
487 referring to Supplementary Fig. S20). The Pie charts on these nodes illustrated the  
488 proportion of gene trees that were concordant with the corresponding clade in the  
489 species tree (blue), the proportion that supported the main alternative (green), the  
490 proportion that supported the first remaining alternative (red), the proportion considered  
491 uninformative (deep gray), and the proportion that are missing in the trees (gray); the  
492 value of partial sampling ICA were also presented below (labeled in green) (details  
493 referring to Supplementary Fig. S19). Campanuloideae was classified into three tribes,  
494 tribe Cyanatheae (orange), tribe Wahlenbergieae (purple), and tribe Campanuleae; tribe

495 Campanuleae was further divided into four clades /subtribes, clade I (subtribe  
496 Phyteumatinae with blue background), clade II (subtribe Campanulinae with yellow  
497 background), clade III (subtribe Musschiinae with pink background), and clade IV  
498 (subtribe Jasioninae with light green background); clade I and II were subdivided into  
499 nine subclades (A-I). All species belonging to the genus *Campanula* were highlighted  
500 in red, indicating the polyphyly of *Campanula*. **b)** Represented species of four subtribes  
501 in tribe Campanuleae, indicating the morphological diversity of flowers. From top to  
502 bottom: subtribe Phyteumatinae (*Adenophora potaninii* subsp. *wawreana* ), subtribe  
503 Campanulinae (*Campanula yunnanensis*), subtribe Musschiinae (*Campanula*  
504 *lactiflora*), and subtribe Jasioninae (*Jasione montana*). Photos credit to You-Pai Zeng,  
505 Hai-Lei Zheng, Ke Cheng, and Jia-Nong Li (from top to bottom).

506         Additionally, all nuclear trees consistently supported the monophyly of four major  
507 clades within the bellflower tribe, such as clade I (blue), clade II (yellow), clade III  
508 (pink), and clade IV (light green) (Fig. 2a; Supplementary Figs. S3-S14). All four  
509 clades were recovered with maximum support and full QS support (1/-/1), except clade  
510 IV which includes five individuals of *Jasione* and one species of *Hesperocodon*  
511 *hederaceus*—received relatively lower support in the three ASTRAL-III coalescent  
512 trees (LPP = 0.97) and strong QS support (0.54/1/1) (Fig. 2a nodes 6-9; Supplementary  
513 Figs. S6, S10, S14, and S20). Monophyly of the clade I, clade II, and clade III were  
514 supported by most of the informative trees: clade I with 296 concordant trees (out of  
515 370 informative trees; ICA = 0.55; Fig. 2a node 9), clade II with 275 concordant trees

516 (out of 325 informative trees; ICA = 0.63; Fig. 2a node 8), and clade III with 479  
517 concordant trees (out of 489 informative trees; Fig. 2a node 7); but clade IV were  
518 supported by only 74 concordant trees out of 223 informative trees (ICA = 0.36; Fig. 2a  
519 node 6).

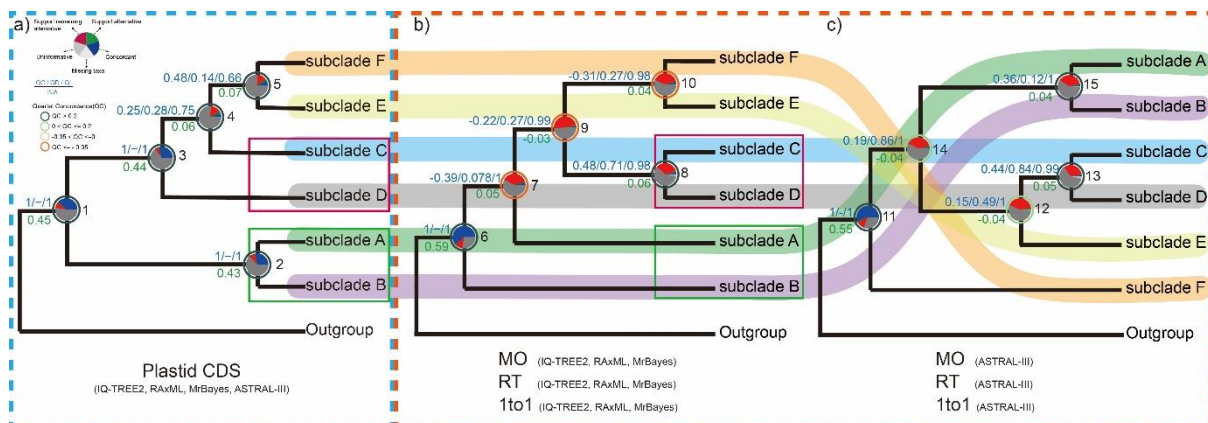
520 The sister group consisting of clade I and clade II was recovered with maximum  
521 support, 203 concordant trees out of 294 informative trees (ICA = 0.44), and full QS  
522 score (1/-/1) (Fig. 2a node 5). Clade III was recovered as sister of clade I + clade II with  
523 relatively high support (SH-aLRT/UFBboot = 100/100; BS = 100; pp = 1; LPP = 0.97),  
524 only 55 concordant trees (out of 208 informative trees; ICA = 0.16), and counter QS  
525 support with a strong majority of quartets supporting an alternative discordant topology  
526 (-0.23/0.19/0.98) (Fig. 2a node 4). Finally, clade IV was recovered as the sister to the  
527 rest of tribe Campanuleae with maximum support, 265 concordant trees out of 361  
528 informative trees (ICA = 0.54), and full QS score (1/-/1) (Fig. 2a node 3).

529 According to the monophyletic groups recovered in the 12 nuclear trees, we  
530 subdivided clade I into six subclades (A-F) and clade II into three subclades (G-I) (Fig.  
531 2a; Supplementary Figs. S3-S14). The phylogenetic relationships in the 12 nuclear trees  
532 were concordant for the three subclades (G-I) in clade II; however, these 12 nuclear  
533 trees revealed significant conflicting topologies in clade I.

534 In both the concatenation and coalescent-based tree of plastid CDSs (Fig. 3a;  
535 Supplementary Figs. S23-S28), the sister relationship between subclade F and E was  
536 recovered by 5 out of 18 informative trees (ICA = 0.07) and strong QS support with



537 discordant skew (0.48/0.14/0.66) (Fig. 3a node 5). Subclade C was recovered as sister  
 538 to a combined clade of subclade F and E with weak QS support with discordant skew  
 539 (0.25/0.28/0.75), and only four concordant trees (out of 18 informative trees; ICA =  
 540 0.06) (Fig. 3a node 4). Subclade D was recovered as sister to a large clade consisting of  
 541 subclade C, subclade E, and subclade F, with 22 concordant trees (out of 27 informative  
 542 trees; ICA = 0.44) and full QS support (1/-/1) (Fig. 3a node 3). The sister group  
 543 composed of subclade A and subclade B was recovered with full QS support (1/-/1) and  
 544 21 concordant trees (out of 28 informative trees; ICA = 0.43) (Fig. 3a node 2), and was  
 545 placed as sister to the rest of clade I with full QS support (1/-/1) and 29 concordant  
 546 trees (out of 36 informative trees; ICA = 0.45) (Fig. 3a node 1).



547 **Figure 3.** Comparative visualization of three conflicting topologies from different  
 548 datasets and inference methods for clade I (equivalent to subtribe Phyteumatinae). **a)**  
 549 The same topology inferred from IQ-TREE2, RAxML, MrBayes, and ASTRAL-III  
 550 based on the plastid CDSs dataset. **b)** The same topology estimated by IQ-TREE2,  
 551 RAxML, and MrBayes across all three nuclear datasets (MO, RT, and 1to1). **c)** The  
 552 same topology estimated by ASTRAL-III across all three nuclear datasets. Quartet

553 Sampling analysis values for QC/QD/QI are displayed above branches (e.g., 1/-/1, in  
554 blue). Pie charts on the nodes, determined by *phyparts*, illustrated the proportion of  
555 gene trees that were concordant with the corresponding clade in the species tree (blue),  
556 the proportion that supported the main alternative (green), the proportion that supported  
557 the first remaining alternative (red), the proportion considered uninformative (deep  
558 gray), and the proportion that are missing in the trees (gray). Below the branches,  
559 values of the partial sampling ICA are presented (in green). Different color bandings  
560 beneath subclades A to F visually highlight the topological discrepancies between them.

561 All the 12 nuclear trees recovered the monophyly of clade I with full QS support  
562 (1/-/1), and almost all the informative gene trees being concordant with these nodes  
563 (219 out of 262 for node 6, ICA = 0.59; 296 out of 370 for node 11, ICA = 0.55).  
564 However, almost all the concerned nodes within the clade I showed conflict with a little  
565 percentage of supporting trees, but QS scores of most nodes revealed supported  
566 alternative topologies (Fig. 3 nodes 6-15). The sister group consisting of subclade C  
567 and subclade D was recovered with strong QS support (i.e., a strong majority of  
568 quartets supported the focal nodes, and the low skew in discordant frequencies  
569 indicated no alternative history was favored) in both concatenation-based (node 8,  
570 0.48/0.71/0.98) and coalescent-based trees (node 13, 0.44/0.84/0.99), moreover, a few  
571 informative trees showed concordance on this nodes (ten out of 139 for concatenation-  
572 based trees, ICA = 0.06, node 8; nine out of 187 for coalescent-based trees, ICA = 0.05,  
573 node 9). The topologies of the rest of clade I showed high level varied among

574 concatenation and coalescent-based trees. In the concatenation-based trees, the sister  
575 group composed of subclade E and subclade F was recovered with only nine  
576 concordant trees (out of 157, ICA = 0.04, node 10), and counter QS support (-  
577 0.31/0.27/0.98) with a skew in discordance suggesting an alternative discordant  
578 topology; furthermore, this group was placed as the sister to subclade C + subclade D,  
579 with five concordant trees (out of 185, ICA = -0.03, node 9), and counter QS support (-  
580 0.22/0.27/0.99). Then subclade A was recovered as sister to the clade consisting of  
581 subclades C to F, with 11 concordant trees (out of 153, ICA = 0.05, node 7) and counter  
582 QS support. Finally, subclade B was the earliest-divergent lineage within clade I. In the  
583 ASTRAL trees based on nuclear, subclade E was placed as the sister to the group  
584 consisting of subclade C and subclade D, with only one concordant tree (out of 284 ,  
585 ICA = -0.04, node 12), and the QS score also showed weak support with discordant  
586 skew (0.15/0.49/1) indicating a possible alternative topology. The group consisting of  
587 subclade A and subclade B with was recovered with nine concordant trees (out of 218,  
588 ICA = 0.04, node 15), and weak QS support with discordant skew (0.36/0.12/1),  
589 moreover, this group was recovered as the sister of the rest subclades within clade I  
590 except subclade F, with six concordant trees (out of 230, ICA = -0.04, node 14) and  
591 weak QS support (0.19/0.86/1). Finally, subclade F was placed as the earliest-divergent  
592 lineage within clade I.

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### *Cytonuclear Discordance*

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The results from different datasets (nuclear and plastid CDSs) and various phylogenetic inference methods (concatenation and coalescent-based) consistently indicated similar topologies on the main clade nodes (Fig. 3; Supplementary Figs. S3-S14, S23-S26). However, some conflicts were observed within clade I. In the concatenation and coalescent-based trees inferred from plastid CDSs, clade I was divided into two major monophyly, subclade A and subclade B consisted the first-branding lineage, the other subclades composed another monophyly (Fig. 3a). Similar to the trees of plastid CDSs, the monophyly composed by subclades C to F was recovered in the concatenation-based trees of nuclear too, with two groups consisting respectively of subclade C + subclade D and subclade E + subclade F, but the subclade A and subclade B were placed as the first and the second branding lineages(Fig. 3b). The topology of the ASTRAL trees was vastly different with trees of plastid CDSs, except the monophyly consisting of subclade A and subclade B, subclade C, subclade D, and subclade E consisted a group, the subclade F was placed as the earliest-divergent lineage which was sister to the other with in clade I (Fig. 3c).

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### *Reticulate Evolution of the Four Major Clades in the tribe Campanuleae*

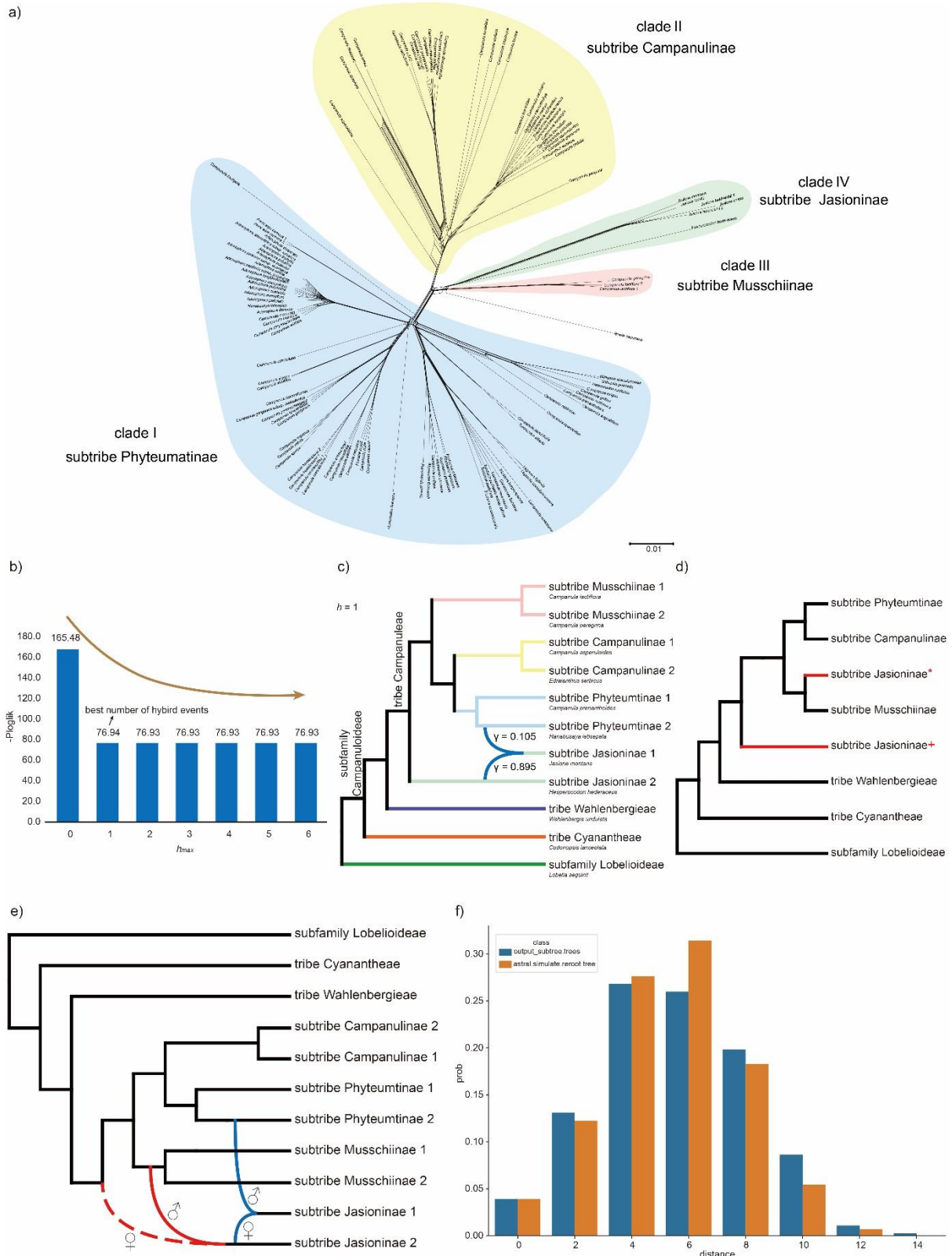
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Reticulate evolution events were analyzed in more detail due to the strong cytonuclear discordance among several branches. We built a split network utilizing sequences from the MO dataset and designated *Roella decurrens* as the outgroup. Similar to the nuclear

613 phylogenetic tree, the split network indicated the separation of four clades in the tribe  
 614 Campanuleae; the clades were each well differentiated from the rest of the tribe, with  
 615 clades III and IV being relatively close together (Fig. 4a). **Figure 4.** Exploring the



616 phylogenetic network of the major clades, including three tribes in the subfamily  
617 Campanuloideae and four subtribes in the tribe Campanuleae. **a)** Split network  
618 estimated from the nuclear MO dataset, showcasing four distinct subtribes. Clade I,  
619 represented in blue, corresponds to the subtribe Phyteumatinae; Clade II, shown in  
620 yellow, aligns with the subtribe Campanulinae; Clade III, depicted in pink, associates  
621 with the subtribe Musschiinae; and Clade IV, in light green, represents the subtribe  
622 Jasioninae. **b)** Bar chart representing the pseudo-loglikelihood scores (-ploglik) across a  
623 range of maximum reticulations (from zero to six). The chart highlights the optimal  
624 number of hybrid events, with  $h_{max} = 1$  being identified as the best number. **c)**  
625 Phylogenetic network inferred from SNaQ analysis with  $h_{max} = 1$  as the optimal  
626 network. On the right, species names are displayed in a reduced font size beneath their  
627 corresponding tribe/subtribe names, indicating the species chosen as representatives  
628 from various subfamilies, tribes, or subtribes in the analysis. **d)** The simplified most  
629 parsimonious multi-labeled trees (MUL-trees) derived from the species tree based on  
630 the nuclear MO dataset that includes all taxa. Branches marked in red signify the  
631 allopolyploid origin of the subtribe Jasioninae, further highlighted by a red asterisk or a  
632 red plus sign from different parents. **e)** Summary of the potential phylogenetic network  
633 among the major clades within the subfamily Campanuloideae. Red curves denote the  
634 potential allopolyploidization events inferred from the GRAMPA analysis, with the  
635 dotted lines indicating extinct ancestors. The symbols ♂ and ♀ on the curving  
636 branches signify paternal and maternal parents. Blue curves highlight potential

637 hybridization events based on the SNaQ analysis. **f)** Distribution of tree-to-tree  
638 distances between empirical gene trees and the species tree inferred from ASTRAL-III  
639 analysis, compared to those from the coalescent simulation.

640 Additionally, it also revealed significant levels of reticulation in the clade,  
641 implying intricate relationships among the species within tribe Campanuleae (Fig. 4a).

642 The coalescent simulation analysis for tribe Campanuleae suggested that ILS  
643 could not fully explain the observed conflict between gene trees and species trees (Fig.  
644 4f). We selected specific species as representatives to investigate possible hybrid events  
645 within tribe Campanuleae. The plot of pseudo-loglikelihood scores suggested that the  
646 optimal number of hybrid events, inferred from the SNaQ network analysis, was one  
647 (Fig. 4b; Supplementary Fig. S29). This indicated the hybrid origin of subtribe  
648 Jasioninae 1, arising between subtribe Jasioninae 2 ( $\gamma = 0.895$ ) and subtribe  
649 Phyteumatinae 2 ( $\gamma = 0.105$ ) (Fig. 4c).

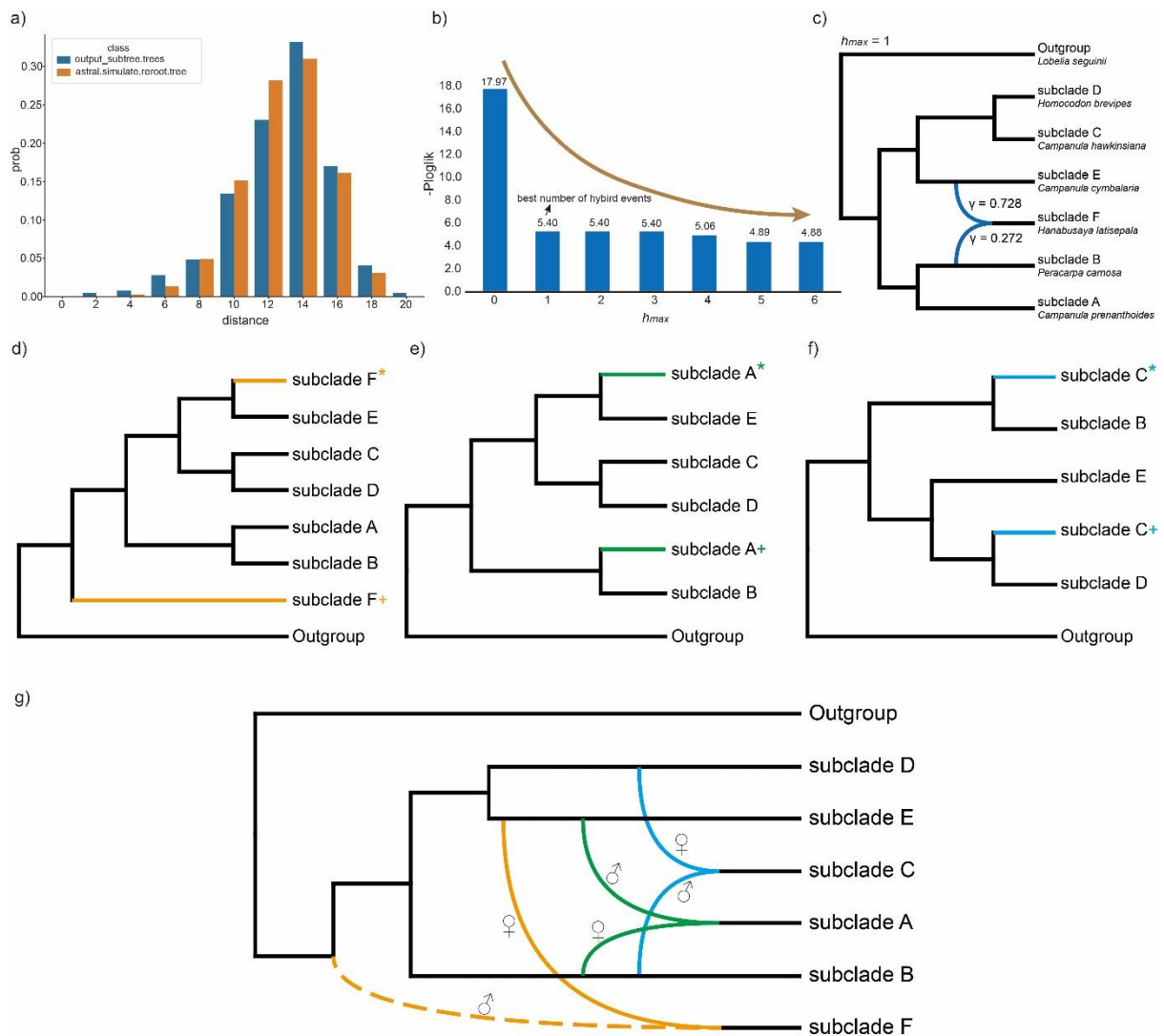
650 GRAMPA was employed to investigate potential allopolyploidy events associated  
651 with the origin of the subtribe Jasioninae. The inferred MUL-tree supported that the  
652 subtribe Jasioninae was of allopolyploid origin between subtribe Musschiinae and an  
653 unsampled or extinct lineage sister to tribe Campanuleae (Fig. 4d; Supplementary Fig.  
654 S30).

#### 655 *Reticulate Evolution of the Six Subclades in the Subtribe Phyteumatinae*

656 Given the intensive discordance among the topologies based on different datasets



657 and alternative phylogenetic inference methods, several analyses were employed to test  
 658 the causes. We used coalescent simulation analysis to assess the impact of ILS, and the  
 659 results indicated that ILS was not the primary factor contributing to the conflict within  
 660 clade I (Fig. 5b). Several species were chosen from different subclades as  
 661 representatives to identify possible hybrid events within clade I. Using the SNaQ  
 662 network analysis, we identified the optimal number of hybrid events ( $h_{max} = 1$ ) with the  
 663 best score (Fig. 5c; Supplementary Fig. S31), revealing the hybrid origin of subclade F,  
 664 with parental lineages subclade E ( $\gamma = 0.728$ ) and subclade B ( $\gamma = 0.272$ ) (Fig. 5d).



665 **Figure 5.** Phylogenetic network exploration of the six monophyletic groups (subclades)



666 within the subtribe Phyteumatinae. **a)** Coalescent simulation analysis showcasing the  
667 distribution of distances between empirical gene trees and the species tree. **b)** Bar chart  
668 showcasing pseudo-loglikelihood scores (-ploglik) over a spectrum of maximum  
669 reticulations, ranging from zero to six. The chart underscores  $h_{max} = 1$  as the optimal  
670 count for hybrid events. **c)** Phylogenetic network inferred from SNaQ analysis with  $h_{max}$   
671 = 1 as the optimal network. To the right, representative species from various subclades  
672 are noted with their names in smaller font sizes, displayed beneath their associated  
673 subclades. **d)** The multi-labeled trees (MUL-trees) based on the species tree inferred  
674 from nuclear MO dataset, encompassing all taxa within clade I (= subtribe  
675 Phyteumatinae). Branches in orange highlight the potential allopolyploid origin of  
676 subclade F, further denoted by an orange asterisk or plus sign. **e)** The MUL-trees  
677 visualized after excluding subclade F, as depicted in d). Branches colored in green  
678 indicate the allopolyploid origin of subclade A, further marked by a green asterisk or  
679 plus sign. **f)** The MUL-trees visualized after removing subclades A and F, as referenced  
680 in e). Branches in blue highlight the allopolyploid origin of subclade C, complemented  
681 by a blue asterisk or plus sign. **g)** Summary of the potential reticulate evolutionary  
682 relationships among the six subclades within the subtribe Phyteumatinae. The orange,  
683 green, and blue curves depict the allopolyploidy events determined by the GRAMPA  
684 analysis, with the dotted line signifying an extinct ancestor. The symbols ♂ and ♀ on  
685 the curving branches signify paternal and maternal parents.

