

1 **A novel phylogenomics pipeline reveals complex pattern of reticulate evolution in**

2 **Cucurbitales**

3 Running Title: **CAPTUS: a novel pipeline for phylogenomics**

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13  
14 *Abstract.*--- A diverse range of high-throughput sequencing data, such as target capture,  
15 RNA-Seq, genome skimming, and high-depth whole genome sequencing, are amenable to  
16 phylogenomic analyses but the integration of such mixed data types into a single  
17 phylogenomic dataset requires a number of bioinformatic tools and significant computational  
18 resources. Here, we present a novel pipeline, CAPTUS, to analyze mixed data in a fast and  
19 efficient way. CAPTUS assembles these data types, allows searching of the assemblies for loci  
20 of interest, and finally produces alignments that have been filtered for paralogs. Compared to  
21 other software, CAPTUS allows the recovery of a greater number of more complete loci across  
22 a larger number of species. We apply CAPTUS to assemble a comprehensive mixed dataset,  
23 comprising the four types of sequencing data for the angiosperm order Cucurbitales, a clade  
24 of about 3,100 species in eight mainly tropical plant families, including begonias  
25 (Begoniaceae) and gourds (Cucurbitaceae). Our phylogenomic results support the currently  
26 accepted circumscription of Cucurbitales except for the position of the holoparasitic

27 Apodanthaceae. Within Cucurbitaceae, we confirm the monophyly of all currently accepted  
28 tribes. However, we also reveal deep reticulation patterns both in Cucurbitales and within  
29 Cucurbitaceae. We show that conflicting results of earlier phylogenetic studies in  
30 Cucurbitales can be reconciled when accounting for gene tree conflict.

31

32 *Keywords*.--- Angiosperms, Cucurbitaceae, Cucurbitales, gene tree discordance, paralog  
33 filtering, genome skimming, phylotranscriptomics, nitrogen-fixing clade

34

35 Modern sequencing technology allows the rapid accumulation of large amounts of  
36 genomic data at low cost. The analysis of these raw data, however, is still a bottleneck and  
37 there is strong demand for skilled bioinformaticians who can handle such complex data in  
38 industry and in research institutions worldwide. Groups who cannot afford to pay for a  
39 bioinformatics expert rely on user-friendly analysis pipelines which are accessible without  
40 specialist training. For target capture data, HYBPIPER (Johnson et al. 2016) and SECAPR  
41 (Andermann et al. 2018) are the most widely used and of enormous importance for the entire  
42 field of phylogenomics. However, they are designed to process one sample at a time, only  
43 making use of multiple computer cores at certain stages [e.g., when assembling sequences *de*  
44 *novo* with SPADES (Bankevich et al. 2012)]. To process multiple samples simultaneously, the  
45 users must rely on external tools such as GNU parallel (Tange 2021) or additional  
46 containerization tools (Jackson et al. 2023). This lack of native parallelization capabilities can  
47 lead to suboptimal utilization of the computing resources in high-performance clusters or  
48 workstations and reduce accessibility for non-experts, potentially causing repeatability issues  
49 due to command inconsistencies across samples or human error. Also, since these pipelines  
50 were optimized for target capture data, their processing times for other data types can be  
51 exceptionally long. In contrast, for restriction site associated DNA (RAD) data, user-friendly  
52 specialized analysis pipelines have been published which efficiently analyze many samples in

53 parallel, e.g., IPYRAD (Eaton and Overcast 2020), Stacks (Rochette et al. 2019), and DDOCENT  
54 (Puritz et al. 2014). For example, IPYRAD (Eaton and Overcast 2020) uses a single or just a  
55 few consistent commands, that, combined with relatively short processing times, allow for  
56 rapid testing of alternative settings. IPYRAD allows users to summarize results of each  
57 processing step so that they can decide on the most appropriate settings needed for following  
58 steps.

59 With these features in mind, we developed CAPTUS, a pipeline written entirely in  
60 Python and aimed at building phylogenomic datasets from multiple types of high-throughput  
61 sequencing (HTS) data (target capture, RNA-Seq, genome skimming, and high-depth whole  
62 genome sequencing) by making extensive use of Python's native parallel computing to  
63 process many samples simultaneously in a consistent manner. Each step has a simple basic  
64 command syntax (although it can be customized with many options), provides complete  
65 reports in HTML to guide the settings of the next step, and is fully logged for repeatability.  
66 CAPTUS is able to extract nuclear, mitochondrial, and plastid proteins as well as any other type  
67 of DNA regions (such as ribosomal RNA, introns, spacers, RAD loci, etc.) in a single  
68 command, unlike HYBPIPER which would need as many runs as marker types or SECAPR  
69 which can only take a reference composed of individual exons in nucleotides. Additionally, as  
70 a feature unique to CAPTUS, it can also be used to search for novel conserved markers by  
71 clustering contigs that received no hits from the reference target loci across samples, thus  
72 making use of data that otherwise would be ignored. During the alignment stage, CAPTUS can  
73 also filter paralogs by taking advantage of reference target files that contain multiple  
74 sequences per locus such as Angiosperms353 (Johnson et al. 2019) and its more  
75 taxonomically comprehensive version Mega353 (McLay et al. 2021). The final outputs of  
76 CAPTUS are multiple sequence alignments (MSAs) for each reference locus found in the  
77 samples. The MSAs are organized by genomic compartment and format (amino acid,  
78 nucleotide, etc.), and multiple versions of each are provided (i.e., unfiltered, paralog-filtered,