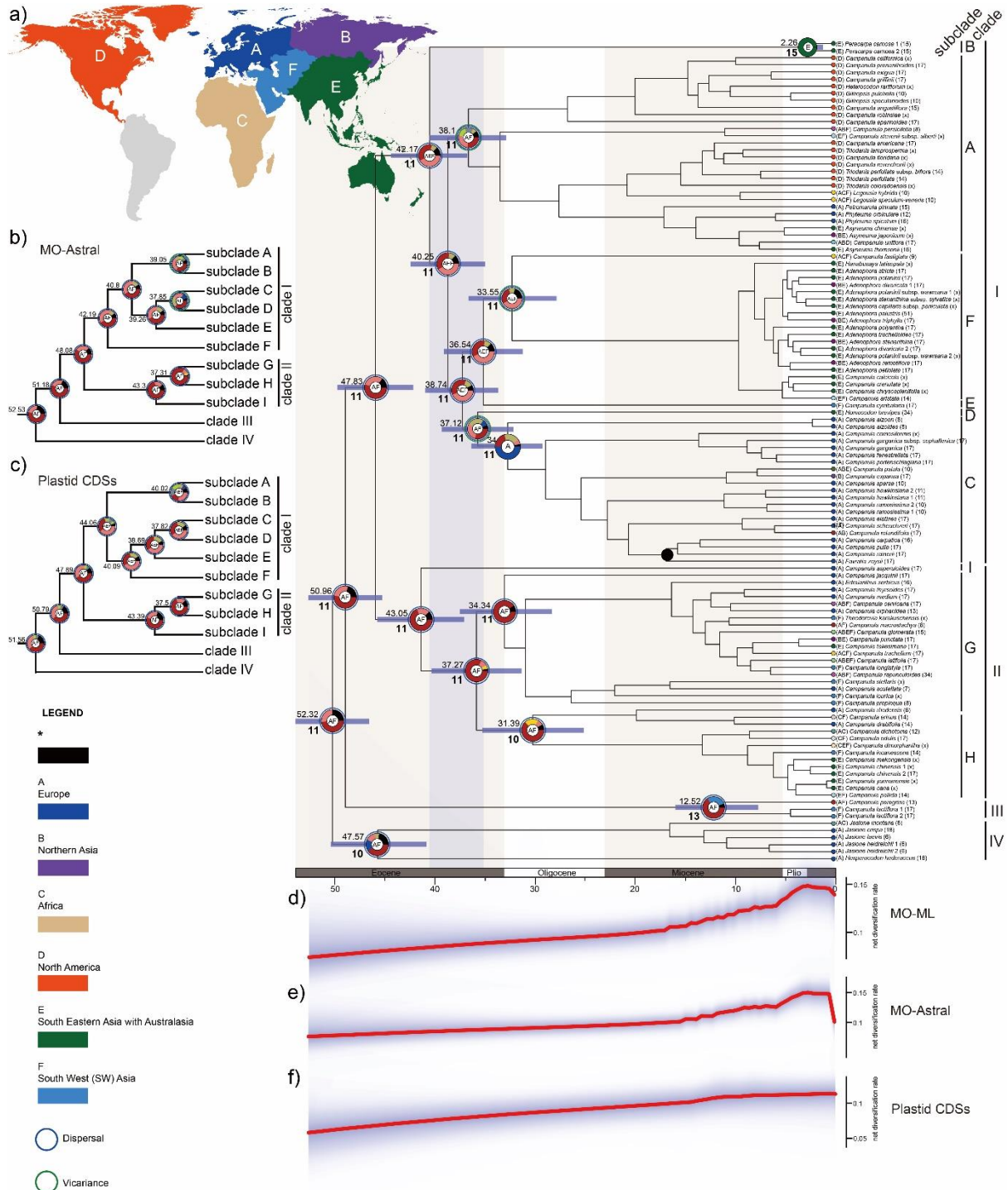
686 The MUL-tree inferred from GRAMPA with the lowest reconciliation score

687 indicated the allopolyploid origin of subclade F, with the subclade E and the common  
688 ancestor of clade I as parental lineages (Fig. 5e; Supplementary Fig. S32). After  
689 removing subclade F (Fig. 5e orange), the analysis revealed subclade A as an  
690 allopolyploid clade, with parental lineages including subclades E and B (Fig. 5f;  
691 Supplementary Fig. S33). Finally, upon removing subclade A (Fig. 5f green), the  
692 analysis found subclade C to have an allopolyploid origin clade between subclade B  
693 and subclade D (Fig. 5g; Supplementary Fig. S34).

694 *Dating Analyses, Ancestral Area, and Chromosome Number Reconstructions*

695 We constrained nodes with a fossil calibration point to estimate divergence times  
696 and diversification dynamics based on three different phylogenies (ML tree and  
697 ASTRAL tree inferred from the MO dataset; ML tree inferred from the plastid CDSs)  
698 within the subfamily Campanuloideae. The result based on all three phylogenetics  
699 indicated that the tribe Campanuleae originated in Europe-South West Asia during the  
700 early Eocene, diverging at *c.* 52.32 million years ago (Mya) (95% highest posterior  
701 density (HPD): 56.19-48.5 Mya) (Fig. 6a; Supplementary Figs. S35-S37).

702 **Figure 6.** Divergence time estimation and geographical range evolution of the  
 703 bellflower tribe Campanuleae. a) Dated chronogram of the tribe Campanuleae inferred  
 704 from PAML based on the nuclear MO dataset. Focal nodes feature estimated



705 divergence times and ancestral geographical ranges, with the purple shading  
706 highlighting the divergence time range for the six subclades within clade I (equivalent  
707 to subtribe Phyteumatinae). The inset map in the upper left outlines the six distribution  
708 areas used for geographical analysis: (A) Europe, (B) Northern Asia, (C) Africa, (D)  
709 North America, (E) Southern East Asia with Australasia, and (F) South West (SW)  
710 Asia. A black circle denotes the fossilized seed constraint. The gametophytic  
711 chromosome count was displayed in brackets next to the relevant species name, and the  
712 boldface numbers represent the reconstructed ancestral chromosome counts. **b)** The  
713 ASTRAL-estimated species tree of the nuclear MO dataset with estimated divergence  
714 time and geographical range marked on focal nodes. **c)** The plastid CDSs-inferred  
715 topology with estimated divergence time and geographical range marked on focal  
716 nodes. **d)** Net diversification rate (species/my) of Campanuleae estimated from the  
717 Maximum Likelihood (ML) phylogeny using the nuclear MO dataset. The red line  
718 represents the median, while the shaded blue area demarcates the 95% credible  
719 intervals for the rate. **e)** The net diversification rate estimated from the species tree of  
720 ASTRAL-III based on the nuclear MO dataset. **f)** The net diversification rate from the  
721 plastid phylogeny.

722 Furthermore, the majority of clades underwent divergence during the Eocene to  
723 the Oligocene period. Clade I had its origins in Europe-South Eastern Asia with  
724 Australasia-South West Asia by the middle Eocene, and diverged at *c.* 42.17 Mya (95%  
725 HPD: 46.22-38.22 Mya) based on the ML-trees inferred from the MO dataset and

726 plastid CDSs (Fig. 6a-c; Supplementary Figs. S35-S43). In contrast, the result based on  
727 the ASTRAL-tree inferred from the MO dataset indicated Europe-South West Asia as  
728 the origin of clade I (Fig. 6b). Within clade I, six subclades underwent divergence  
729 during the Eocene by around *c.* 42.17-36.54 Mya based on the MO dataset (Fig. 6a light  
730 purple shadow, 6b), but a little earlier based on the plastid CDSs which indicated the  
731 divergence by *c.* 44.06-37.82 Mya (Fig. 6c). In addition, clade II diverged around *c.*  
732 43.05 Mya (95% HPD: 47.62-38.55 Mya), and clade IV followed suit at *c.* 47.57 Mya  
733 (95% HPD: 52.5-42.48 Mya). In contrast, clade III experienced diversification in the  
734 Miocene, occurring approximately *c.* 12.52 Mya (95% HPD: 16.46-7.9 Mya) (Fig. 6a).  
735 The diversification rates, as inferred from both the MO dataset and the plastid CDSs,  
736 exhibited an increasing trend from the Eocene to the present. Moreover, the rates  
737 inferred from the MO dataset showed sudden increases in the late Miocene, which  
738 indicated rapid speciation (Fig. 6d-f).

739 The model CONST\_RATE\_DEMI\_EST, as determined by ChromEvol, was  
740 identified as the best fit for chromosome evolution (AIC = 503.2). Using this model, we  
741 deduced that the most probable ancestral haploid chromosome number ( $n = 11$ ) was  
742 shared by the subfamily Campanuloideae and the tribe Campanuleae. This count was  
743 consistently observed in the MRCA of the tribes Cyanantheae, Wahlenbergieae, and  
744 Campanuleae. Within the tribe Campanuleae, the MRCA of both clades I and II  
745 maintained the ancestral haploid chromosome number 11. In contrast, the MRCA of  
746 clade III presented an ancestral chromosome number of 13, and clade IV had a count of

747 10. For most subclades within clades I and II, the ancestral number remained 11, with  
748 the exception of subclade B, which exhibited a chromosome number of 15 (Fig. 6a;  
749 Supplementary Fig. S44).

750

## 751 DISCUSSION

752 By integrating multiple genomic data sources and various phylogenetic inference  
753 methods, we produced a robust phylogenetic backbone for the bellflower tribe  
754 Campanuleae within the subfamily Campanuloideae. Our analyses of deep reticulate  
755 evolution suggest that hybridization and allopolyploidization have played a pivotal role  
756 in the diversification of the Campanuleae tribe, especially in the early diversification of  
757 the subtribe Phytentinae. We demonstrated that fully untangling deep reticulation is  
758 optimally accomplished by leveraging data from multiple genomic sources, especially  
759 when inferring the phylogenetic backbone of non-model plant lineages. Additionally,  
760 thorough taxon sampling is also critically important to fully dissect deep reticulation.  
761 Moreover, it is essential to note that conflicting topologies derived from separate  
762 genomic datasets and inference methods can significantly influence downstream  
763 inferences, such as dating analyses, ancestral area determination, and diversification  
764 rate estimates.

765 *Exploring the Reticulate Phylogenetic Backbone of the Bellflower Tribe Campanuleae*

766 In traditional phylogenetic models, evolutionary relationships are portrayed as  
767 bifurcating branches, suggesting that species or lineages diverge and proceed to evolve  
768 independently. However, this strictly branching framework often fails to capture the  
769 complexity of evolutionary dynamics as they occur in nature (Jin et al. 2023). In many  
770 instances, evolutionary processes are better represented as being network-like, where  
771 species or lineages may engage in various forms of hybridization or experience  
772 intricate, web-like interactions (Stull et al. 2023). Such network-like patterns can arise  
773 from various complex processes involving multiple and ongoing events, such as ILS,  
774 introgression, hybridization, and/or allopolyploidization.

775 In this study, we undertook a comprehensive approach integrating extensive taxon  
776 sampling with diverse genomic data types, including DGS, Hyb-Seq, RNA-Seq, and  
777 WGS. Utilizing state-of-the-art automated tree-based orthology inference methods  
778 (Yang and Smith 2014; Morales-Briones et al. 2022), we carefully estimated paralogs  
779 and generated three alternative nuclear data matrices (1to1, MO, and RT), along with a  
780 complete dataset of 79 plastid CDSs for phylogenomic analysis. Employing various  
781 phylogenomic inference methods, such as concatenated and coalescent-based methods,  
782 we produced 16 phylogenetic trees—12 nuclear and four plastid. These trees confirmed  
783 the classification of three tribes as previously outlined by Hong and Wang (2015) and  
784 recovered four strongly supported major clades—namely Clade I, II, III, and IV—in the  
785 tribe Campanuleae (Fig. 2; Supplementary Figs. S3-S14, S23-S26). Nevertheless, our

786 findings also highlighted the ambiguous phylogenetic position of Clade IV (or the  
787 subtribe Jasioninae), which appears to be either a sister group to the other three clades  
788 in Campanuleae (Fig. 2; Supplementary Figs. S3-S14, S23, S25 and S26) or related to a  
789 combined clade (Clade I + II, as shown in Supplementary Fig. S24). We hypothesized  
790 that the evolutionary relationships among these four major clades in the Campanuleae  
791 tribe may not be strictly bifurcating but could exhibit a more network-like structure.  
792 This hypothesis was corroborated by our SplitsTree network analysis (Fig. 4a).

793 Tree incongruence has been a prevalent and challenging issue in molecular  
794 phylogenetic studies (Wendel and Doyle 1998; Som 2015; Kapli et al. 2020; Steenwyk  
795 et al. 2023). Such incongruence can arise from both biological and non-biological  
796 factors. Non-biological factors primarily encompass limited taxon and/or gene  
797 sampling, the use of paralogous genes, and errors in sequence alignment (Steenwyk et  
798 al. 2023). In our study, these factors seem insufficient to explain the observed  
799 incongruence. Our extensive taxon sampling and use of genomic-level data largely rule  
800 out stochastic uncertainties from insufficient taxon and/or gene sampling. Furthermore,  
801 we used three strategies of orthology inference to reduce the effect of paralogs, and it is  
802 unlikely the observed topological inconsistencies among individual gene trees can be  
803 attributed solely to paralogy. In addition, sequence alignments have undergone  
804 meticulous visual examination and are mostly well-aligned. Tools like trimAl help trim  
805 low-quality alignments, and TreeShrink filters out samples with aberrantly long  
806 branches. This suggests that our sequence alignment is unlikely a major factor in tree



807 incongruence. Biologically, tree incongruences can result from ILS,  
808 hybridization/introgression, or horizontal gene transfer (HGT; Kapli et al. 2020). While  
809 HGT is more common in bacteria and archaea, it is less so in eukaryotes (Kurland et al.  
810 2003; Keeling and Palmer 2008; Boto 2010), making it an unlikely cause of the tree  
811 incongruence in our case. On the other hand, both ILS and hybridization/introgression  
812 are established contributors to tree incongruence across the tree of life (Degnan and  
813 Rosenberg 2009; Mallet et al. 2016). Thus, it is plausible that these factors, individually  
814 or in combination, are behind the observed tree incongruence in Campanuleae.

815 Distinguishing between ILS and hybridization/introgression based solely on  
816 patterns of tree incongruence is challenging. Both processes can produce similar  
817 incongruence patterns, complicating the identification of the specific cause of  
818 incongruence in phylogenetic studies (Degnan and Rosenberg 2009; Degnan 2018). To  
819 further dissect the potential events contributing to this network-like evolution, we  
820 performed step-by-step analyses to distinguish the possible events in this net-like  
821 evolution. Coalescent simulation analyses indicated that ILS alone could not account  
822 for the observed reticulate patterns (Fig. 4f). Subsequent PhyloNetwork analyses using  
823 the SNaQ algorithm and polyploidy assessments with the GRAMPA reconciliation  
824 algorithm were carried out to explore the potential roles of hybridization and  
825 polyploidy.

826 Given the strongly supported gene tree and species tree discordance for the  
827 phylogenetic position of clade III and IV in different datasets and inference methods

828 (Fig. 1), the phylogenetic position of subtribe Jasioninae remains a subject of debate.  
829 As we delved into the diversification of the three tribes and four subtribes, we  
830 pinpointed an allopolyploidy event (Fig. 4b,c; Supplementary Fig. S31) and a distinct  
831 hybridization event (Fig. 4d; Supplementary Fig. S30). The GRAMPA analysis  
832 unveiled the allopolyploid origin of subtribe Jasioninae, resulting from the MRCA of  
833 the tribe Campanuleae (acting as the maternal lineage) and the MRCA of the subtribe  
834 Musschiinae (serving as the paternal lineage, Fig. 4d,e). Our analyses of dating and  
835 ancestral area reconstruction consistently identified the likely occurrence of this  
836 allopolyploidization event in Europe and SW Asia (around the Tethys Sea) during the  
837 early Eocene, as supported by both nuclear (52.32 Mya from the ASTRAL species tree  
838 and 52.53 Mya from the ML tree) and plastid (51.56 Mya) topologies (Supplementary  
839 Figs. S35-S43). Further, our PhyloNetwork results confirmed the hybrid origin of  
840 *Jasione montana* between the MRCA of *Hanabusaya latisepala* (paternal lineage) and  
841 the MRCA of *Hesperocodon hederaceus* (maternal lineage). Nevertheless, the dating  
842 analyses of the stem clade of *Jasione montana* showed disparities between nuclear  
843 (17.08 Mya estimated from the ML tree and 16.53 Mya from the ASTRAL species tree)  
844 and plastid data (2.08 Mya). Considering these strongly supported but discordant time  
845 estimates, we hypothesize that the MRCA of *Jasione montana* might have captured the  
846 chloroplast genome from a particular *Jasione* species, an event possibly occurred in  
847 Europe during the early Quaternary.

848 Within the subtribe Phyteumatinae, we conducted thorough taxon sampling

849 encompassing all 15 clades, as confirmed by Xu and Hong (2021). Significant  
850 nuclear/plastid gene trees and cytonuclear discordance were detected among the 16  
851 generated trees (Fig. 2; Supplementary Figs. S3-S14, S23-S26). We classified the 15  
852 clades of subtribe Phyteumatinae into six monophyletic groups, labeled as subclades A-  
853 F (Figs. 3, 5). We utilized an iterative strategy to estimate the potential polyploidy  
854 origin for each subclade. The first GRAMPA reconciliation identified an allopolyploid  
855 origin of subclade F, arising between the MRCA of subclade E and the MRCA of  
856 subtribe Phyteumatinae (Fig. 5d). The close relationship of subclade F with subclade E  
857 in the plastid topologies (Fig. 3a) suggests that the MRCA of subclade E likely served  
858 as the maternal parent, while the MRCA of subtribe Phyteumatinae may have acted as  
859 the paternal parent. This allopolyploidization is partially consistent with the hybrid  
860 origin proposed by the SNaQ analysis, designating subclade E ( $\gamma = 0.728$ ) as the  
861 maternal parent and subclade B ( $\gamma = 0.272$ ) as the paternal parent (Fig. 5b,c).  
862 Interestingly, these two methodologies inferred different paternal origins. This  
863 discrepancy, marked by an uneven inheritance probability ( $\gamma = 0.728$  vs.  $0.272$ ), could  
864 clarify the conflicting topologies of subclade F in both the nuclear concatenated-based  
865 analyses and species tree (Fig. 3b,c). As a result, we concluded that the origin of the  
866 subclade F could be traced to multiple, undistinguishable hybrid swarms involving the  
867 MRCA of subclade E and the MRCA of subtribe Phyteumatinae, all of which were  
868 accompanied by a whole genome duplication (WGD) event. After excluding subclades  
869 F and A, the origins of subclades A and C were identified as allopolyploid, arising from

870 the combination of subclades E and B, and B and D, respectively (Fig. 5e,f). Our dating  
871 analysis, based on three distinct topologies depicted in Figure 6abc, supports the rapid  
872 radiation hypothesis for the six subclades (A to F) highlighted in the purple-shaded area  
873 of Figure 6a. This radiation possibly took place during the Middle to Late Eocene, with  
874 date ranges of 42.17-36.54 Mya from the nuclear ML topology, 42.19-37.85 Mya from  
875 the nuclear species tree, and 44.06-37.82 Mya from the plastid ML topology. Notably,  
876 discrepancies were observed between our nuclear and plastid topologies regarding the  
877 ancestral areas of the subtribe Phyteumatinae. The nuclear topologies suggest Europe &  
878 SW Asia (the Ancient Mediterranean region) as the ancestral areas, while the plastid  
879 topology points to a broader region encompassing Europe, SW Asia, and Southern East  
880 Asia and Australasia—essentially spanning much of Southern Eurasia. Given that  
881 plastid data may have a narrower genetic diversity (due to uniparental inheritance), they  
882 might not capture the full range of ancestral distributions compared to nuclear data. We  
883 concluded that the possible ancestral areas of the subtribe Phyteumatinae included the  
884 Ancient Mediterranean region. The European and Southwest Asian regions experienced  
885 pivotal transitions in both climate and tectonics. This era marked a departure from the  
886 extremely warm in the early Eocene to a slightly cooler, yet still warm global  
887 environment. It was also a time of significant tectonic events; the collision between the  
888 African, Eurasian, and Anatolian tectonic plates continued during this period, resulting  
889 in the formation of the Alpine mountain chain and uplift of mountain ranges like the  
890 Taurus and the Zagros. Additionally, the closure of the Tethys Sea also had significant

891 impacts on ocean circulation patterns and climate. The extreme climate and tectonic  
892 changes may have promoted the rapid diversification of the six major clades in subtribe  
893 *Phyteumatinae*.

894 The notable cytonuclear inconsistency in dating analyses, ancestral area  
895 reconstruction, net diversification rates analyses (Fig. 6) underscores a critical lesson:  
896 relying solely on a singular genomic data source might not furnish a comprehensive or  
897 precise depiction of biogeographic evolutions for potential reticulate lineages (Dong et  
898 al. 2022; Liu et al. 2022). This disparity underscores the need for integrative and multi-  
899 faceted approaches in phylogenomic research, ensuring a more holistic understanding  
900 of evolutionary histories and events. Utilizing multiple data sources can bridge  
901 knowledge gaps, validate findings, and offer richer insights into the complexities of  
902 lineage diversification and adaptation.

### 903 *Multi-source Genomic Data for Phylogenomic Analyses of Non-model Plants*

904 Phylogenomics has revolutionized our understanding of the evolutionary  
905 relationships among organisms by incorporating hundreds to thousands of nuclear  
906 genes in advanced phylogenetic inference methods. However, applying phylogenomics  
907 to non-model plants presents several challenges. Non-model plants display vast  
908 diversity, making it difficult to obtain representative samples that encompass the entire  
909 phylogenetic and geographical diversity of a particular group. Researchers from  
910 specific regions may have an advantage in collecting taxon samples from their areas,

911 but conducting comprehensive species sampling, especially for cosmopolitan lineages,  
912 becomes a challenge for a single laboratory. As a result, previous phylogenomic studies  
913 have mainly focused on resolving the phylogenetic backbone of major lineages at the  
914 family or even higher level, such as Xiang et al. (2017), Zhao et al. (2021), Huang et al.  
915 (2022), Hu et al. (2023), and Zhang et al. (2023), due to the ease of conducting  
916 comprehensive taxon sampling covering most major clades. However, incomplete  
917 taxon sampling has substantially hindered shallow-level phylogenomic studies of non-  
918 model plants, except for some ecologically and economically important lineages, such  
919 as apples (Liu et al. 2022) and *Rhododendron* (Xia et al. 2022). Moreover, limited  
920 sampling can lead to biased inferences and inaccurate estimation of evolutionary  
921 relationships (Heath et al. 2008; Nabhan and Sarkar 2011; Young et al. 2020). Limited  
922 or biased sampling can be particularly problematic when reticulation is prevalent  
923 because recent hybridization can obscure accurate inference of more ancient  
924 reticulation events.

925 To address these challenges, Guo et al. (2021) proposed several criteria for an  
926 optimal sequencing strategy for non-model plants, which recommended generation of  
927 hundreds to thousands of SCNs from a large number of samples. As Next-Generation  
928 Sequencing (NGS) technologies have developed, researchers in plant taxonomy,  
929 evolutionary biology, and horticulture have sequenced various types of genomic-level  
930 data from different species within their respective countries. Most of these raw data  
931 have been deposited in public data repositories, such as Sequence Read Archive (SRA)

932 in the National Center for Biotechnology Information (NCBI), National Genomics Data  
933 Center (NGDC), and European Nucleotide Archive (ENA). Since a single sequencing  
934 technology does not entirely fulfill the requirements for non-model plant  
935 phylogenomics, our objective is to propose an alternative method by integrating all  
936 available genomic data and employing a feasible bioinformatic pipeline. Liu et al.  
937 (2021, 2022) introduced a practical and innovative approach for assembling SCN genes  
938 and plastomes, as well as conducting phylogenomic discordance analyses in non-model  
939 plants. The researchers identified three primary data types that proved to be particularly  
940 suitable for their methodology: DGS, WGS, and RNA-Seq data. Each data type offered  
941 unique advantages in contributing to a comprehensive understanding of the genomic  
942 landscape and evolutionary history of non-model plants. DGS data, which were  
943 generated with approximately 10× coverage, and WGS data, with higher coverage at  
944 around 20×, were both derived from whole genome DNA libraries. These two  
945 approaches complemented each other, with DGS providing a cost-effective means of  
946 obtaining broader genomic coverage and WGS offering higher sequencing depth for  
947 more accurate variant calling and detection of rare genetic variations. The combination  
948 of both DGS and WGS data allowed for a robust analysis of genomic diversity and  
949 evolutionary patterns within the studied plant lineages. In addition to DGS and WGS,  
950 the researchers utilized RNA-Seq data, which represented the complete set of RNA  
951 transcripts of expressing coding regions. Moreover, the focus on nuclear protein-coding  
952 genes, particularly nuclear SCN genes, in current phylogenomic studies offered an

953 effective strategy to resolve the phylogenetic relationships of non-model plants. The  
954 conserved nature of nuclear SCN gene ensures their suitability for inferring  
955 evolutionary relationships across a wide range of plant species. Leveraging these genes  
956 provided a stable and well-supported phylogenetic backbone for the studied lineages,  
957 allowing researchers to confidently infer the evolutionary history of non-model plants.

958 Collectively, the integration of DGS, WGS, and RNA-Seq data, along with the  
959 emphasis on nuclear SCN genes, has opened up new avenues of research in the  
960 phylogenomics of non-model plants. This integration of multi-source genomic data  
961 approach has empowered researchers to explore the rich genomic diversity and  
962 evolutionary patterns of diverse plant lineages more comprehensively and accurately.  
963 By leveraging the various types of available data and employing innovative  
964 bioinformatic pipelines, researchers can now gain deeper insights into the evolutionary  
965 relationships, historical biogeography, and functional implications of genetic variations  
966 in non-model plants, ultimately advancing our understanding of plant evolution and  
967 diversification. Continued advancements in sequencing technologies and analytical  
968 methods are expected to further enhance the potential of phylogenomics, opening up  
969 exciting possibilities for unraveling the mysteries of plant biodiversity and adaptation.

970 *An updated infra-tribal classification of the tribe Campanuleae*

971 **Campanulaceae** Juss., Gen. Pl. [Jussieu] 163 (1789), nom. cons. Type: *Campanula* L.



- 972 Subfamily **Campanuloideae** Burnett, *Outlines Bot.* 942, 1094, 1110 (1835). Type:
- 973 *Campanula* L.
- 974 Tribe **Campanuleae** Dumort., *Fl. Belg.* 58. 1827. Type: *Campanula* L.
- 975 = tribe Jasioneae Dumort., *Fl. Belg.* 59. 1827. Type: *Jasione* L.
- 976 = tribe Phyteumateae Dumort., *Fl. Belg.* 59. 1827, ‘Phyteumeae’. Type: *Phyteuma* L.
- 977 = tribe Peracarpeae Fed., *Fl. URSS* 24: 471. 1957. Type: *Peracarpa* Hook.f. &
- 978 Thomson.
- 979 = tribe Michauxieae Fed., *Fl. URSS* 24: 472. 1957. Type: *Michauxia* L’Hér.
- 980 = tribe Edraiantheae Fed., *Fl. URSS* 24: 475. 1957. Type: *Edraianthus* A.DC.
- 981 = tribe Annaeae Kolak., *Bot. Zhurn. (Moscow & Leningrad)* 72(12): 1575. 1987,
- 982 ‘Annaea’. Type: *Annaea* Feer.
- 983 = tribe Azorineae Kolak., *Bot. Zhurn. (Moscow & Leningrad)* 72(12): 1575. 1987.
- 984 Type: *Azorina* Feer.
- 985 = tribe Echinocodoneae Kolak., *Bot. Zhurn. (Moscow & Leningrad)* 72(12): 1575.
- 986 1987, nom. illeg. Type: *Echinocodon* Kolak., *Soobshch. Akad. Nauk Gruz. SSR*,
- 987 121(2): 387. 1986, hom. illeg. non D.Y. Hong, *Acta Phytotax. Sin.*, 22(3): 183.
- 988 1984.
- 989 = tribe Gadellieae Kolak., *Bot. Zhurn. (Moscow & Leningrad)* 72(12): 1576. 1987.
- 990 Type: *Gadellia* Schulk.
- 991 = tribe Muehlbergelleae Kolak., *Bot. Zhurn. (Moscow & Leningrad)* 72(12): 1575.
- 992 1987. Type: *Muehlbergella* Feer.

- 993 = tribe *Musschieae* Kolak., Bot. Zhurn. (Moscow & Leningrad) 72(12): 1575. 1987.
- 994 Type: *Musschia* Dumort.
- 995 = tribe *Mzymteleae* Kolak., Bot. Zhurn. (Moscow & Leningrad) 72(12): 1578. 1987.
- 996 Type: *Mzymtella* Kolak.
- 997 = tribe *Neocodoneae* Kolak., Bot. Zhurn. (Moscow & Leningrad) 72(12): 1577. 1987.
- 998 Type: *Neocodon* Kolak. & Serdyuk.
- 999 = tribe *Sachokieleae* Kolak., Bot. Zhurn. (Moscow & Leningrad) 72(12): 1578. 1987.
- 1000 Type: *Sachokiella* Kolak.
- 1001 = tribe *Sergieae* Kolak., Bot. Zhurn. (Moscow & Leningrad) 72(12): 1577. 1987. Type:
- 1002 *Sergia* Fed.
- 1003 = tribe *Theodorovieae* Kolak., Bot. Zhurn. (Moscow & Leningrad) 72(12): 1575. 1987.
- 1004 Type: *Theodorovia* Kolak.
- 1005 = tribe *Echinococonieae* Kolak., Bot. Zhurn. (Moscow & Leningrad) 79(1): 114. 1994.
- 1006 Type: *Echinocodonia* Kolak.
- 1007 = tribe *Pseudocampanuleae* Kolak., Bot. Zhurn. (Moscow & Leningrad) 79(1): 115.
- 1008 1994. Type: *Pseudocampanula* Kolak.
- 1009 I. subtribe **Campanulinae** R.Schönland (clade II) in H.G.A. Engler & K.A.E. Prantl,
- 1010 Nat. Pflanzenfam. IV, 5(48). 1889. Type: *Campanula* L.
- 1011 Included genera: *Azorina*, *Campanula*, *Edraianthus*, *Michauxia*, *Muehlbergella*,
- 1012 *Sachokiella*, *Theodorovia*, *Trachelium*, and *Zeugandra*.

- 1013 II. subtribe **Phyteumatinae** Caruel (clade I), *Epit. Fl. Europ.* 2: 248. 1894,  
1014 ‘Phyteumeae’. Type: *Phyteuma* L.  
1015 Included genera: *Adenophora*, *Astrocodon*, *Asyneuma*, *Brachycodonia*,  
1016 *Cryptocodon*, *Cylindrocarpa*, *Decaprisma*, *Eastwoodiella*, *Favratia*, *Githopsis*,  
1017 *Hanabusaya*, *Hayekia*, *Heterocodon*, *Homocodon*, *Legousia*, *Loreia*, *Melanocalyx*,  
1018 *Palustricodon*, *Peracarpa*, *Petromarula*, *Physoplexis*, *Phyteuma*, *Poolea*, *Protocodon*,  
1019 *Ravenella*, *Rotantheta*, *Sergia*, *Smithiastrum*, and *Triodanis*.  
1020 III. subtribe **Musschiinae** B.B.Liu (clade III), **stat. nov.** basionym: tribe Musschieae  
1021 Kolak. *Bot. Zhurn. (Moscow & Leningrad)* 72: 1575. 1987. Type: *Musschia* L.  
1022 Included genera: *Echinocodonia*, *Gadellia*, and *Musschia*.  
1023 IV. subtribe **Jasioninae** Endl. (clade IV), *Gen. Pl. [Endlicher]*. 514. 1838, ‘Jasioneae’.  
1024 Type: *Jasione* L.  
1025 Included genera: *Feeria*, *Hesperocodon*, and *Jasione*.

## 1026 CONCLUSIONS

1027 We focused on the bellflower tribe Campanuleae, a non-model plant lineage  
1028 known for its extensive history of hybridization and introgression. We presented a  
1029 comprehensive and versatile framework for deciphering the network-like phylogenetic  
1030 relationships within such lineages, relying on various genomic data sources and  
1031 analytical methods. Our results unveiled compelling evidence supporting the role of

1032 allopolyploidization and hybridization in promoting the early diversification of the  
1033 Campanuleae tribe. Notably, the rapid radiation of six major subclades in the subtribe  
1034 Phyteumatinae may have been driven by multiple and continuous allopolyploidization  
1035 events, taking place in the ancient Mediterranean region during the Middle to Late  
1036 Eocene epochs. Furthermore, our study emphasizes a significant challenge in  
1037 evolutionary biology research: conflicting tree topologies derived from different  
1038 genomic datasets and phylogenetic inference methods can lead to substantial variation  
1039 in our downstream estimates of the timing of evolutionary events, ancestral geographic  
1040 origins, and diversification rates.

#### 1041 SUPPLEMENTARY MATERIAL

1042 Data available from the Dryad Digital Repository:

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1051

AUTHOR CONTRIBUTIONS

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B.B.L. designed the project and supervised the study. B.B.L., Z.T.J., S.Y.X., and

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C.X. wrote the draft manuscript. Z.T.J., S.Y.X., and Y.Z. carried out the phylogenomic

1054

analyses. C.X. performed the deep genome skimming sequencing. B.L. provided

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S.H.J., L.Z., C.R., and D.Y.H. provided suggestions for structuring the paper. All the

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authors contributed to the writing and interpreting of the results and approved the final

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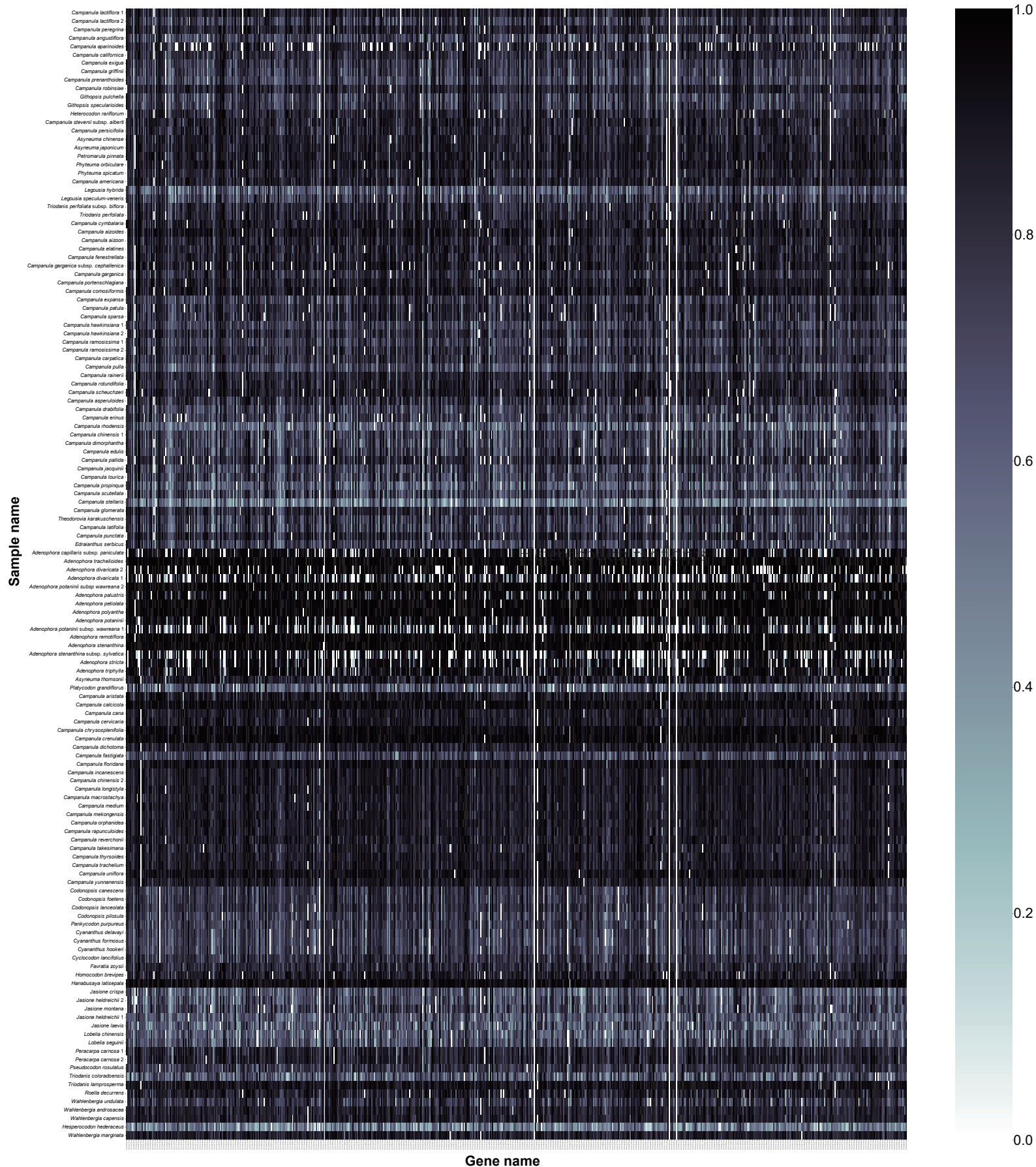
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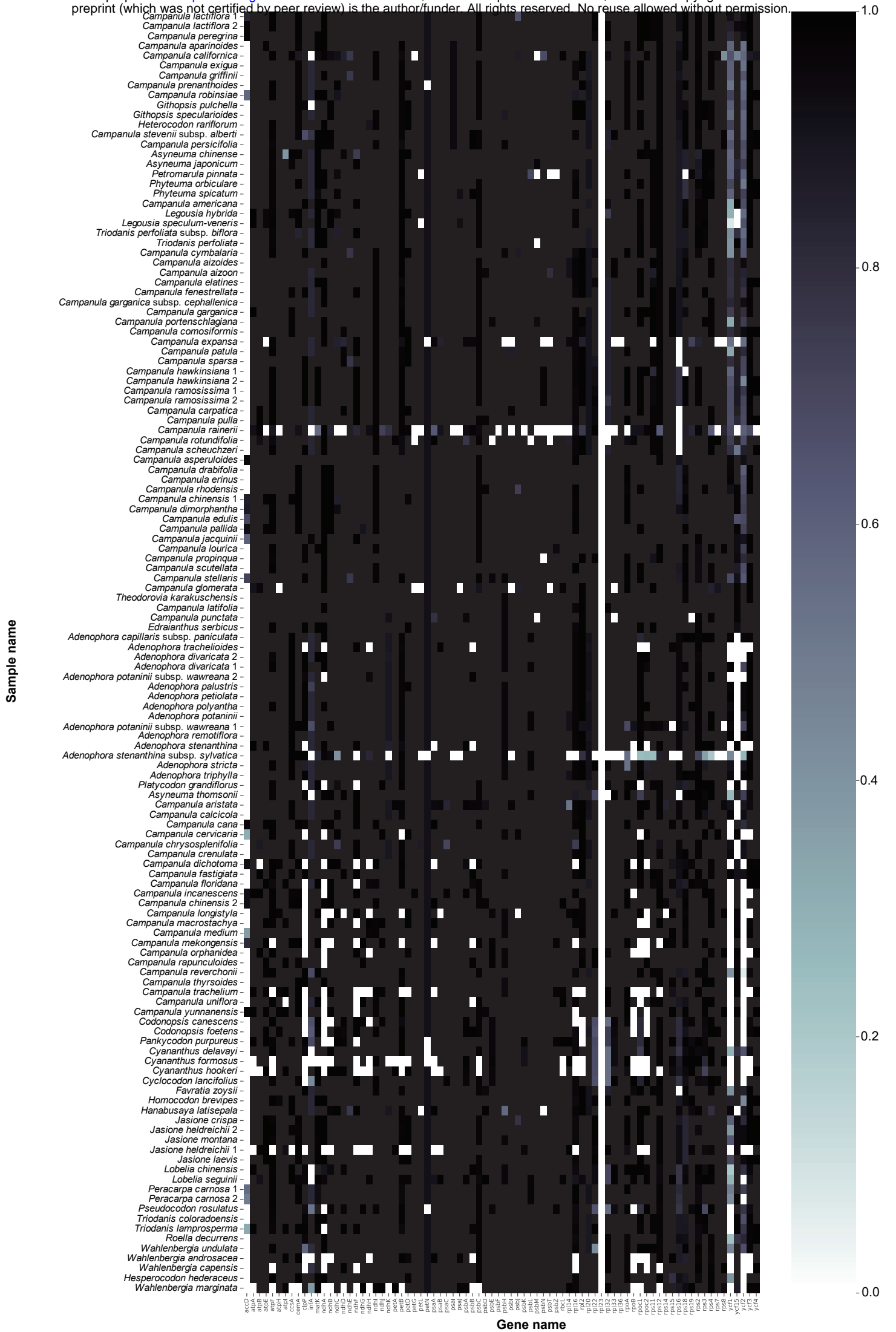
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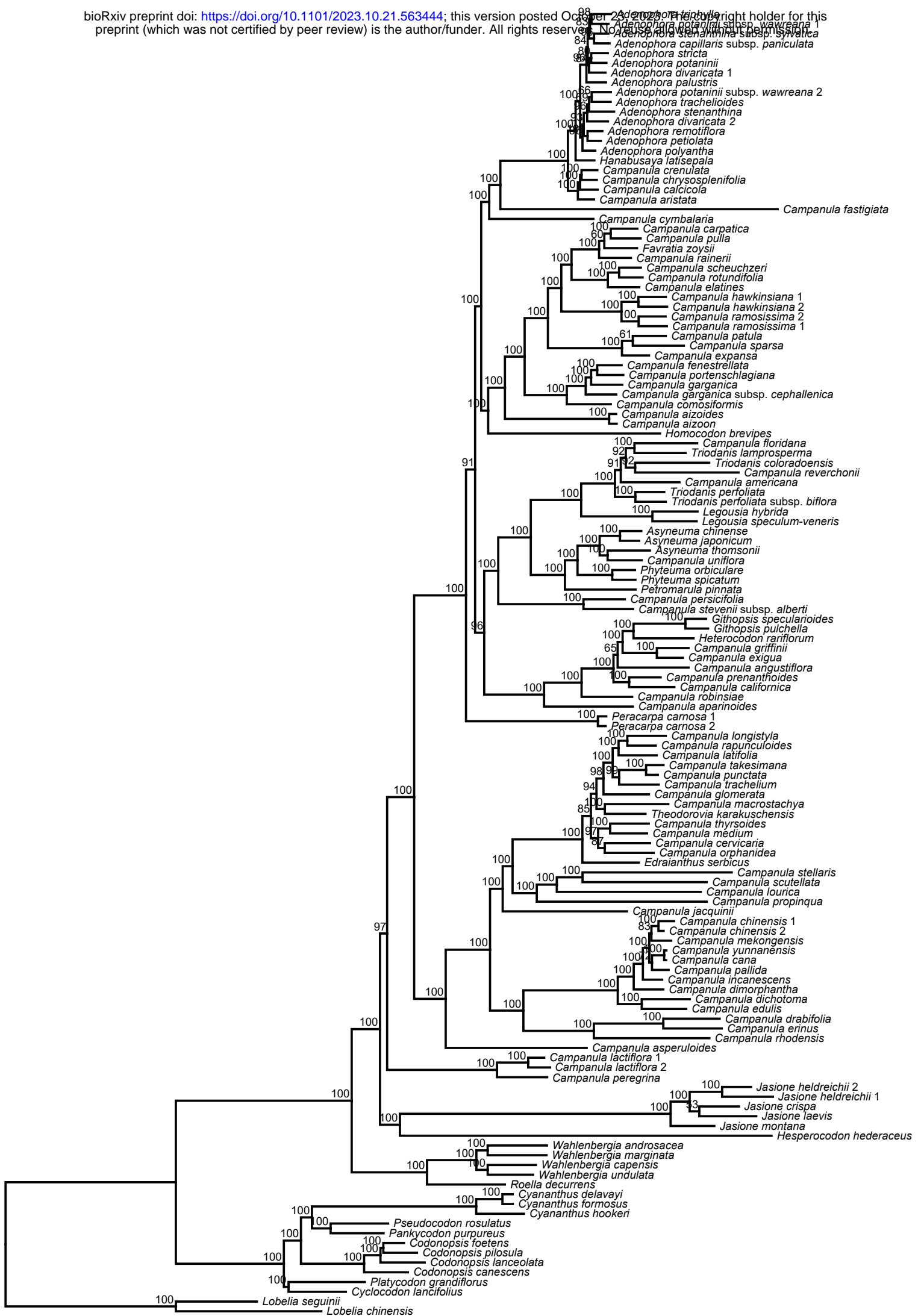
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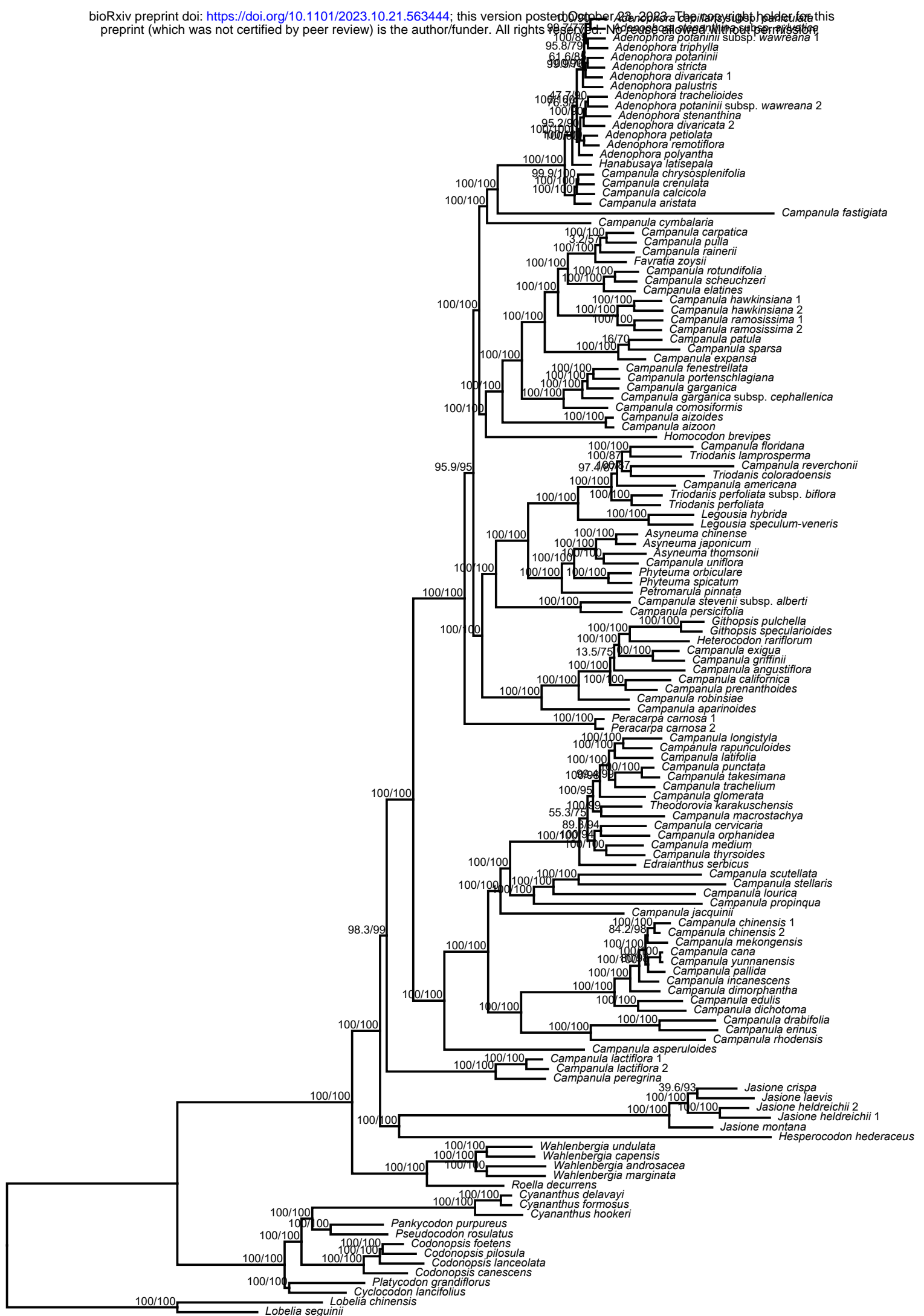
Percentage length recovery for each gene, relative to mean of targetfile references