79 untrimmed, and trimmed) so the user can select which type of alignment to analyze for  
80 phylogenetic inference.

81 We demonstrate the potential of CAPTUS in a test case focusing on the flowering plant  
82 order Cucurbitales, a clade of eight plant families, which have their diversity centers in the  
83 Tropics: Anisophylleaceae, Apodanthaceae, Begoniaceae, Coriariaceae, Corynocarpaceae,  
84 Cucurbitaceae, Datisceae, and Tetramelaceae (Zhang et al. 2006). Together, they include  
85 110 genera with more than 3,100 species, about 2,000 of them in the mega-diverse genus  
86 *Begonia* in Begoniaceae (Goodall-Copestake et al. 2009) and 1,000 in Cucurbitaceae (Stevens  
87 2001; Schaefer 2020). The genus *Begonia* is of great horticultural importance while  
88 Cucurbitaceae include some of the most important vegetable and fruit crops worldwide, like  
89 cucumber and watermelon (Schaefer and Renner 2011a). Morphologically, the taxa of  
90 Cucurbitales are rather diverse ranging from the holoparasitic Apodanthaceae (Bellot and  
91 Renner 2014), to annual and perennial herbs in Datisceae and Begoniaceae, to trees and  
92 shrubs in Anisophylleaceae, Corynocarpaceae, Coriariaceae, and Tetramelaceae and finally to  
93 woody or herbaceous climbers and creepers in Cucurbitaceae (Schaefer and Renner 2011a;  
94 Schaefer 2020).

95 A number of studies addressed phylogenetic problems in Cucurbitales in the past two  
96 decades. Zhang et al. (2006) produced the first comprehensive phylogeny estimate for the  
97 order based on nine plastid, nuclear and mitochondrial loci. Schaefer and Renner (2011b)  
98 inferred phylogenetic relationships in the order based on 14 DNA regions from all three  
99 genomes. Other studies targeted individual families: Zhang et al. (2007) provided the first  
100 comprehensive phylogeny estimate for Anisophylleaceae, Kocyan et al. (2007) and Schaefer  
101 et al. (2009) for Cucurbitaceae, Goodall-Copestake et al. (2009) for Begoniaceae, and Renner  
102 et al. (2020) for Coriariaceae. The results of Filipowicz and Renner (2010) suggested that the  
103 holoparasitic Apodanthaceae are best placed in Cucurbitales as sister lineage to all other taxa.  
104 In recent years, several phylogenomic studies contributed to an even better understanding of

105 the evolutionary relationships in Cucurbitaceae. Bellot et al. (2020) analyzed entire plastomes  
106 plus a set of 57 single-copy nuclear genes and the ITS region for 29 species from all but one  
107 tribe and detected four nodes with conflicting phylogenetic signal. With an impressive set of  
108 136 transcriptomes and full genomes of Cucurbitaceae, representing 52 of the 97 genera, Guo  
109 et al. (2020) produced a well-supported (albeit incomplete) phylogeny estimate which  
110 conflicted with earlier studies in several positions (Kocyan et al. 2007; Schaefer et al. 2009;  
111 Schaefer and Renner 2011b). Finally, the angiosperm-wide genus level analysis (Zuntini et al.  
112 in review) in the framework of the Plant and Fungal Trees of Life (PAFTOL) project (Baker  
113 et al. 2022) with an almost complete representation of Cucurbitales places the holoparasitic  
114 Apodanthaceae outside Cucurbitales in Malpighiales and finds a sister group relationship  
115 between Cucurbitales and Rosales, challenging the results of earlier studies.

116 In this study, we demonstrate how our new analysis pipeline CAPTUS can be used not  
117 only to extract the Angiosperms353 genes (Johnson et al. 2019), but also to derive a new set  
118 of thousands of nuclear genes from transcriptomic data and to extract entire plastomes. The  
119 resulting nuclear phylogenomic datasets were analyzed with coalescent and concatenation  
120 methods to infer a complete genus-level phylogeny for the Cucurbitales. We chose to test the  
121 performance and efficiency of CAPTUS against HYBPIPER since, in contrast to the other  
122 available pipelines, these two are able to use amino acid sequences as reference targets and  
123 can handle multiple reference target sequences per locus. Overall, we demonstrate that  
124 CAPTUS is more efficient and user-friendly than currently available pipelines and thus, a good  
125 choice for most data types and users with different levels of informatics skills.