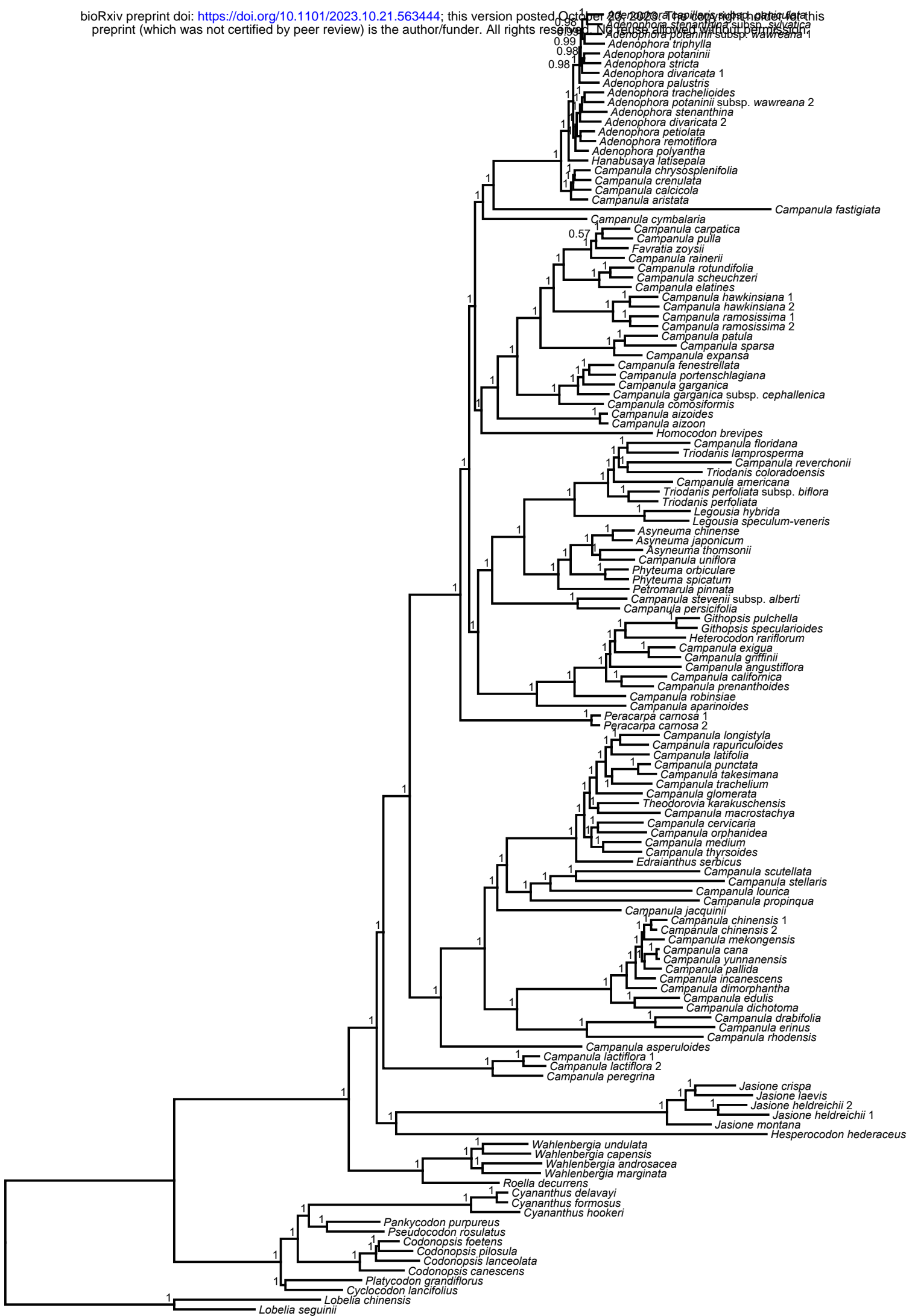


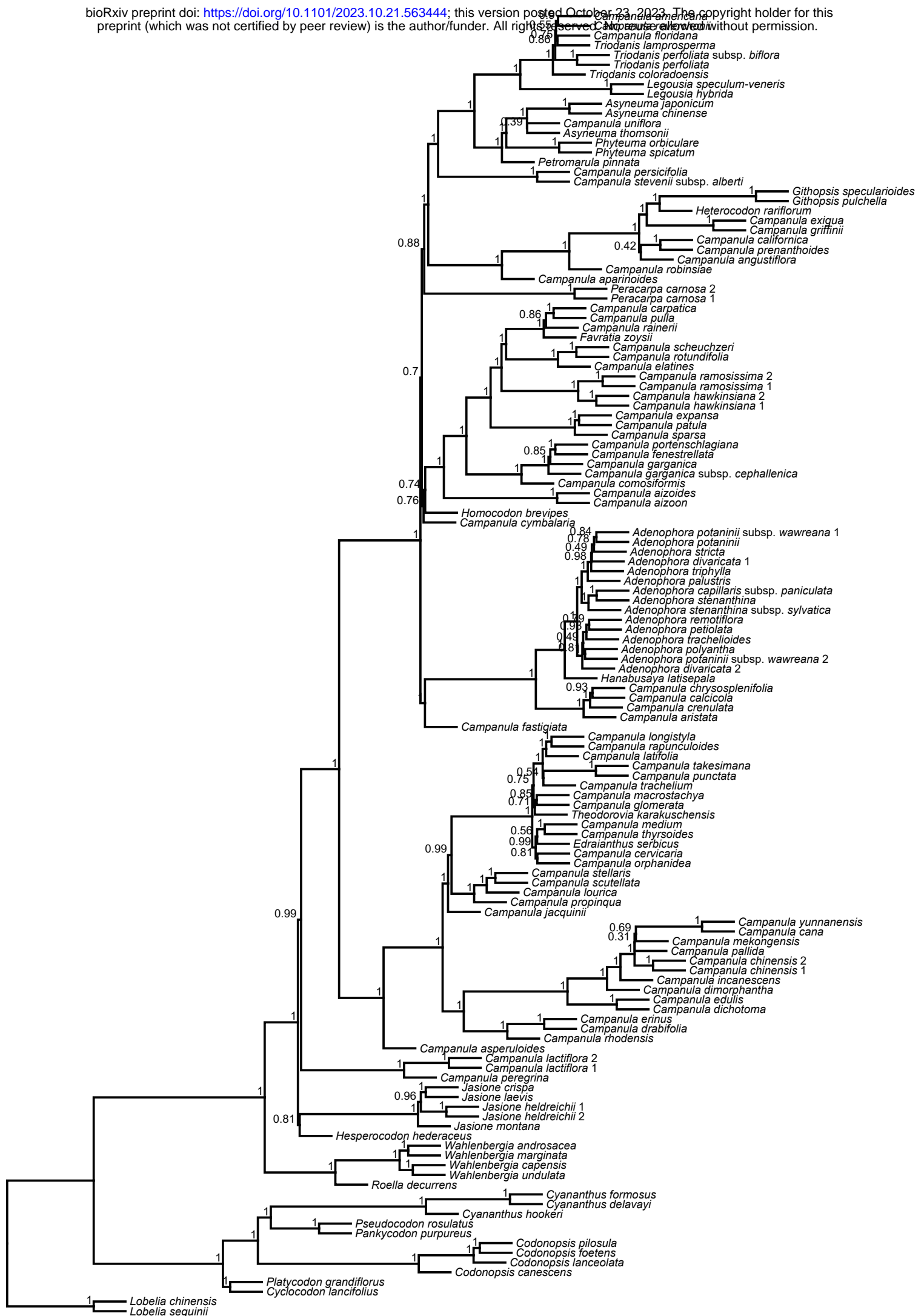


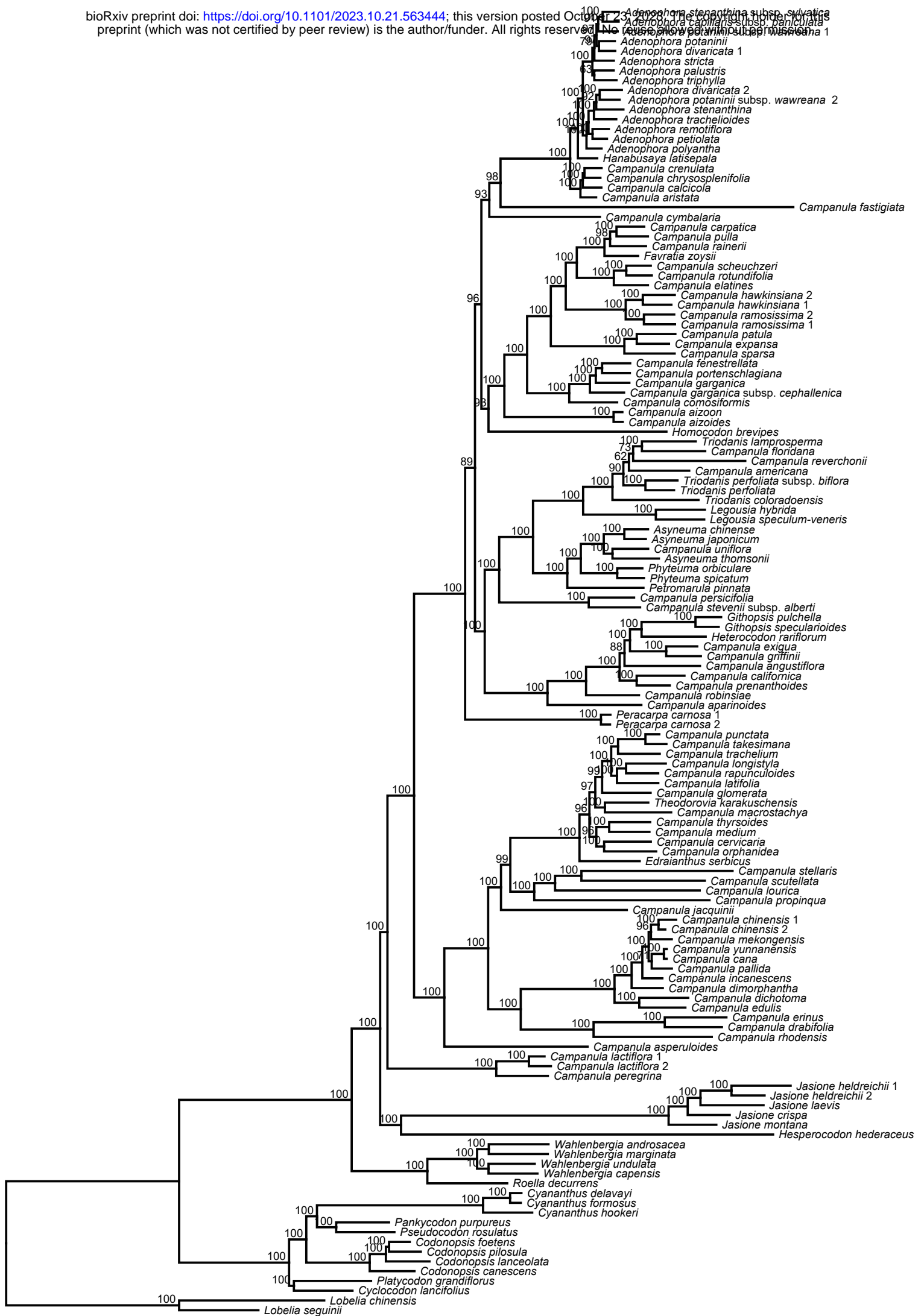




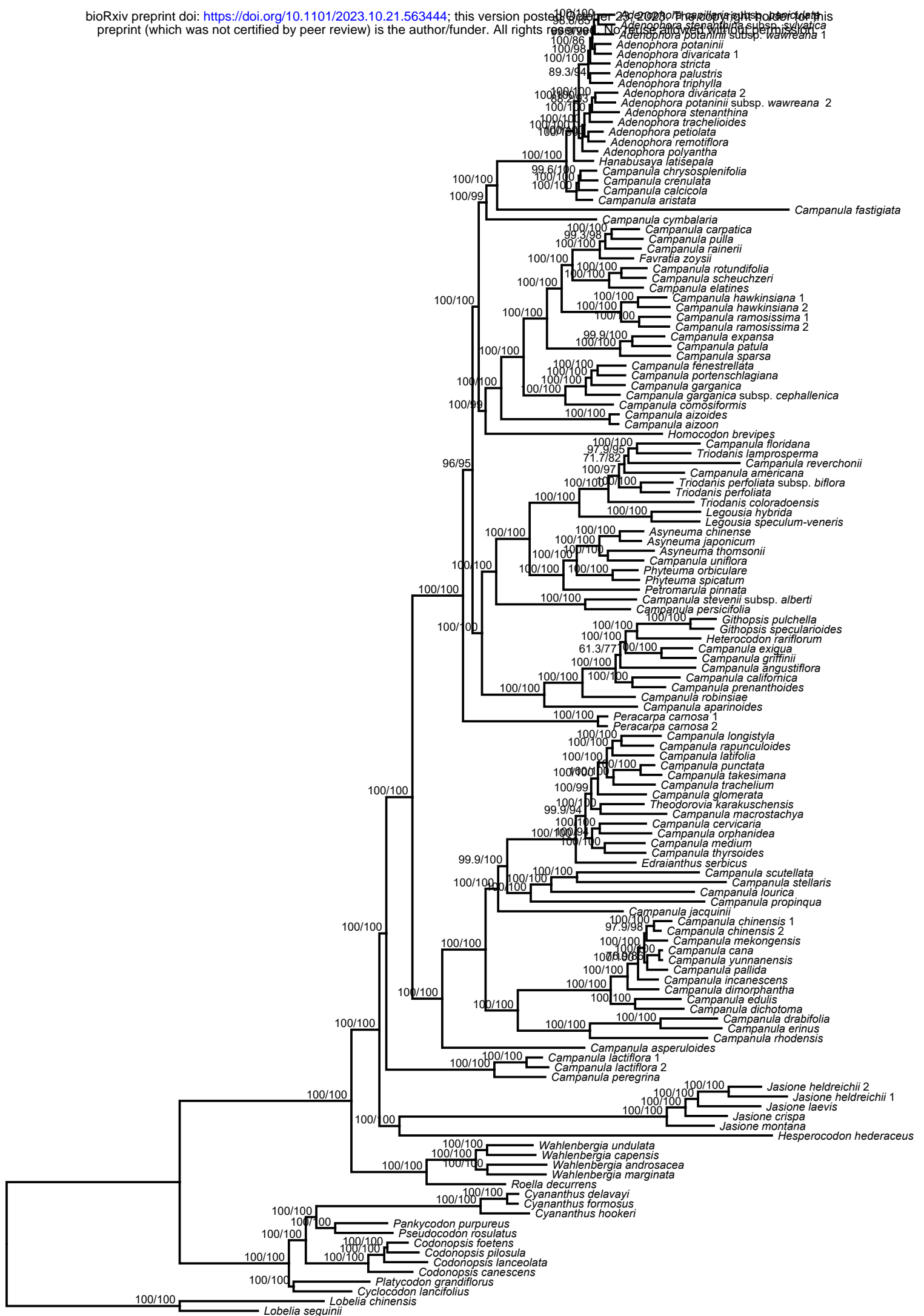


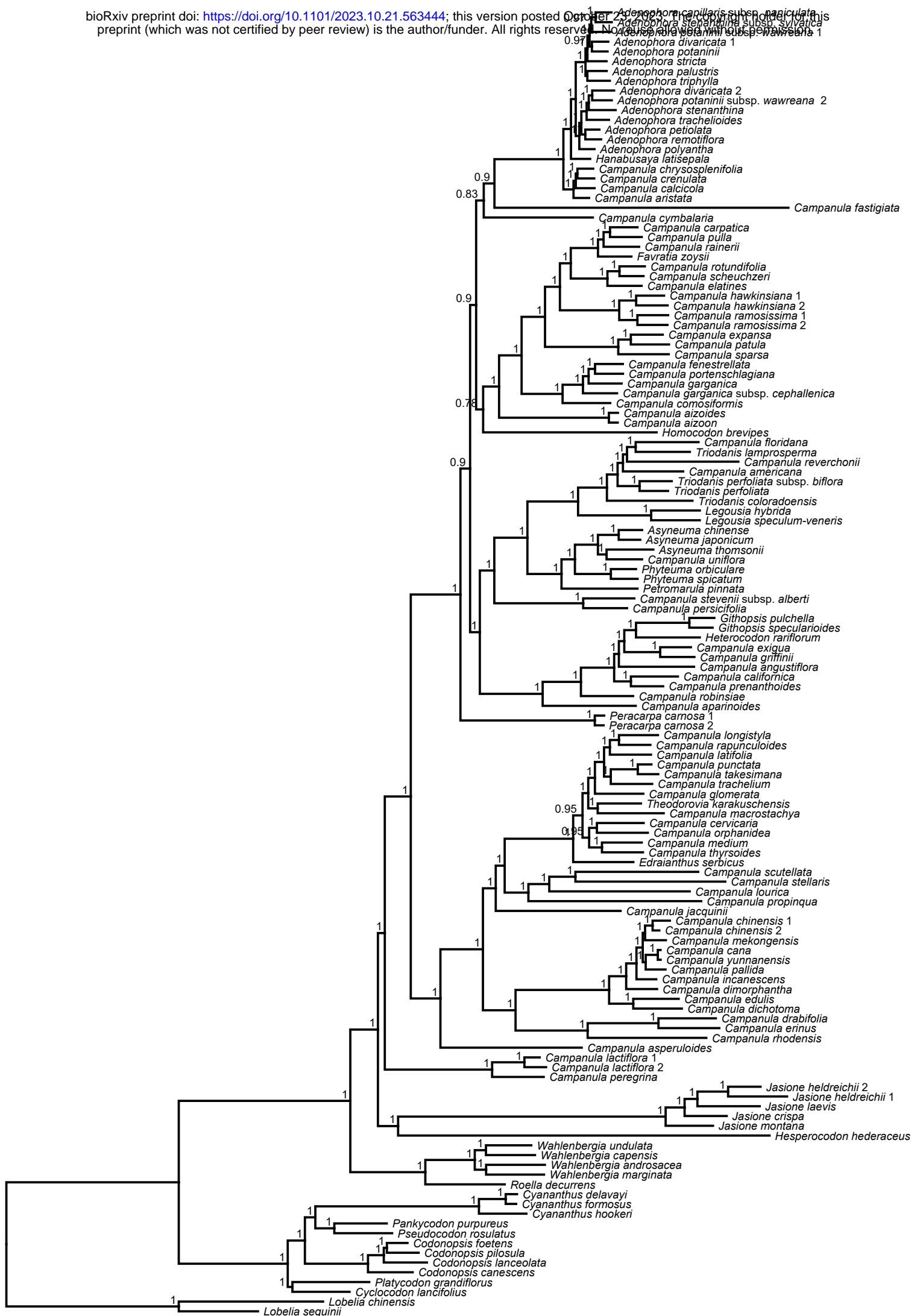


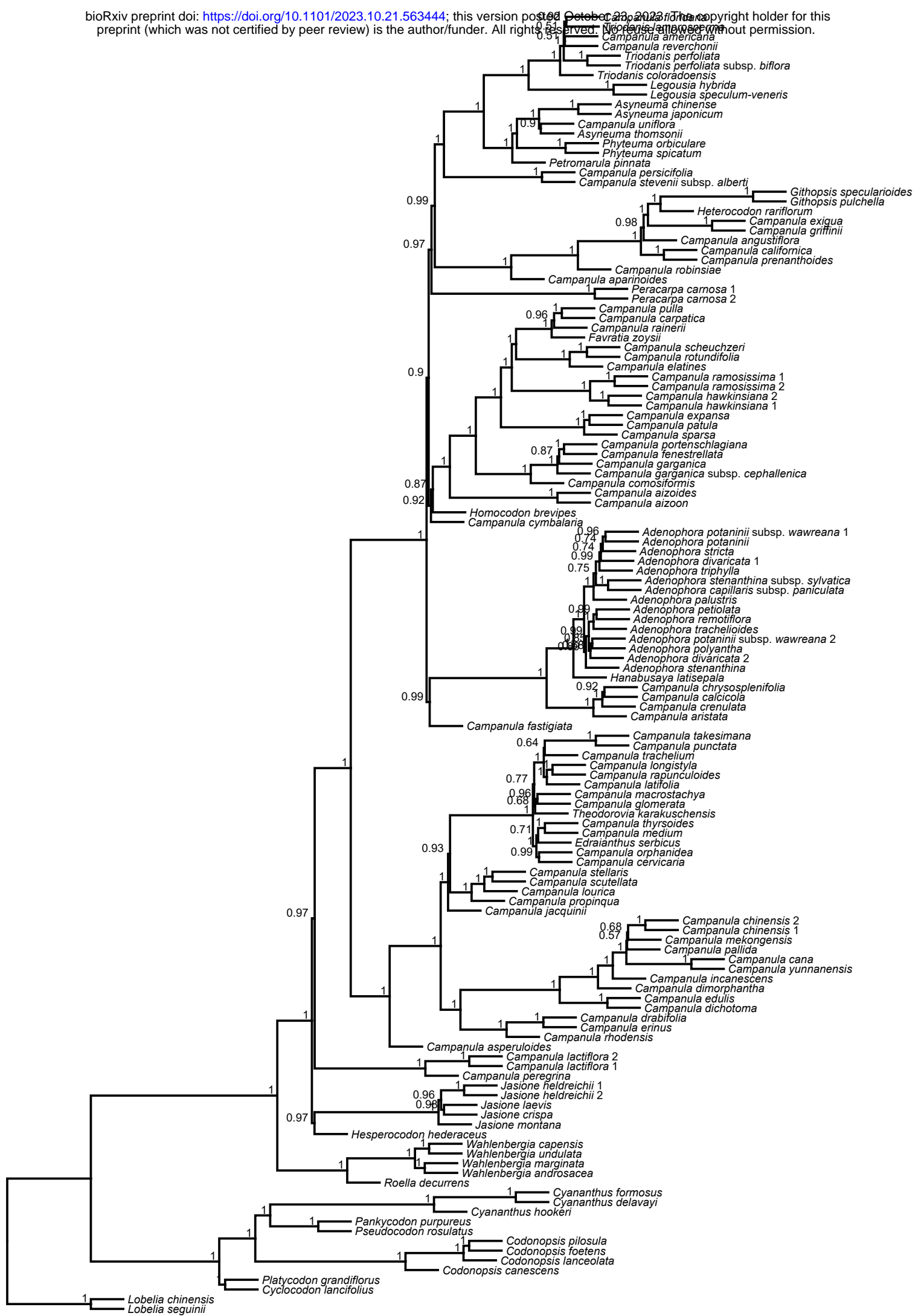




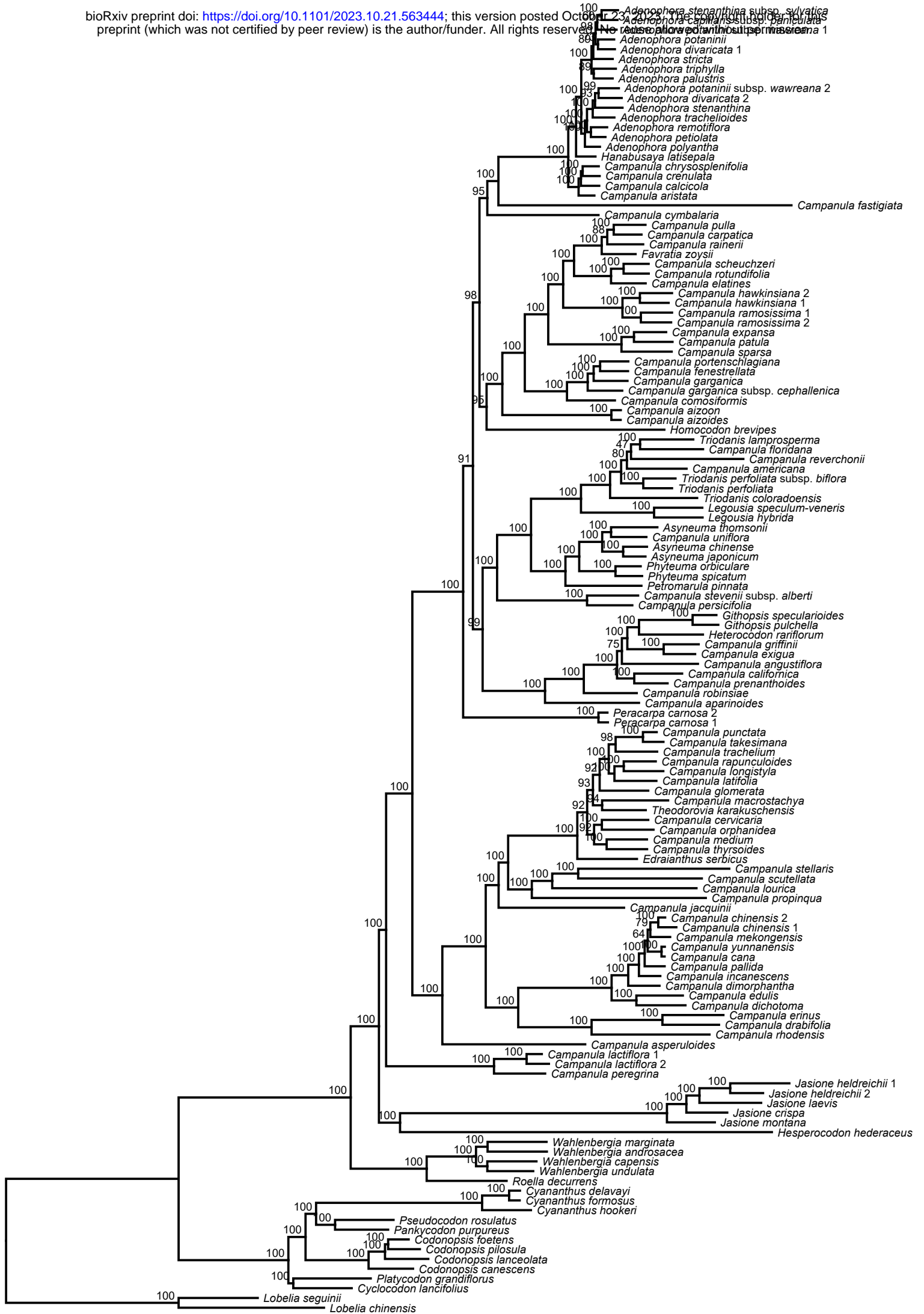


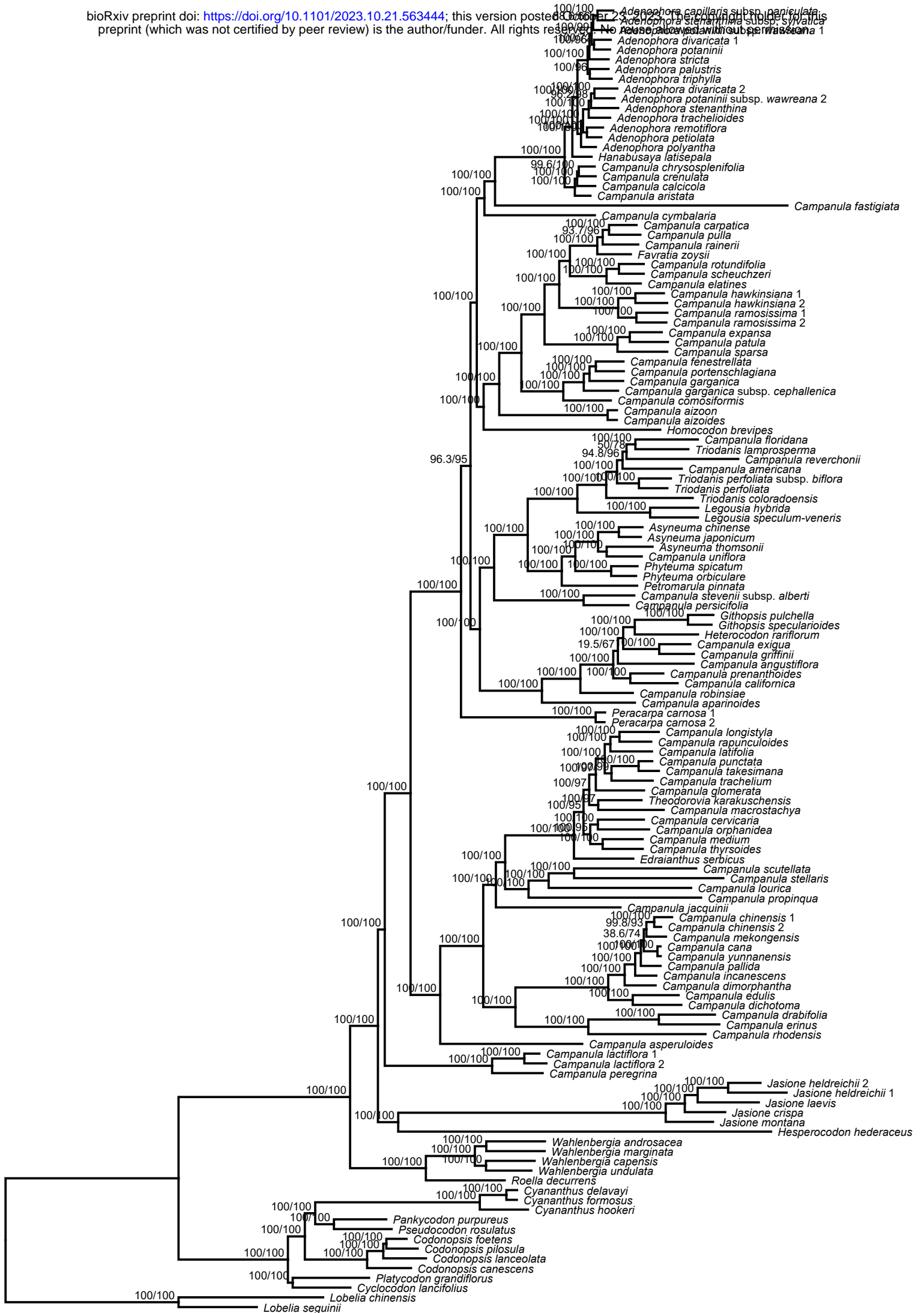


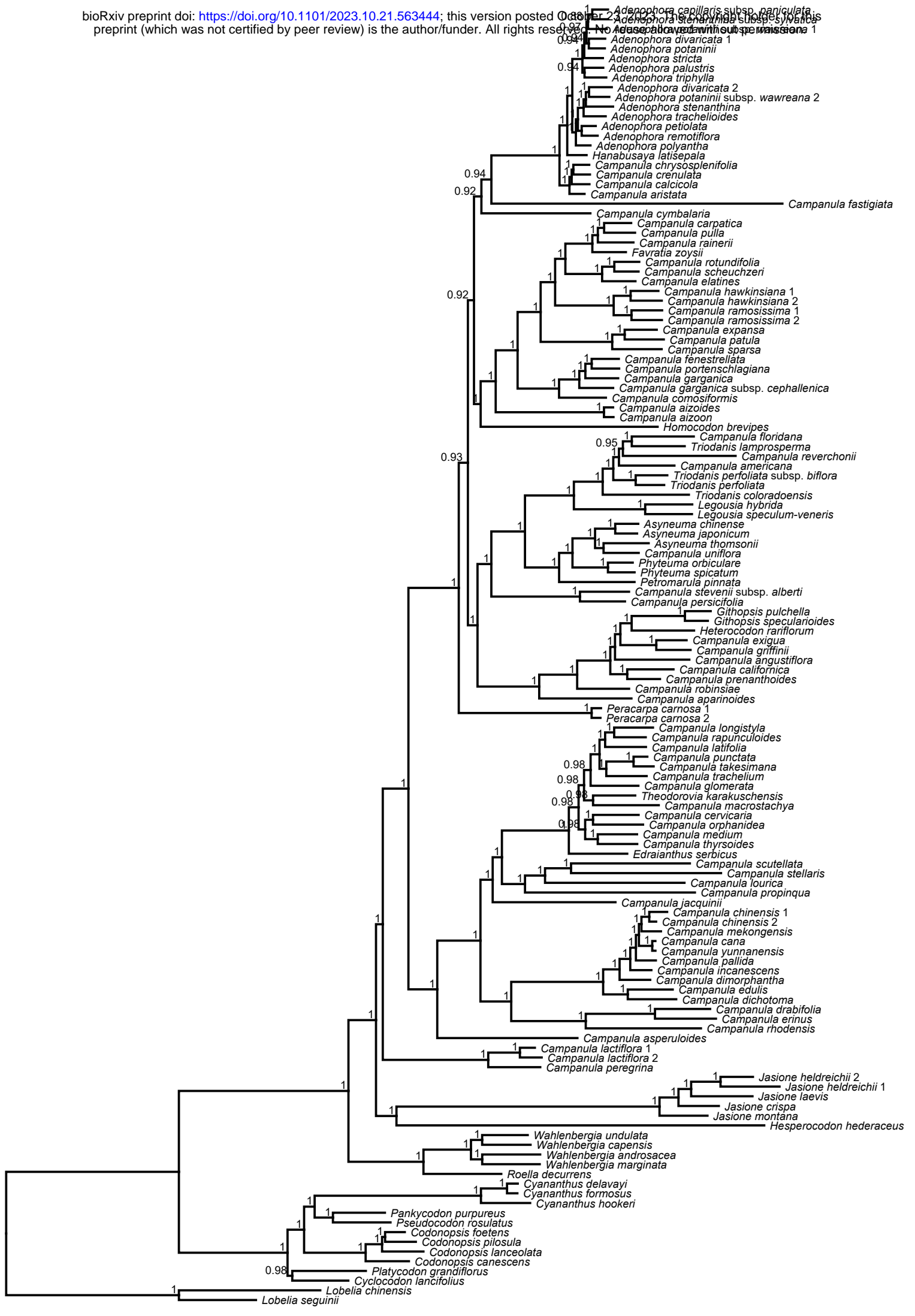


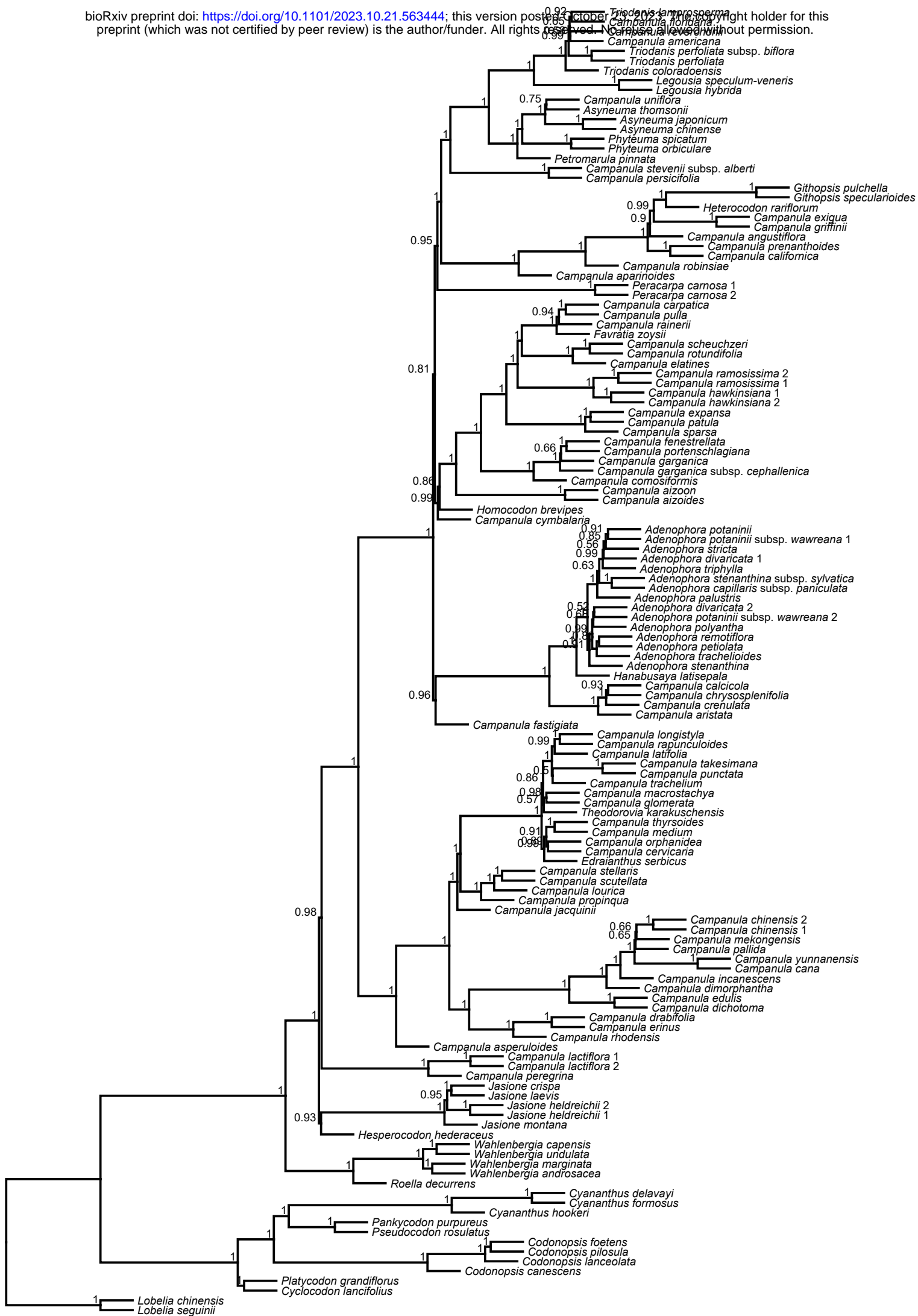


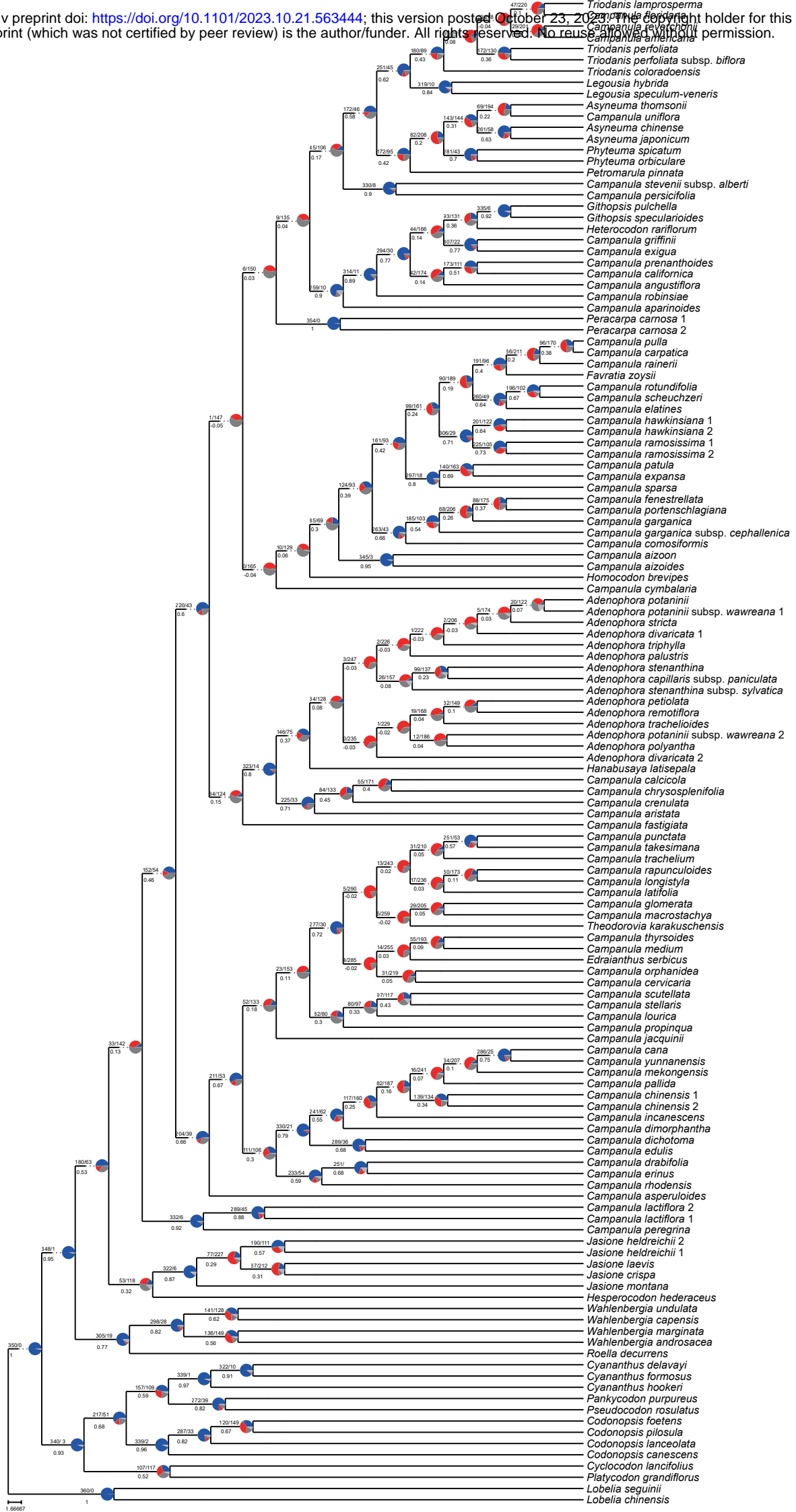










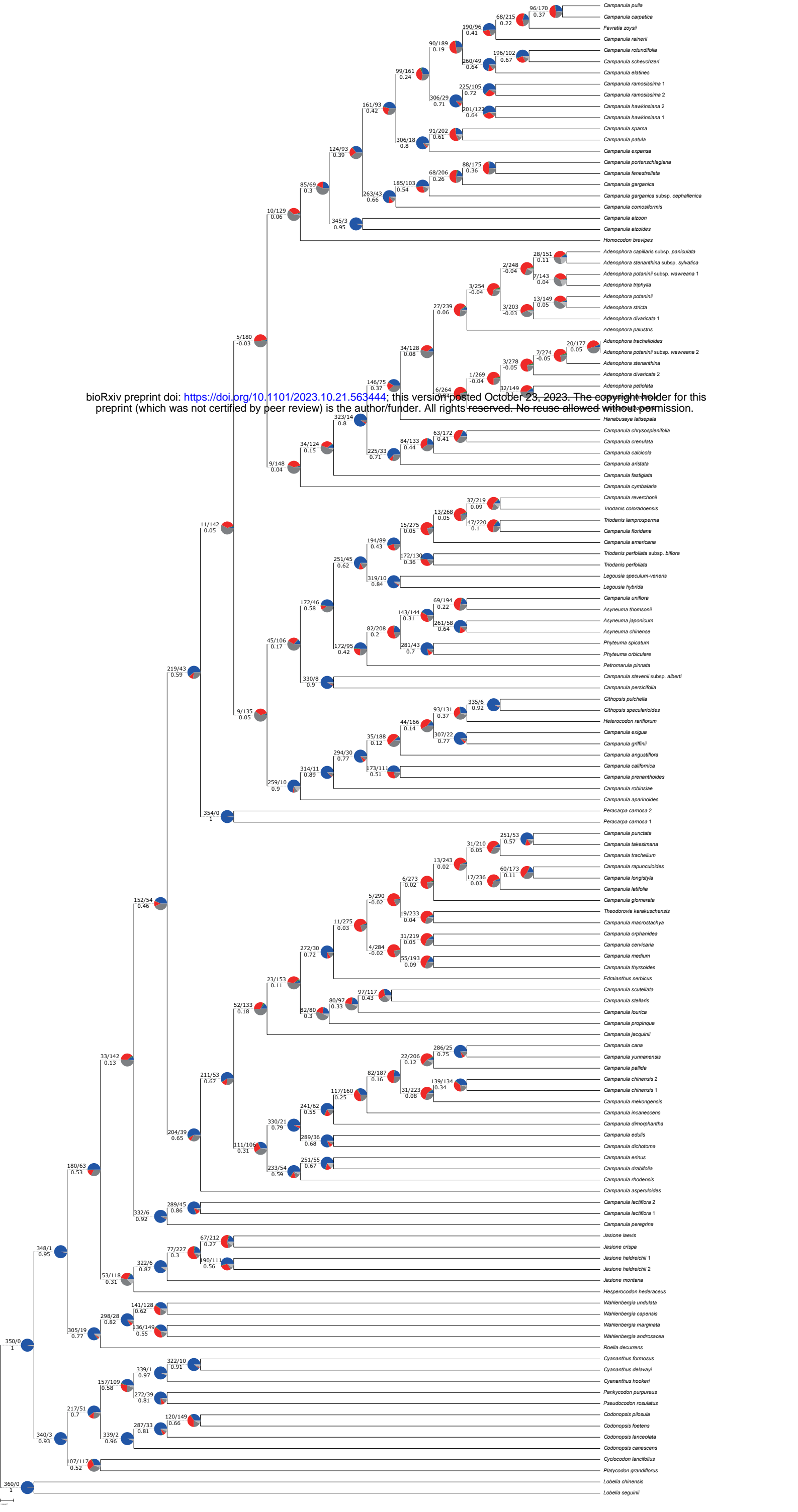


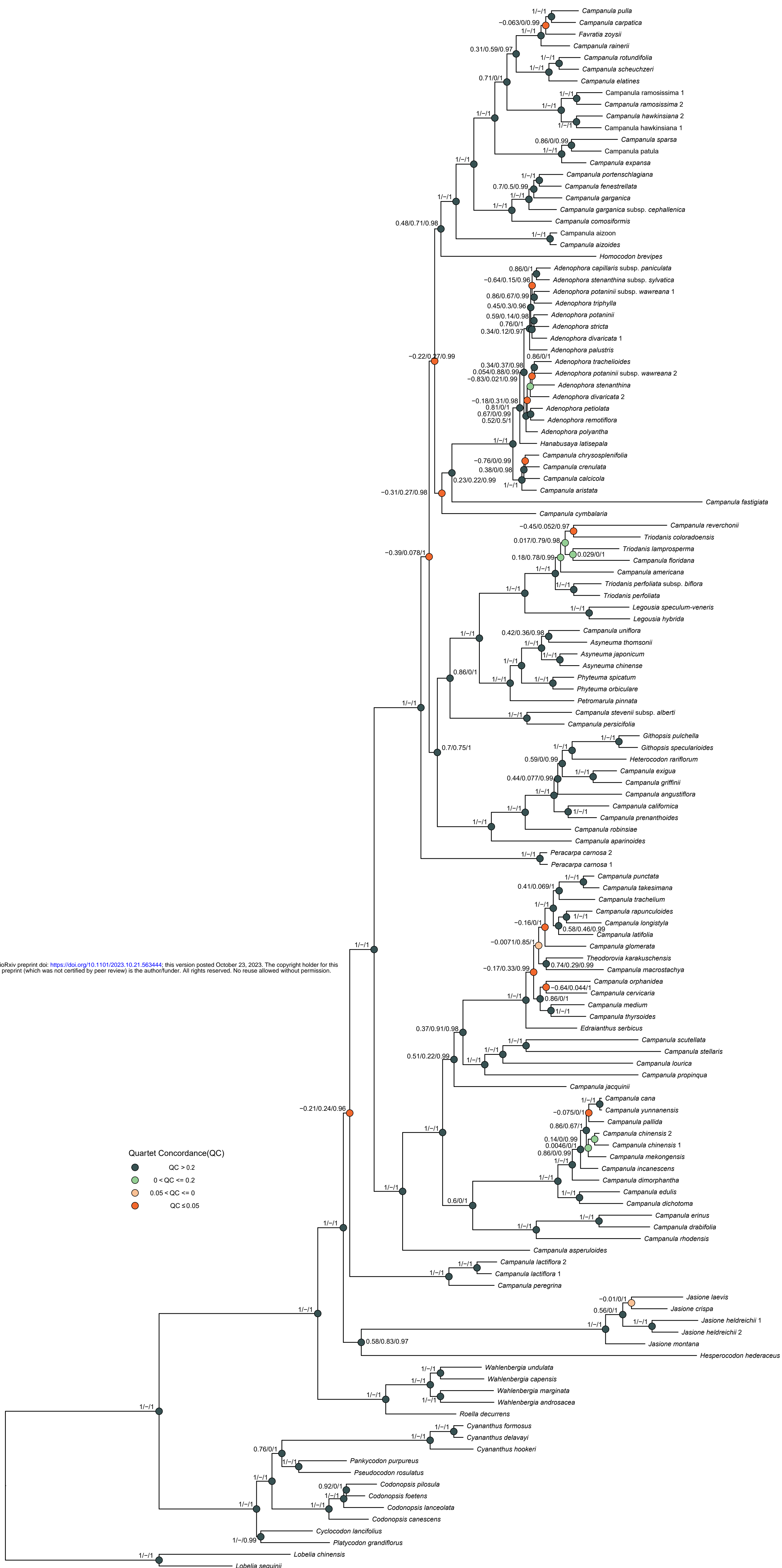


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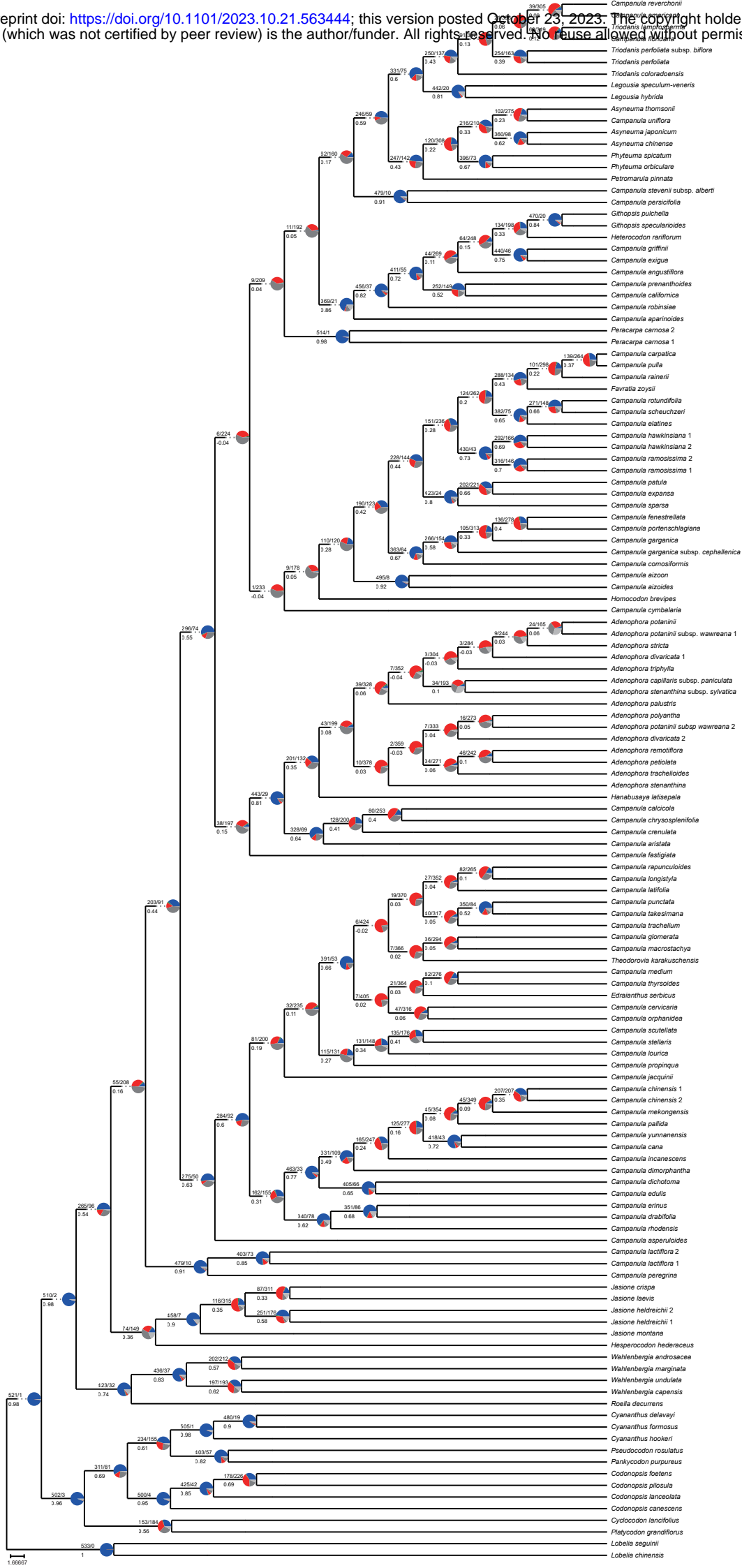
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- 0 < QC ≤ 0.2
- -0.05 < QC ≤ 0
- QC ≤ -0.05



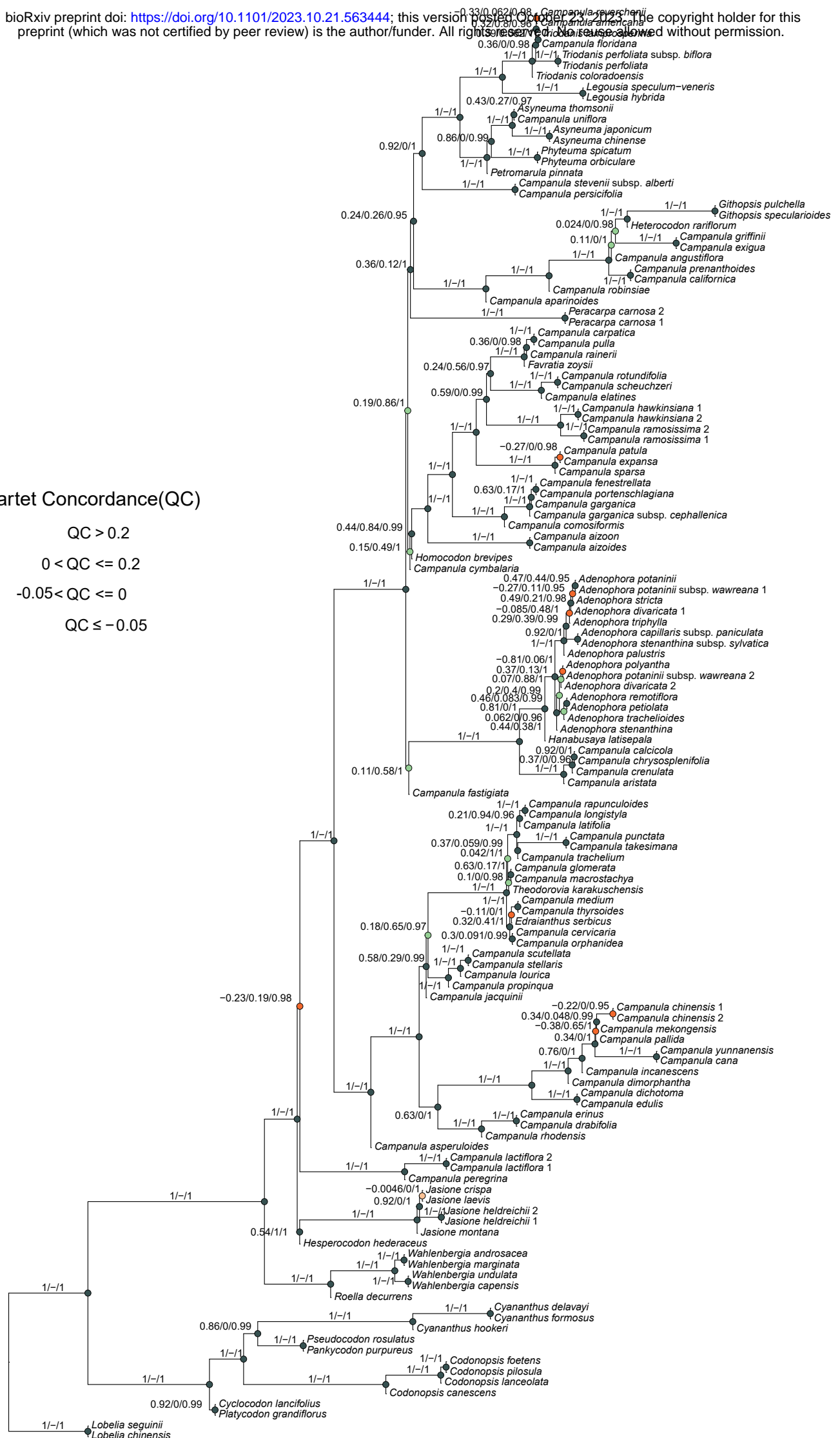
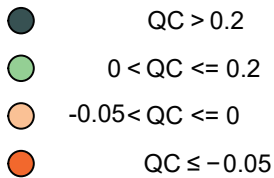


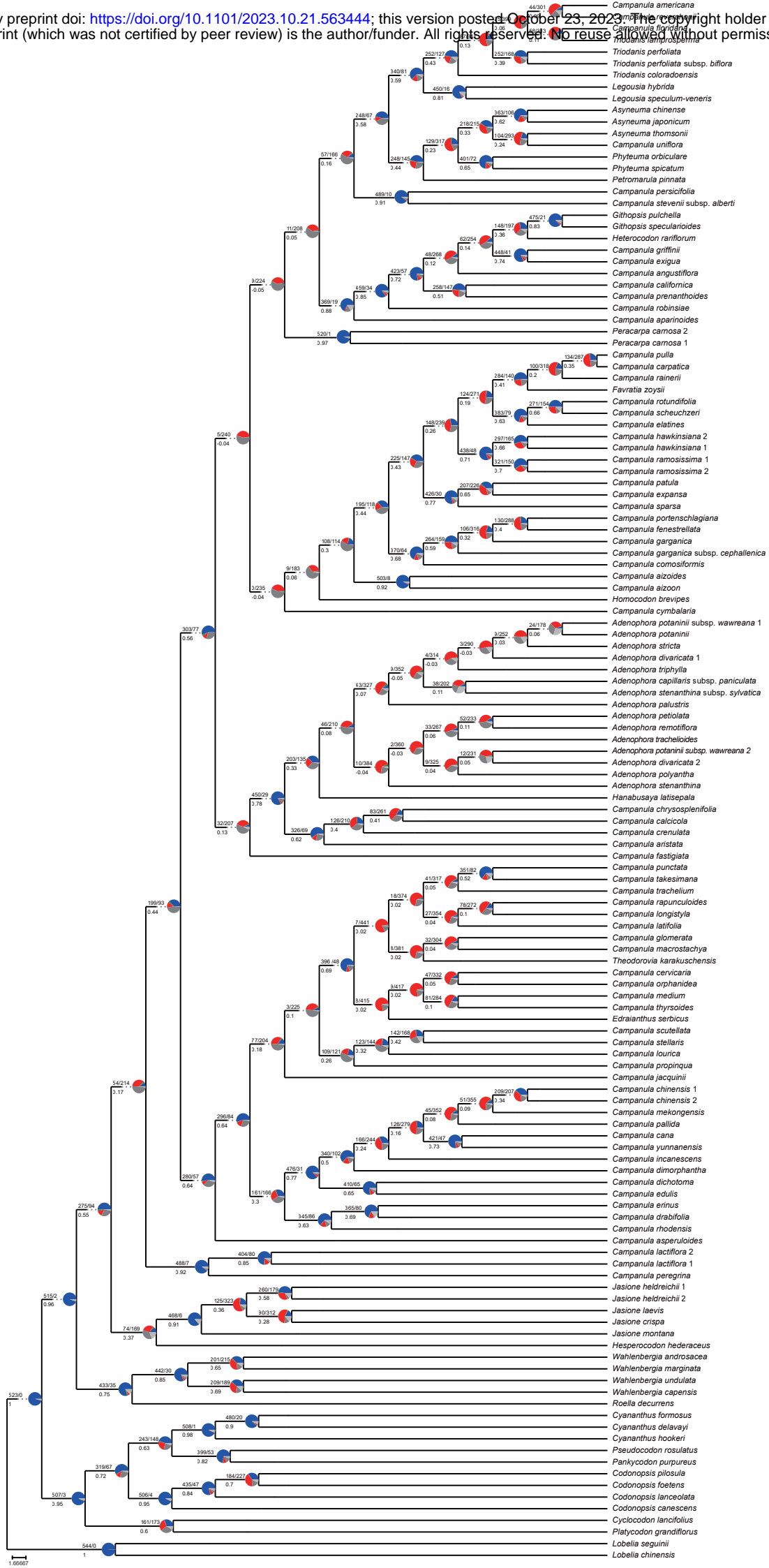




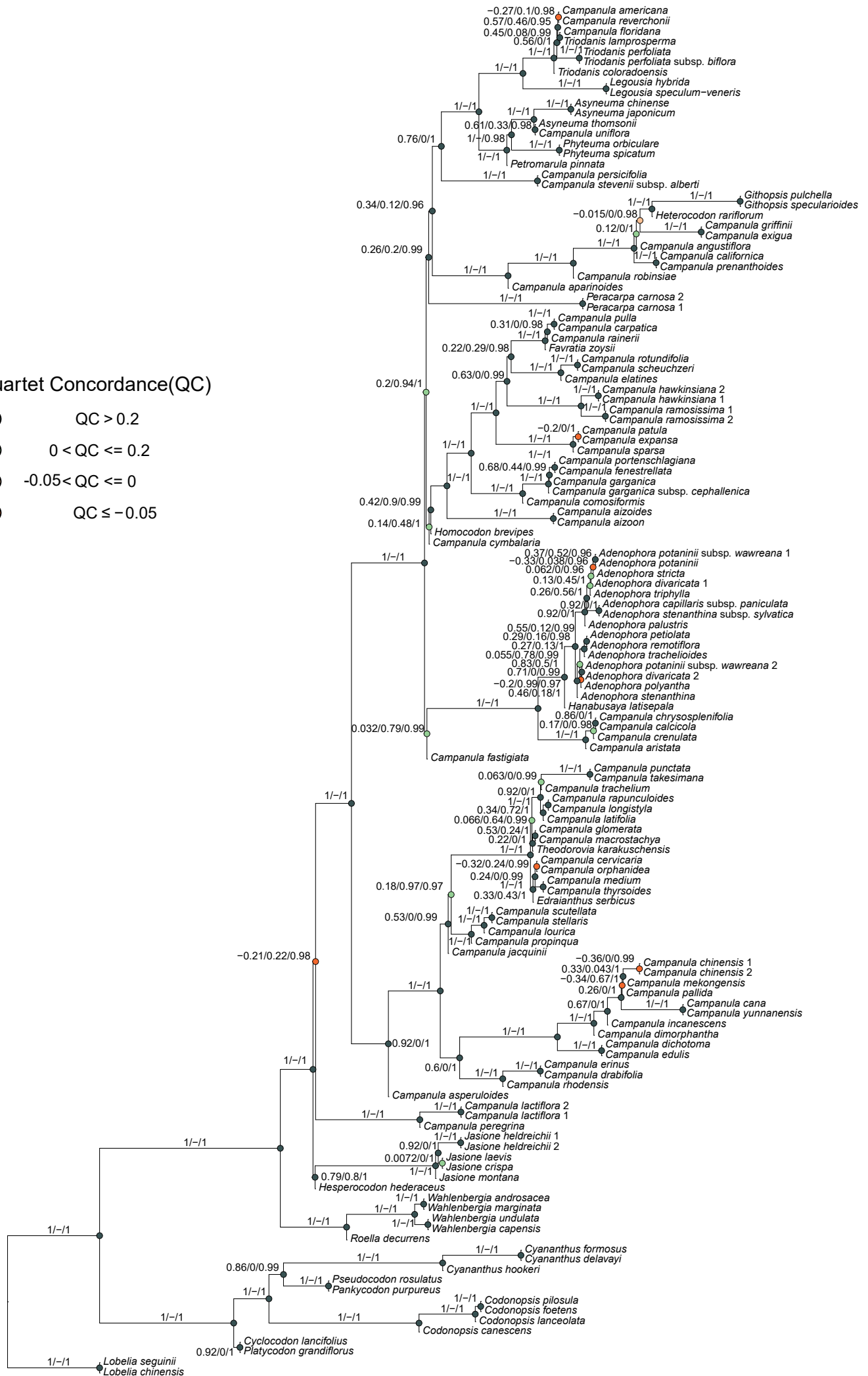
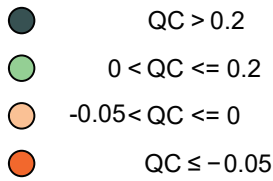


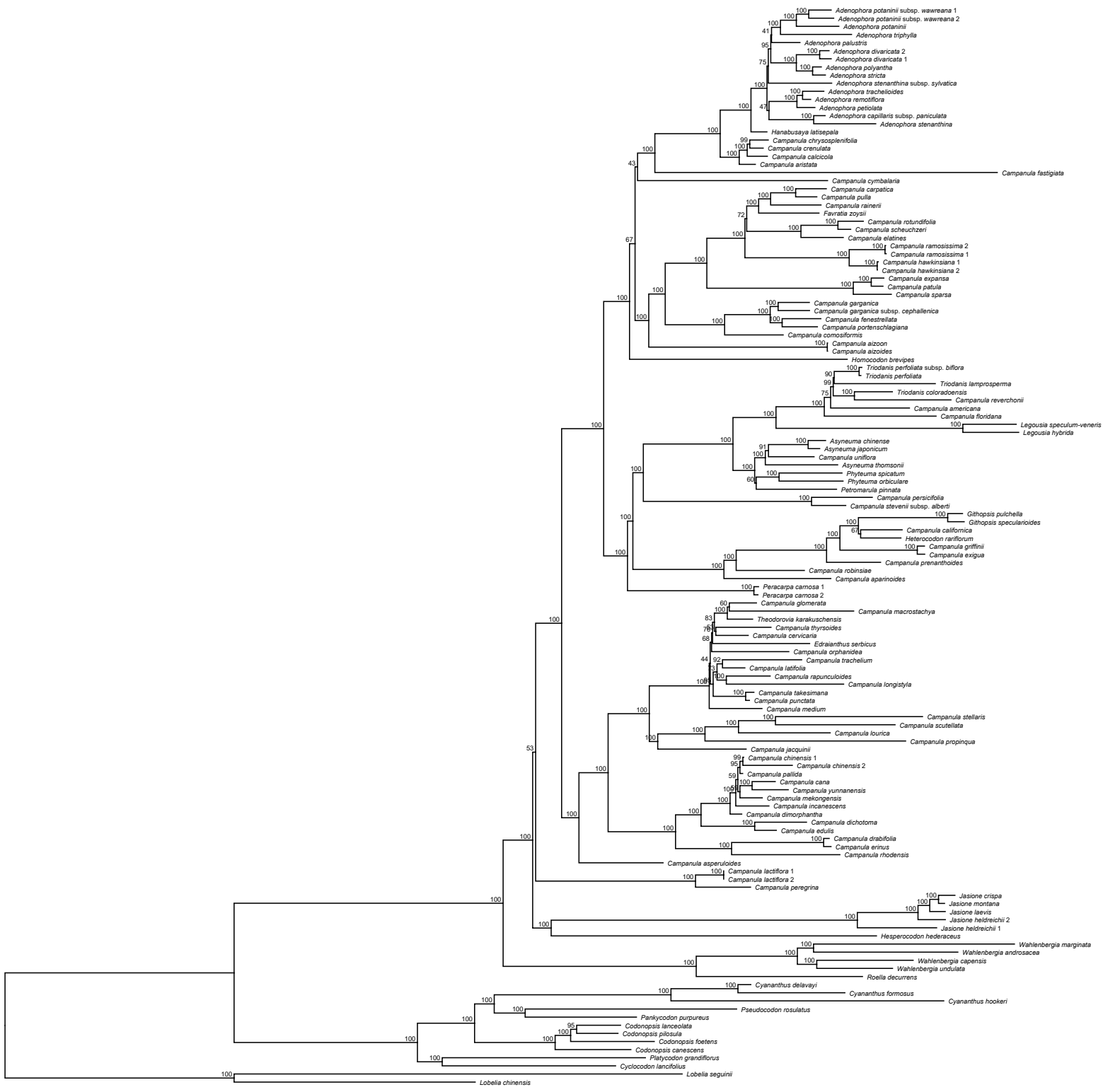
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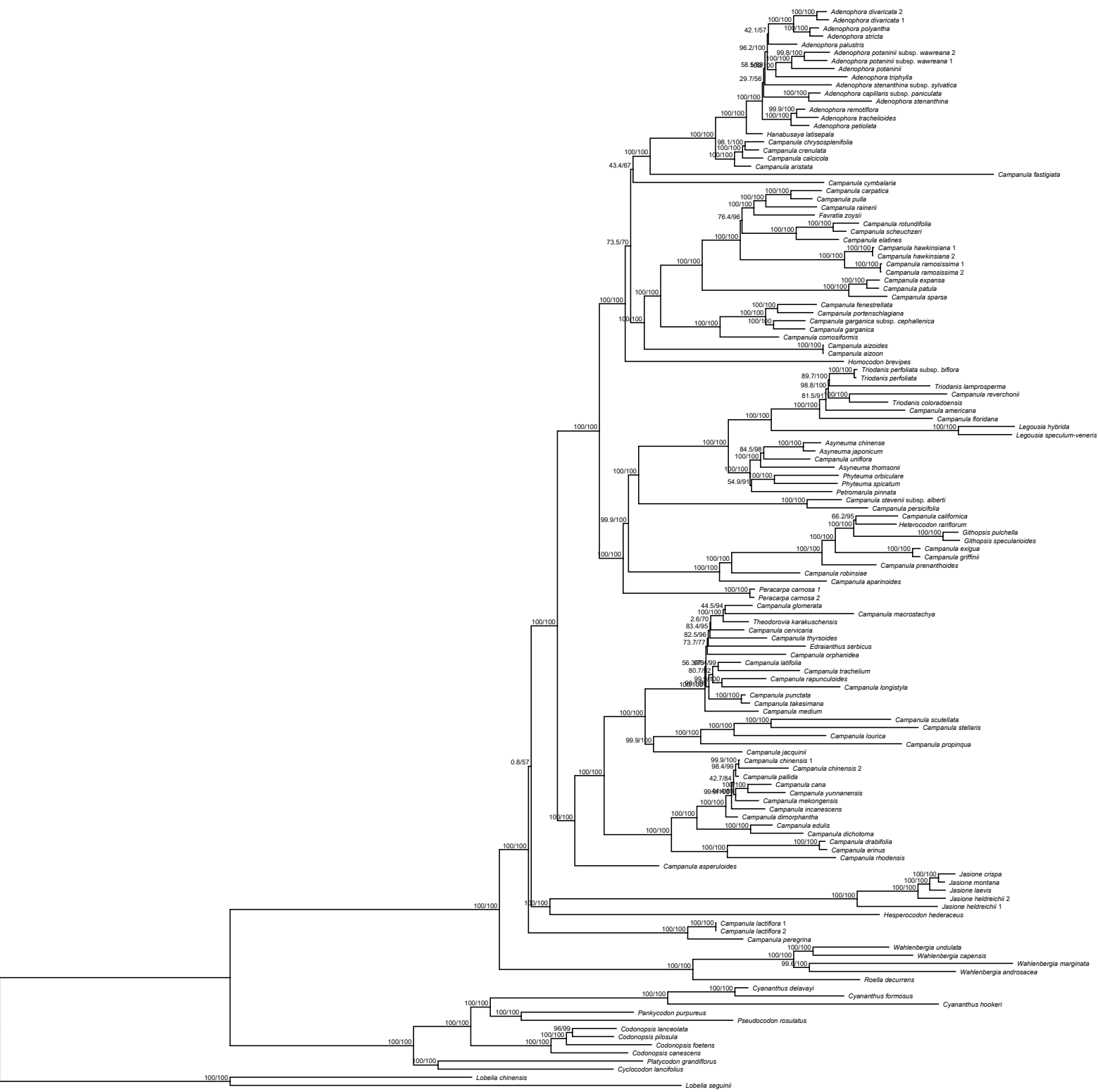


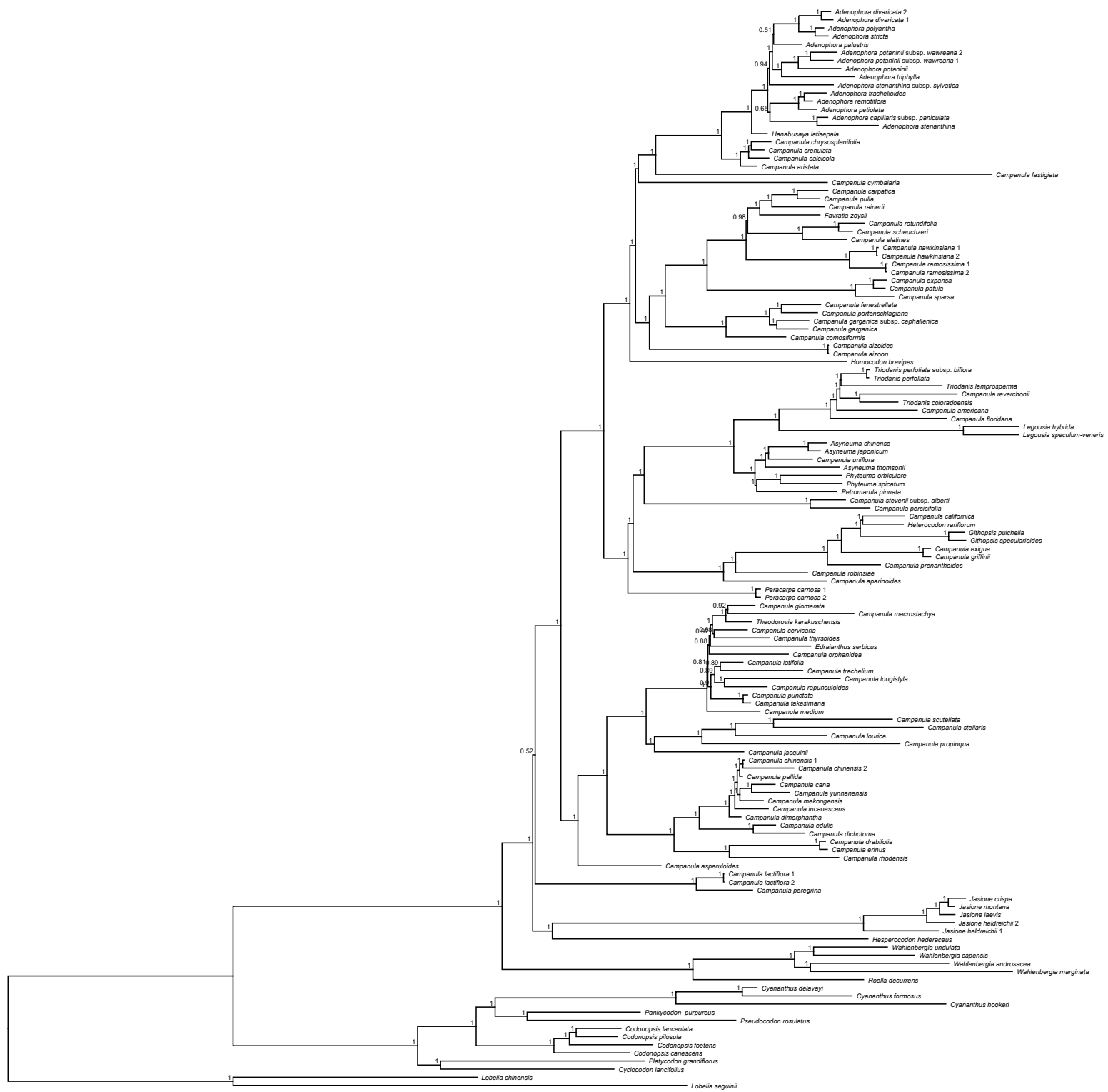


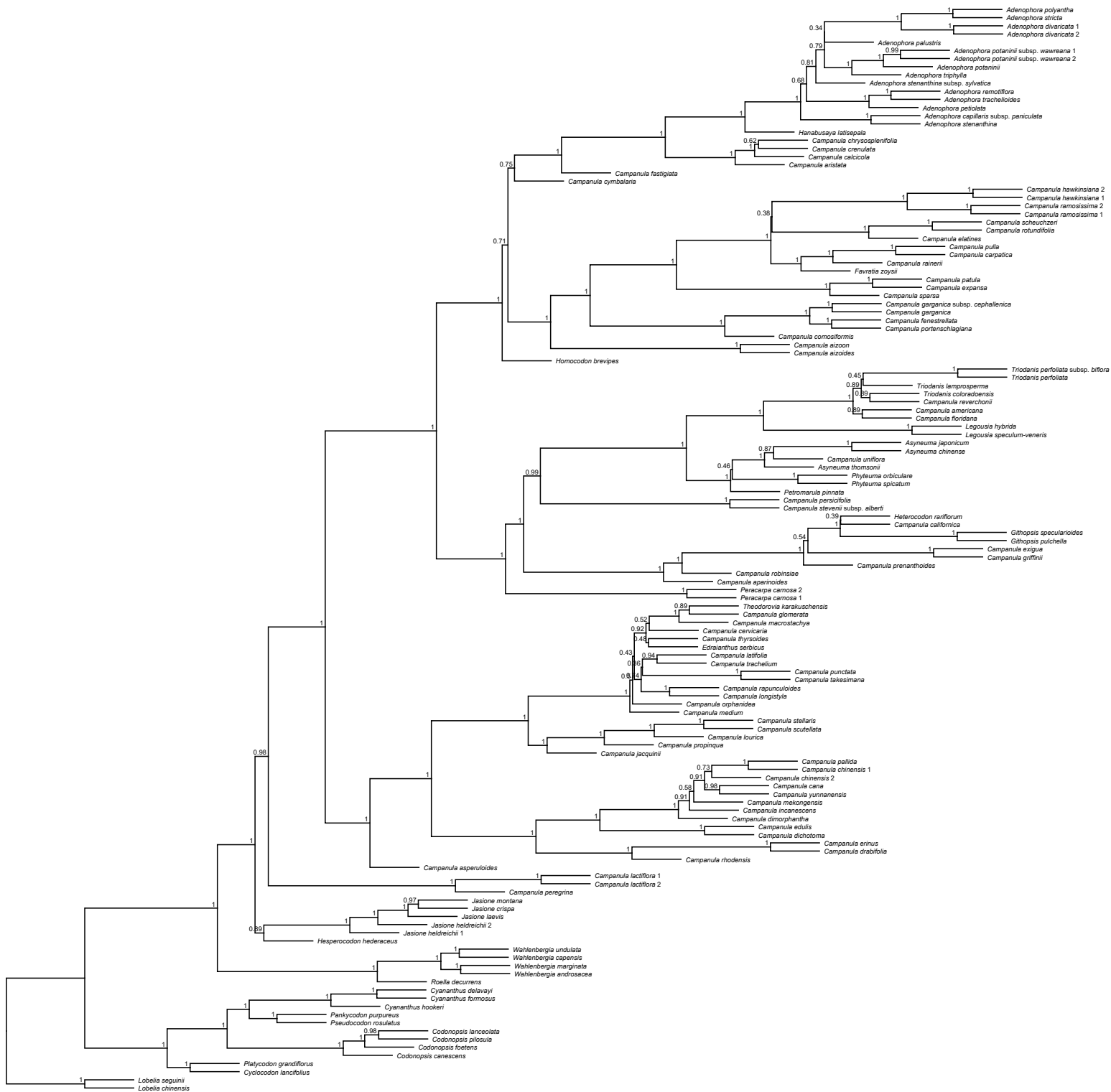
Quartet Concordance(QC)



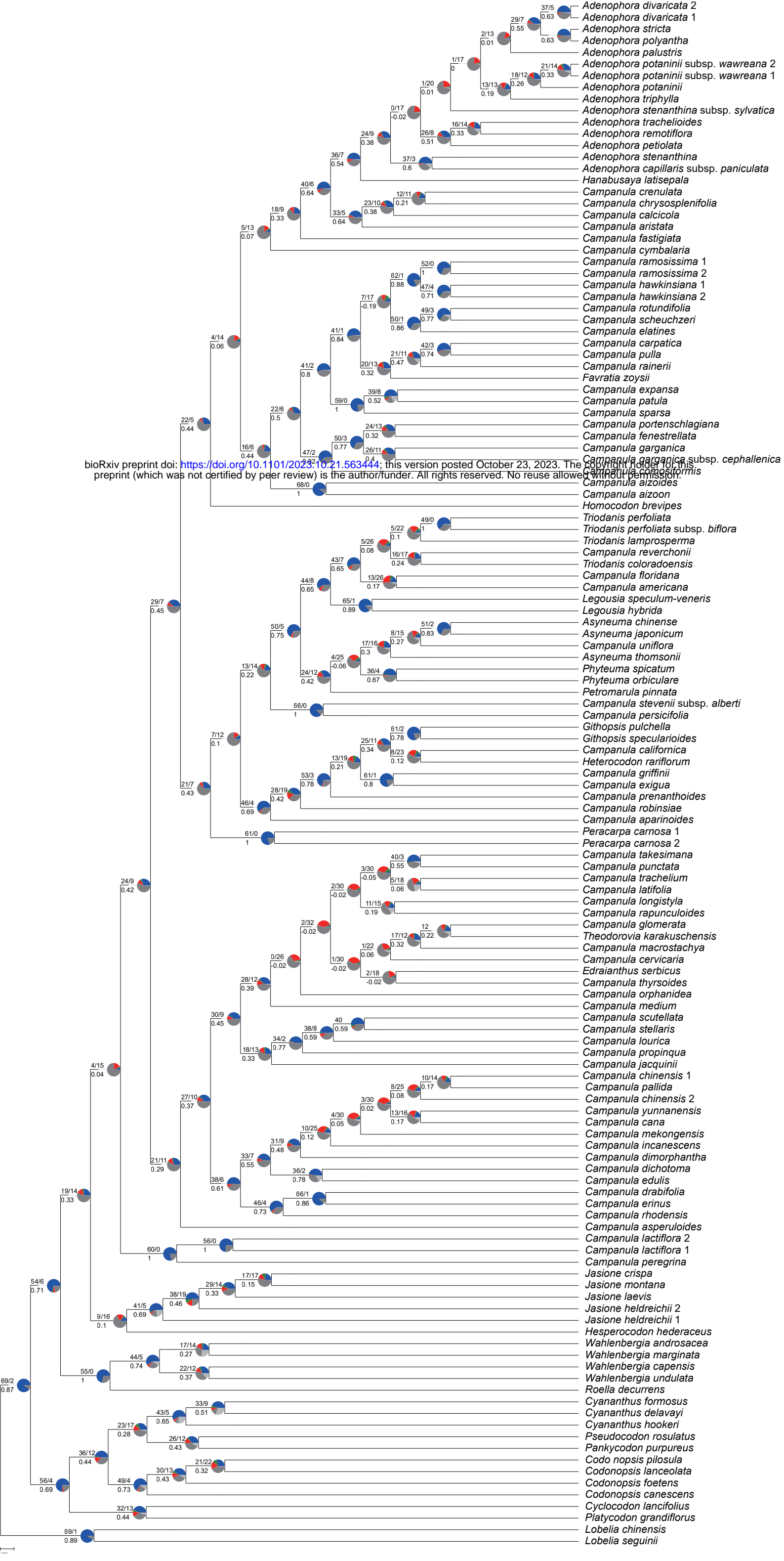






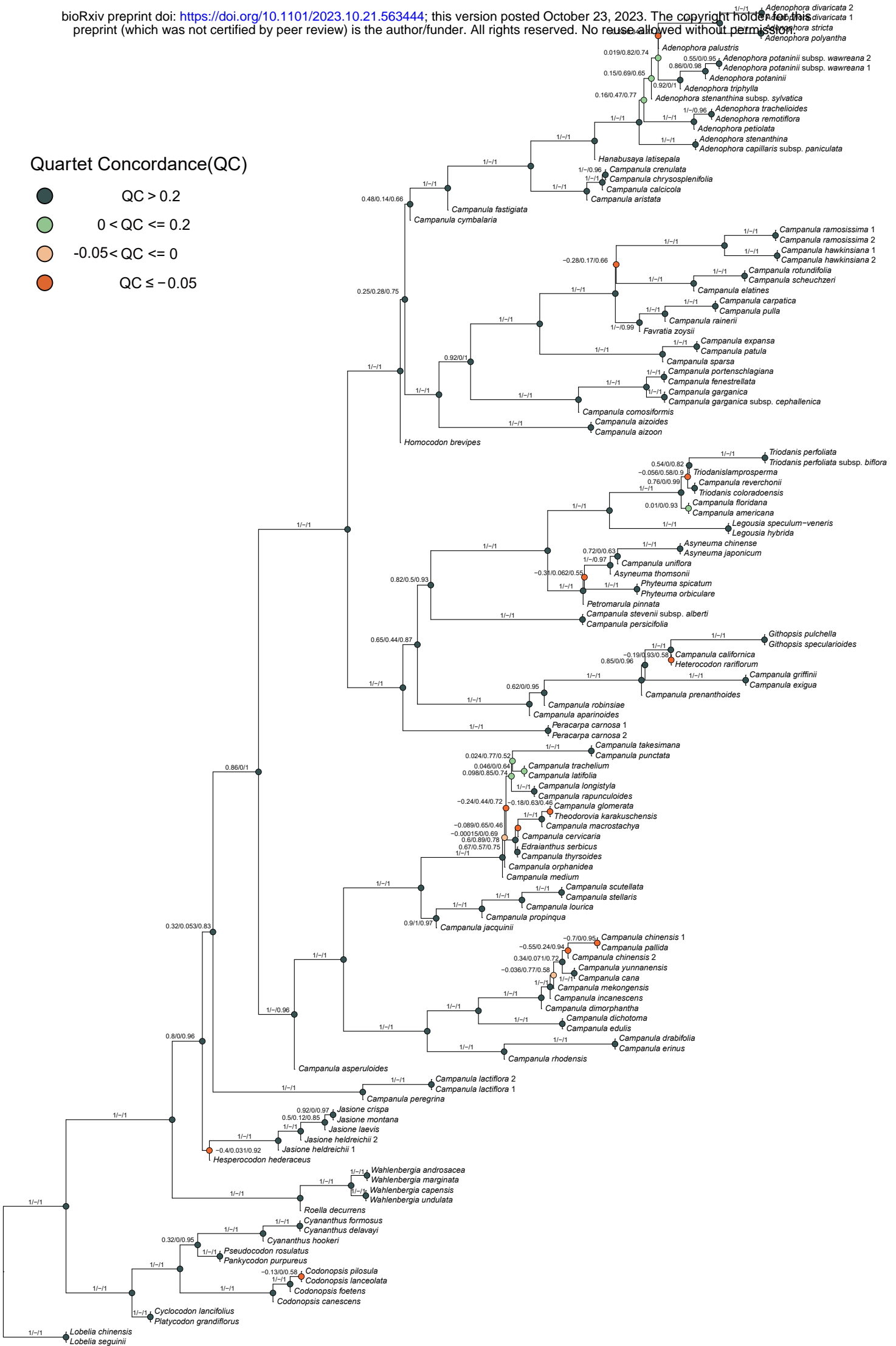


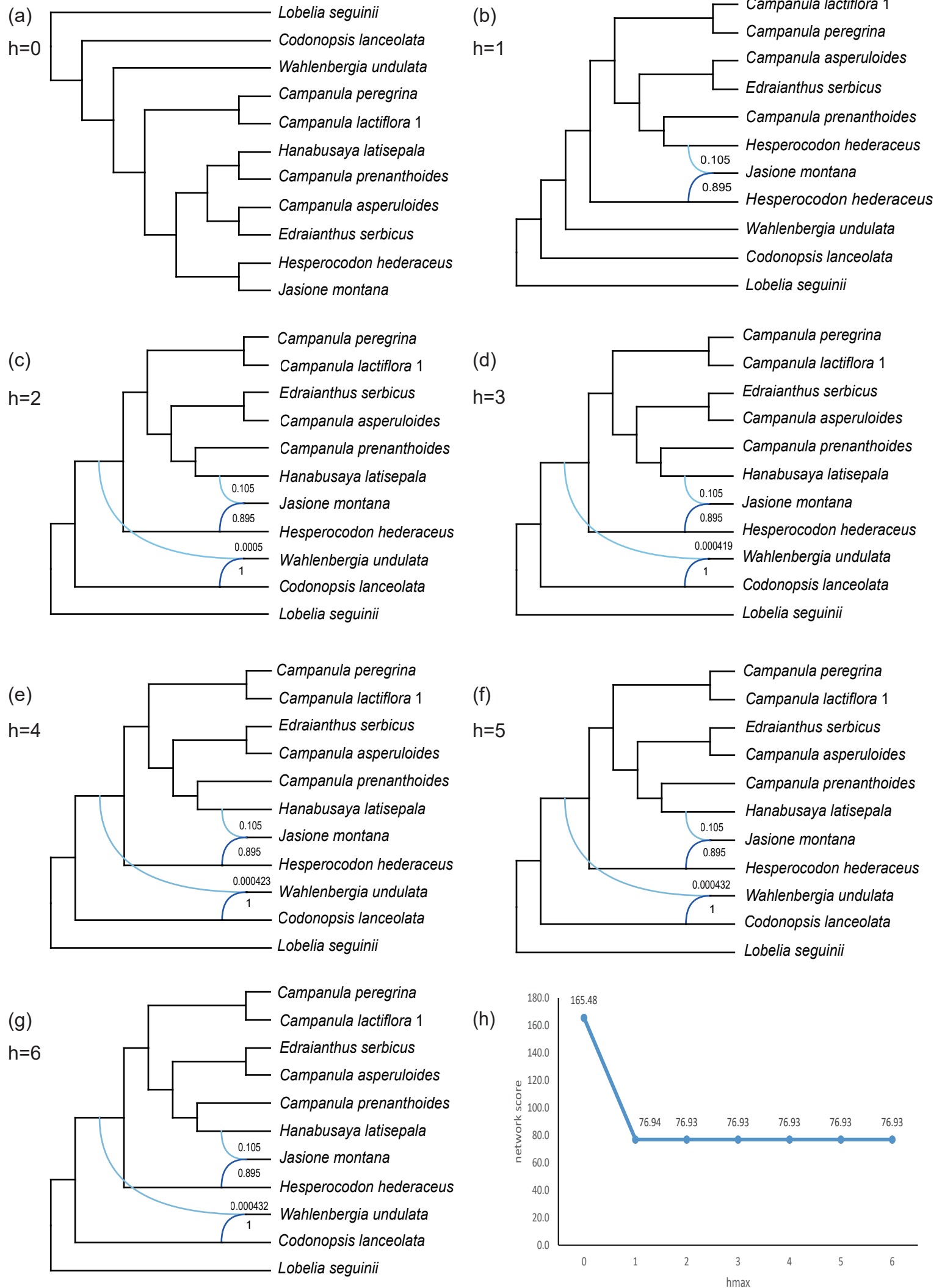


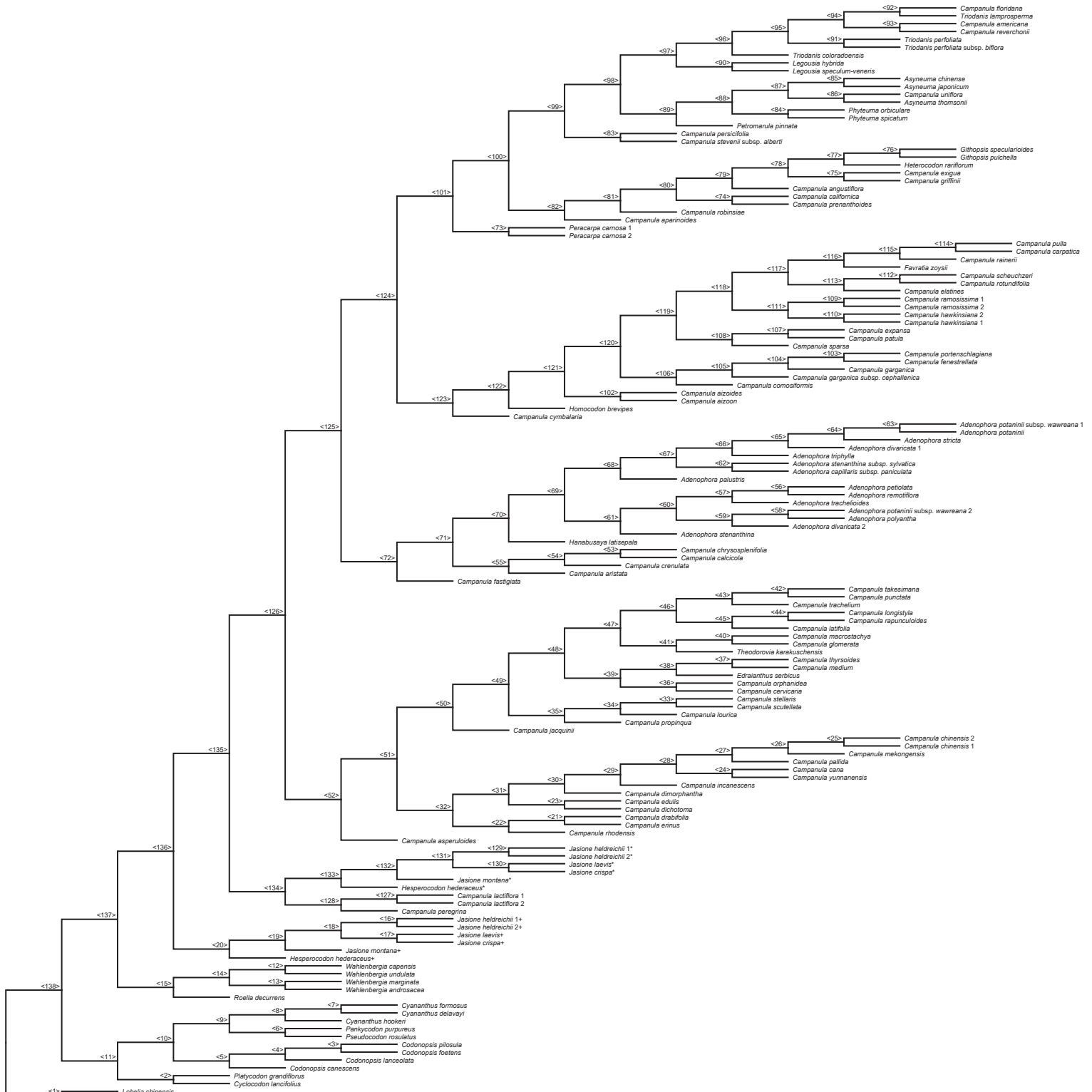


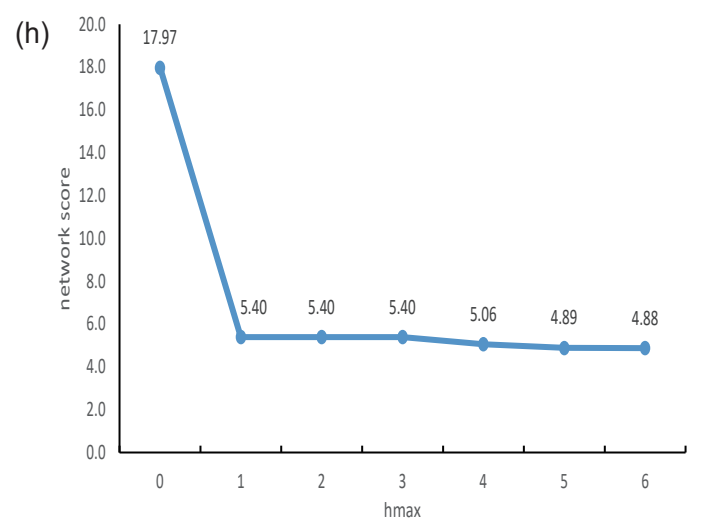
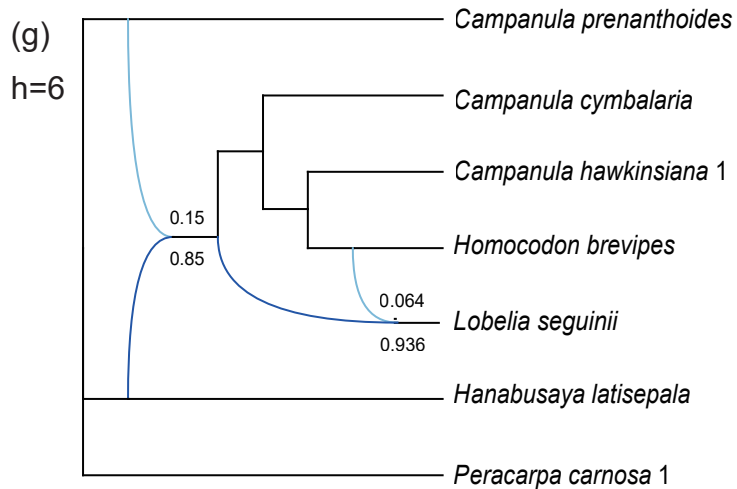
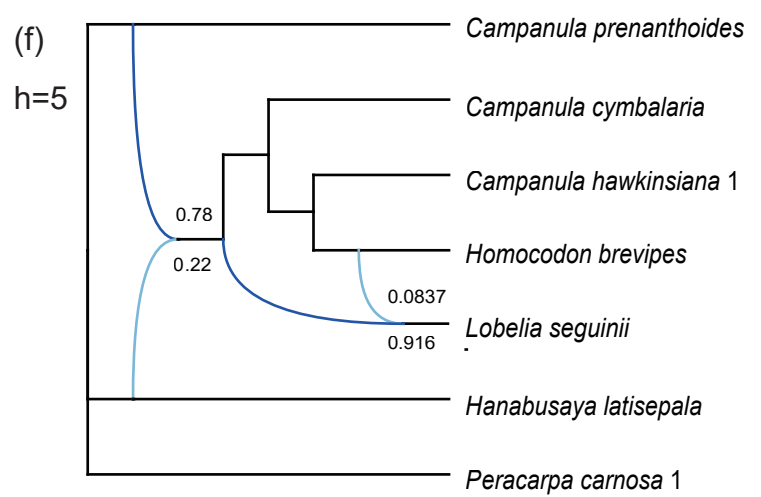
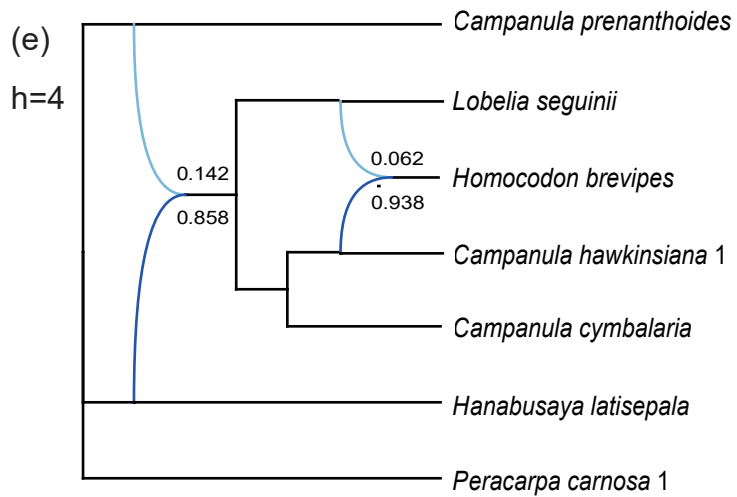
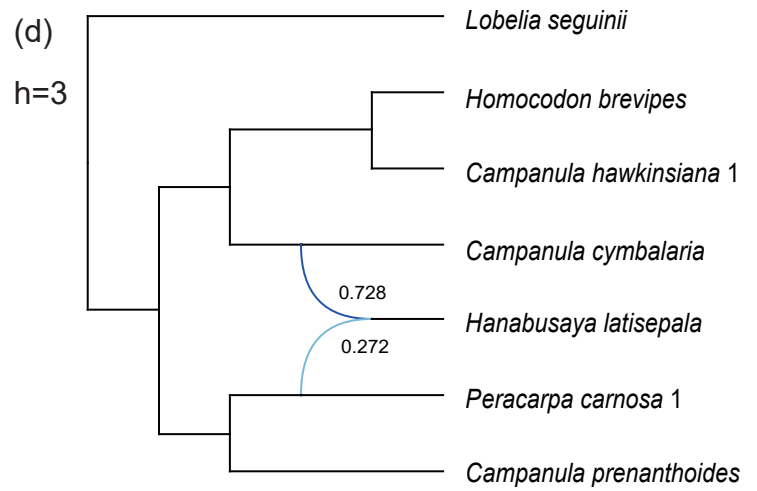
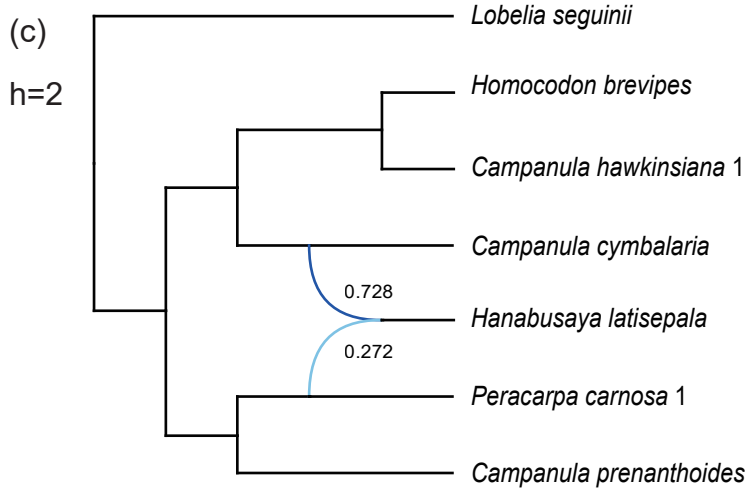
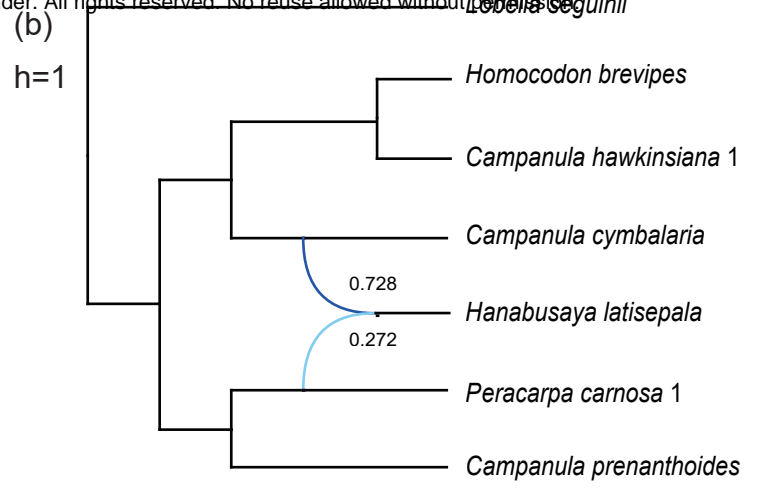
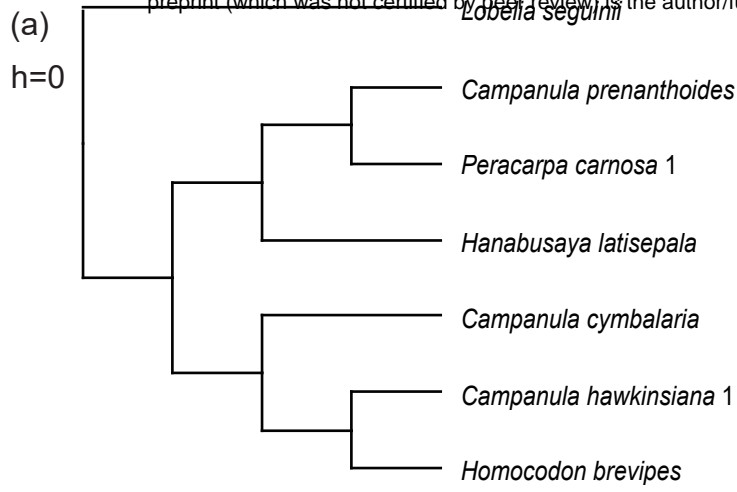
### Quartet Concordance(QC)

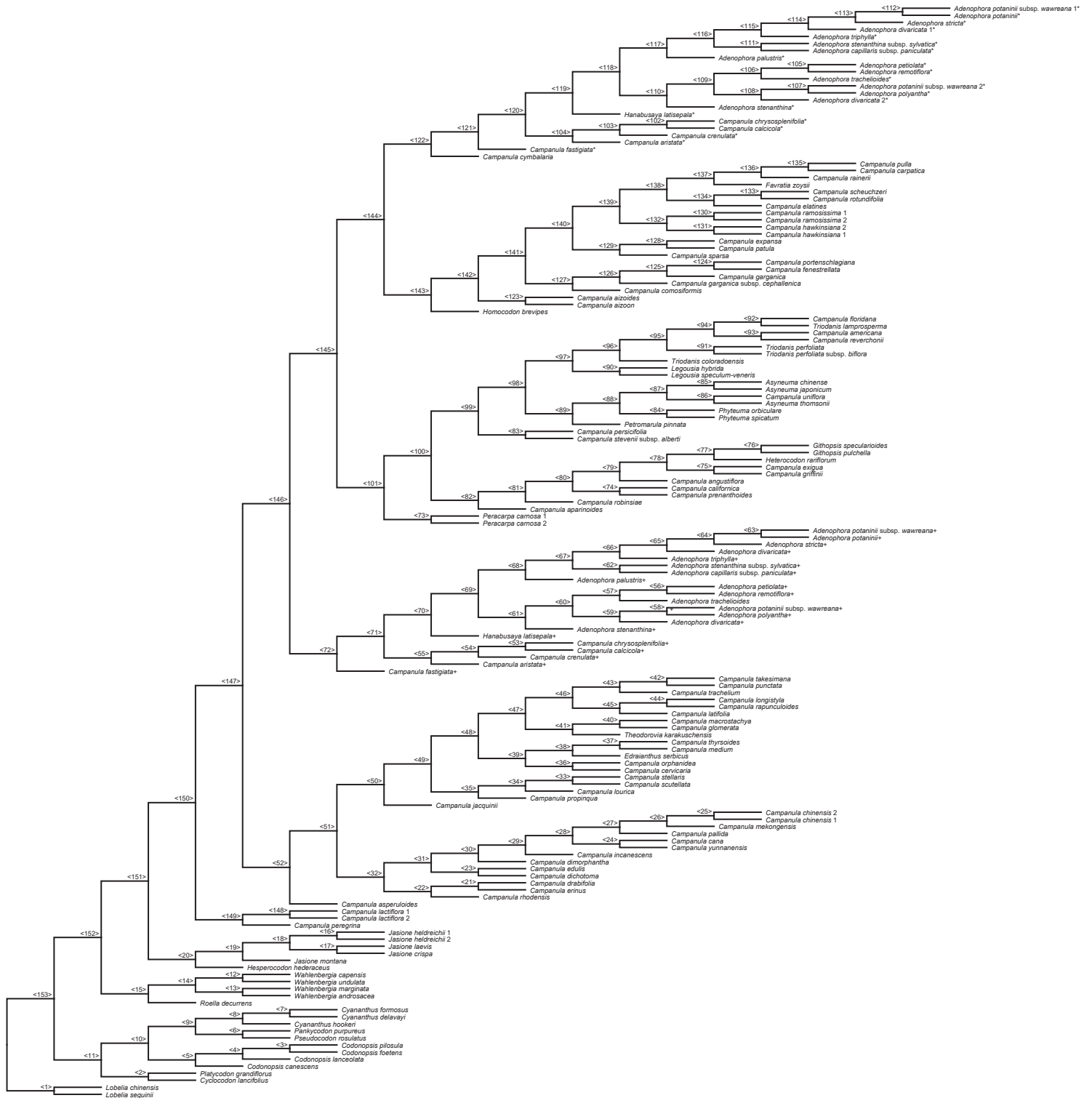
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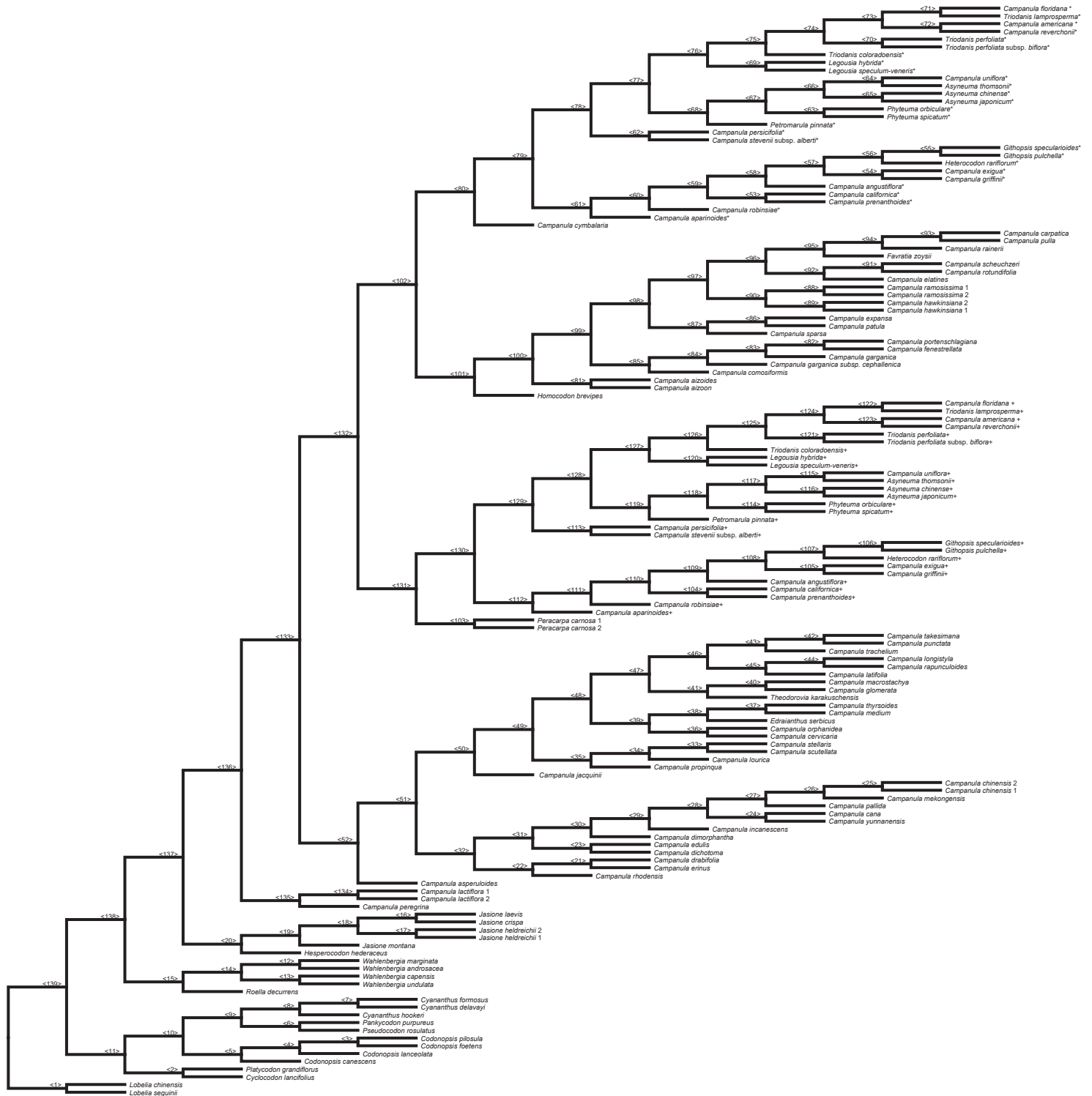








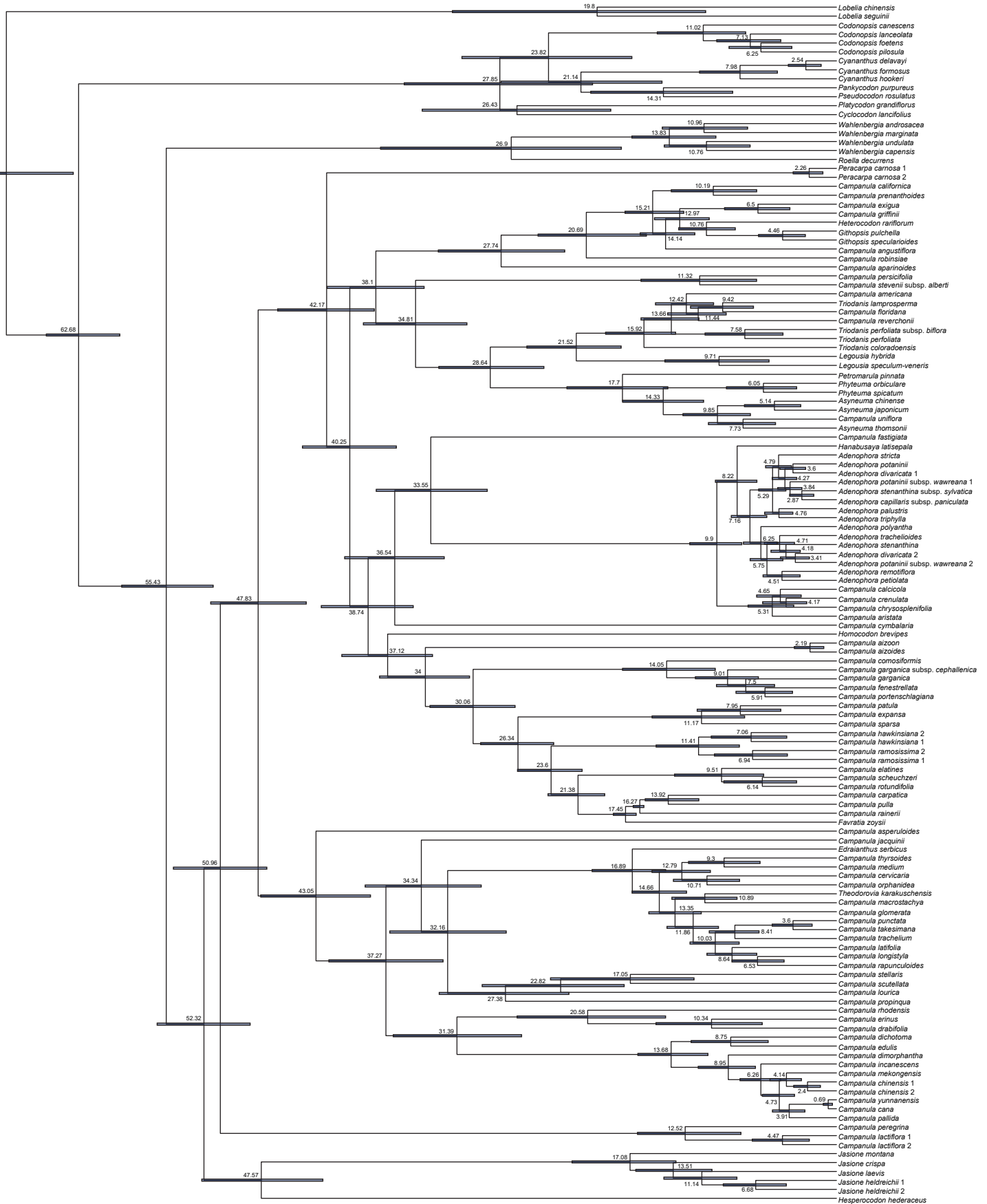


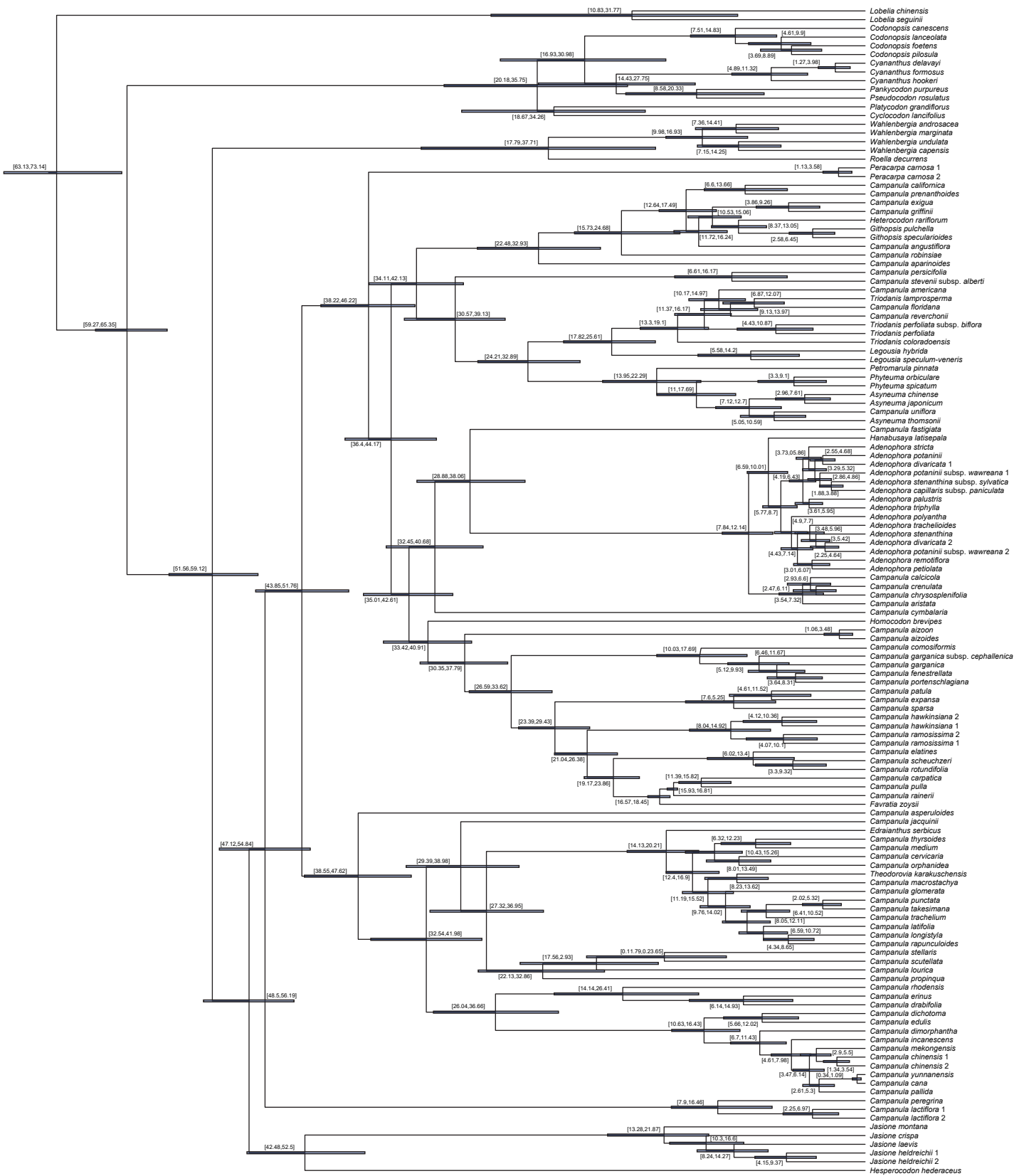




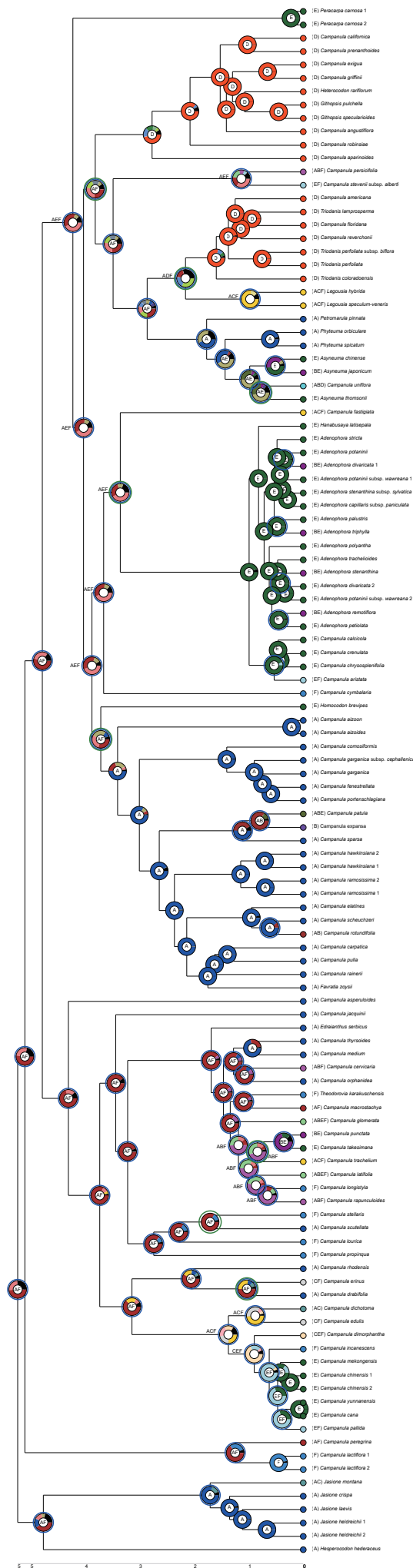
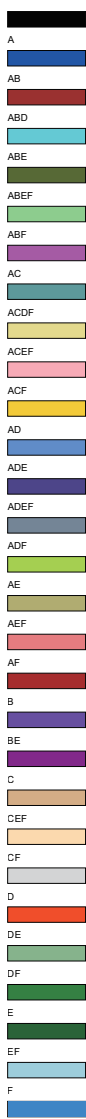


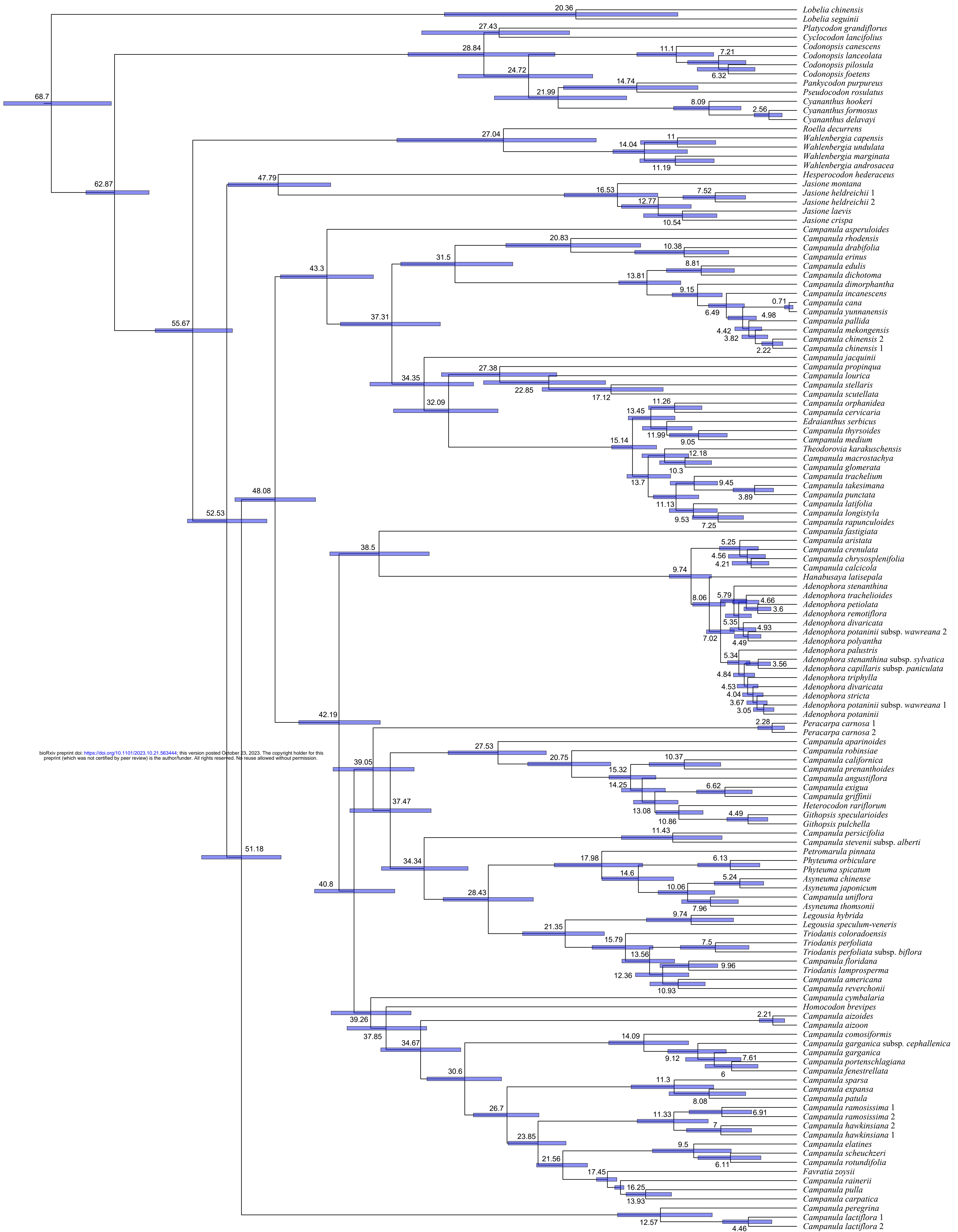






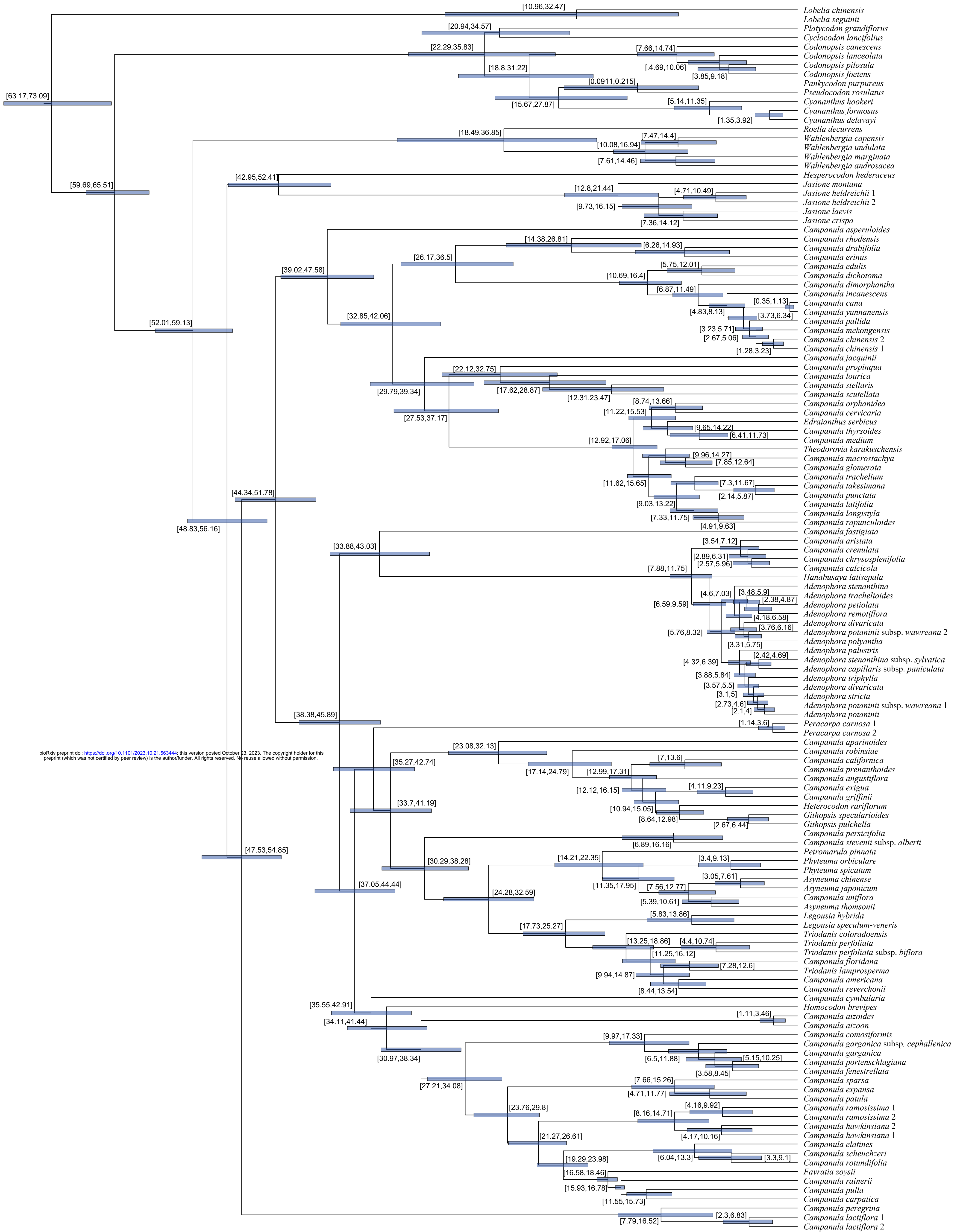
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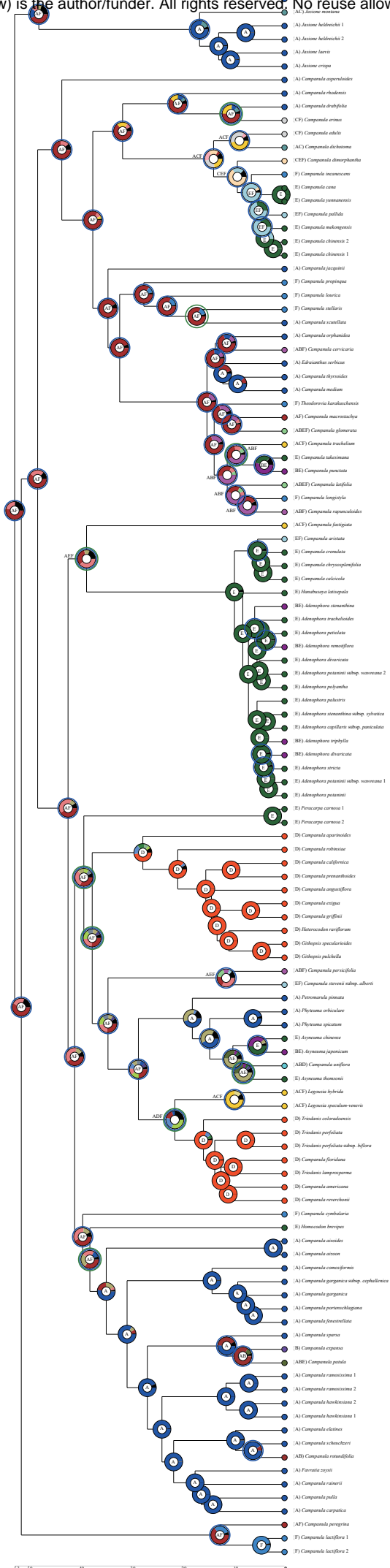
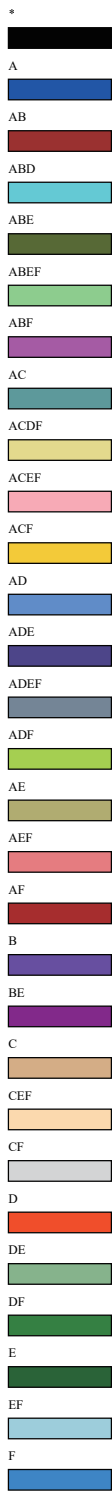
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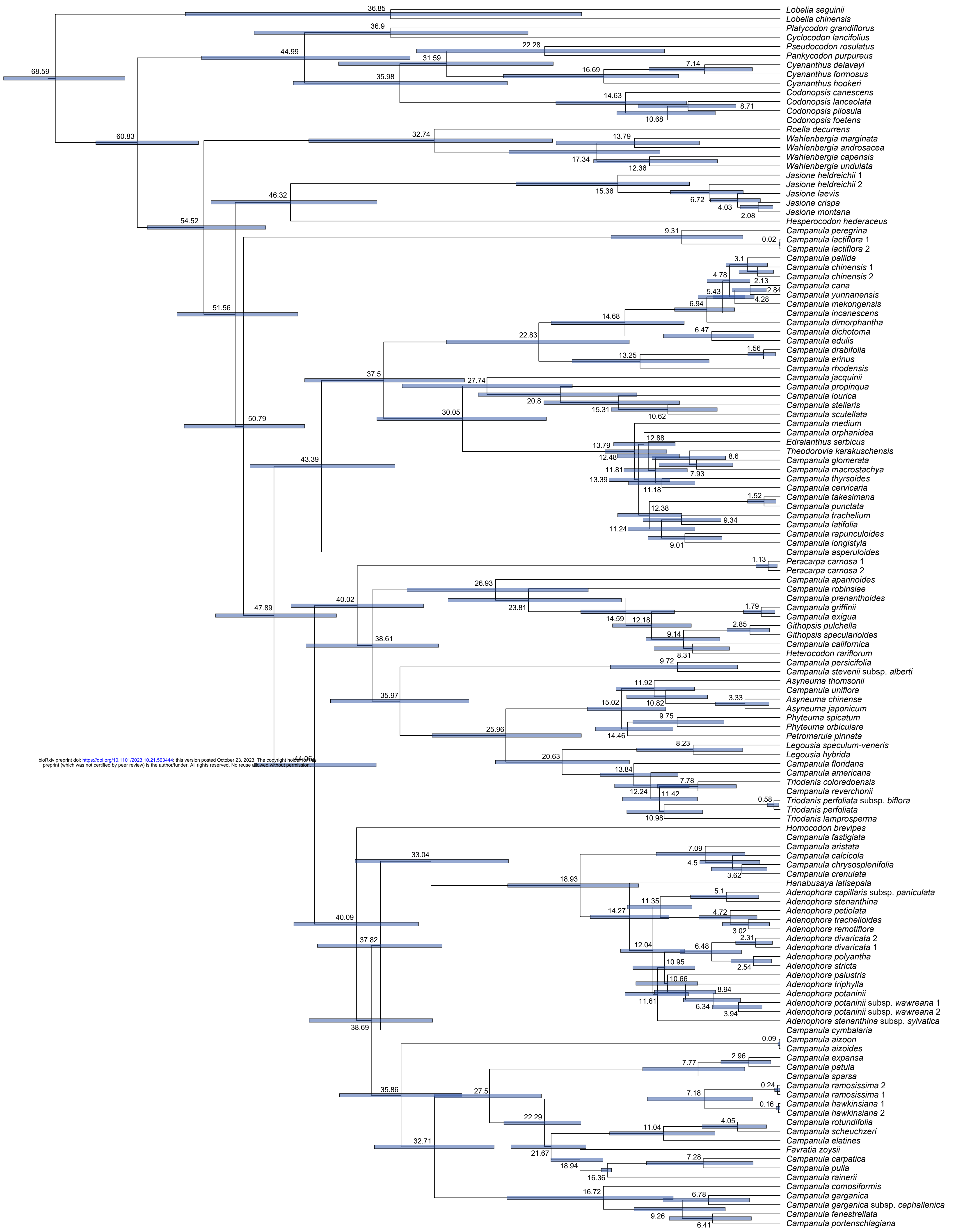


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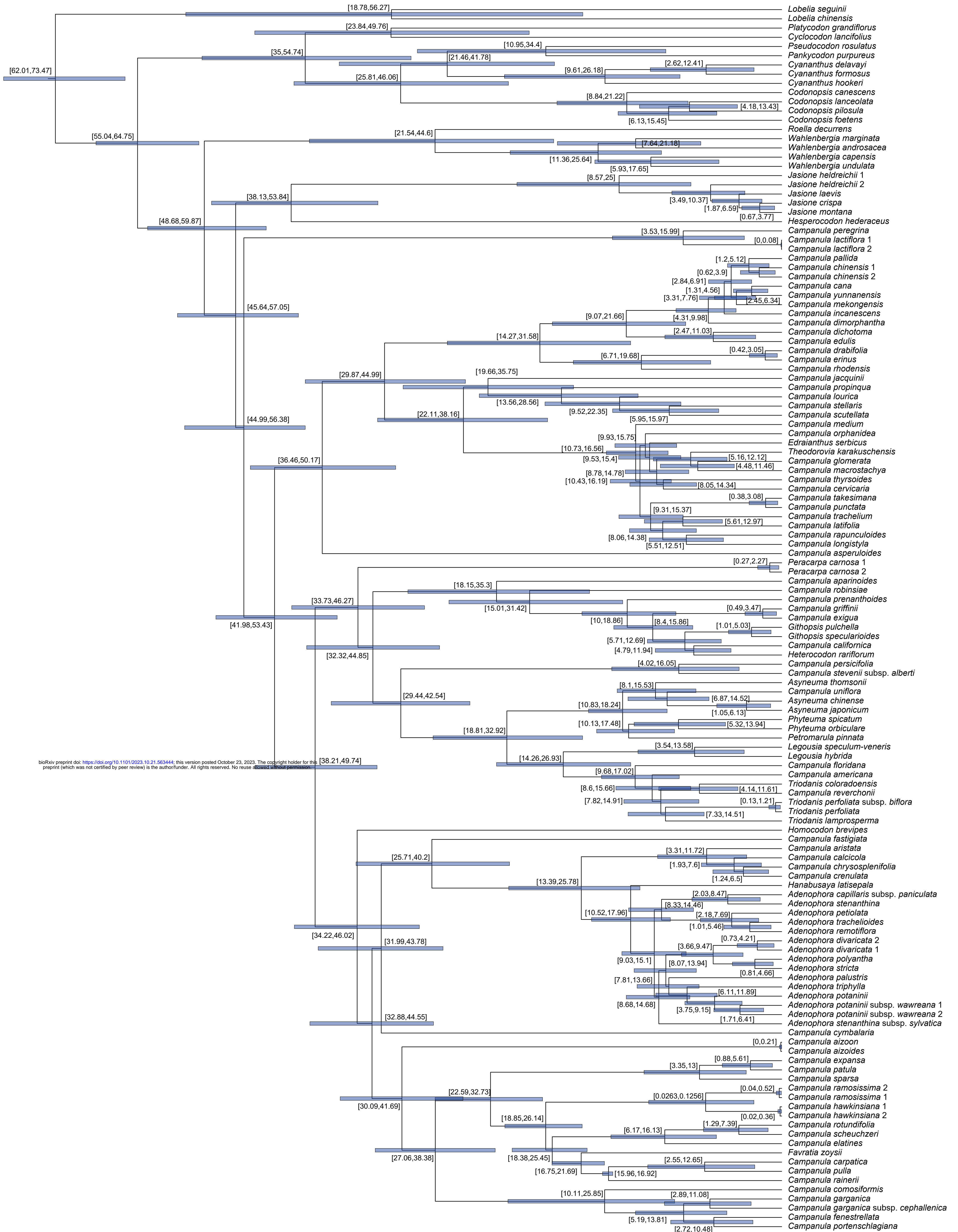






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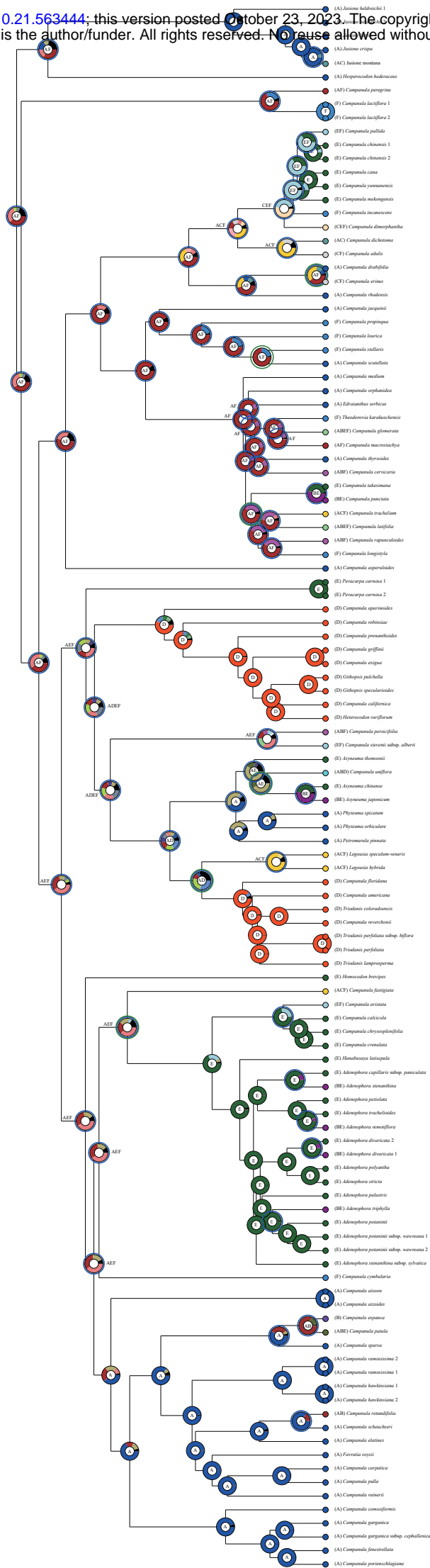


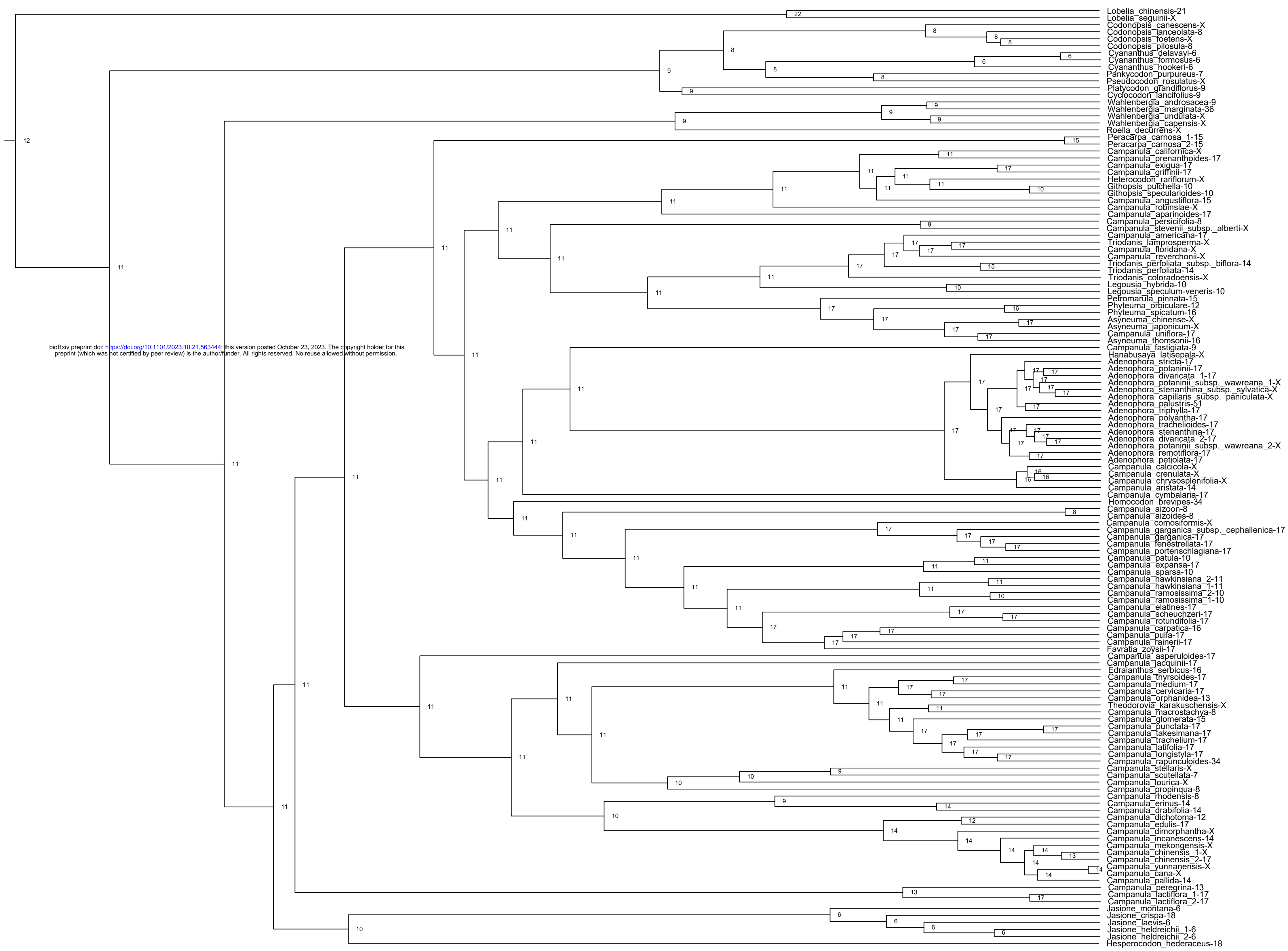


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