126

## 127 MATERIALS & METHODS

### 128 *Sampling*

129 We sequenced a total of 125 Cucurbitales samples, 118 samples for target capture, and  
130 seven for high-depth whole genome sequencing (Table S1). Additionally, we downloaded 327

131 samples available in NCBI's SRA for a total of 240 RNA-Seq samples, 48 genome skimming  
132 samples (<50M reads), 31 high-depth whole genome sequencing samples (>50M reads), and  
133 eight additional target capture samples (Table S2). The entire dataset comprises 342 samples  
134 of Cucurbitales (including Apodanthaceae), representing the 110 currently accepted genera  
135 and 249 species. In order to confirm the results of the recent angiosperm-wide analysis of  
136 Zuntini et al. (in review), who found Apodanthaceae placed outside Cucurbitales, we decided  
137 to use a rather broad outgroup selection. We included 110 additional samples covering taxa of  
138 the nitrogen-fixing clade (representing 25 species in eight families of Rosales, 11 species in  
139 six families of Fagales, and 28 species in three families of Fabales), as well as 31 species in  
140 23 families of Malpighiales, six species in six families of Malvales, three species of Huaceae  
141 (Oxalidales), and three species of Vitaceae (Vitales).

142

#### 143 *DNA Extraction and Sample Preparation*

144 DNA samples were taken from the Royal Botanic Gardens, Kew DNA bank and the  
145 DNA Bank of Biodiversity of Plants, Technical University of Munich. Tissue samples for  
146 further DNA extractions came from the herbaria of Royal Botanic Gardens, Kew (K),  
147 Muséum National d'Histoire Naturelle in Paris (P), Botanische Staatssammlung München  
148 (M), and Technical University of Munich (TUM).

149 Several methods were used for DNA extraction from herbarium material intended for  
150 target capture. These included: (i) a CTAB-chloroform-based protocol with ethanol washes  
151 and a caesium chloride/ethidium bromide density gradient cleaning and dialysis, (ii) a  
152 modified CTAB protocol (Doyle and Doyle 1987) followed by an Agencourt AMPure XP  
153 bead clean-up (Beckman Coulter, Indianapolis, Indiana, USA), (iii) an SDS-based protocol  
154 using Magen HiPure SF Plant DNA Kit (Angen Biotech Co., Ltd, Guangzhou, China) using  
155 magnetic beads for extraction and cleaning, and (iv) the manufacturers protocol of the CTAB-  
156 based NucleoSpin Plant II Extraction Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren,

157 Germany) using silica columns for binding the DNA. Depending on the available herbarium  
158 material, we used 20-160 mg of material for extraction.

159 The seven samples intended for high-depth whole genome sequencing were extracted  
160 with the following methods: (i) the standard protocol of the CTAB-based NucleoSpin Plant II  
161 Extraction Kit, and (ii) the standard protocol of the MagBind® Plant DNA plus 96 Kit from  
162 Omega (<https://www.omegabiotek.com/product/mag-bind-plant-dna-plus-96-kit/>). These  
163 seven DNA extractions were sent for library preparation and sequencing to GENEWIZ  
164 Germany GmbH (Leipzig, Germany).

165 To measure DNA quality, we evaluated fragment size distribution using an Agilent  
166 Technologies 4200 TapeStation System with Genomic DNA ScreenTapes (Agilent  
167 Technologies, Santa Clara, California, USA) or by electrophoresis in 1% agarose gel. DNA  
168 was quantified using a Quantus™ Fluorometer (Promega Corporation, Madison, WI, USA) or  
169 with a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts,  
170 USA).

171

### 172 *Library Preparation*

173 Shearing was only performed for samples with fragment sizes above 350 bp using a  
174 Covaris M220 Focused-ultrasonicator with Covaris microTUBES AFA Fiber Pre-Slit Snap-  
175 Cap (Covaris, Woburn, Massachusetts, USA). Libraries were prepared with the DNA  
176 NEBNext Ultra™ II Library Prep Kit, including end-repair/end-prep, NEBNext Adapter  
177 ligation, size selection of preferred DNA fragments with a length of 300 to 400 bp using SPRI  
178 beads (Agencourt AMPure XP Bead Clean-up; Beckman Coulter, Indianapolis, IN, USA) and  
179 amplification with NEBNext Multiplex Oligos for Illumina (Dual Index Primer Set 1 and Set  
180 2, New England BioLabs, Ipswich, Massachusetts, USA) as indices for sample identification.

181

### 182 *Pooling, Hybridization, Target Enrichment, and Sequencing*

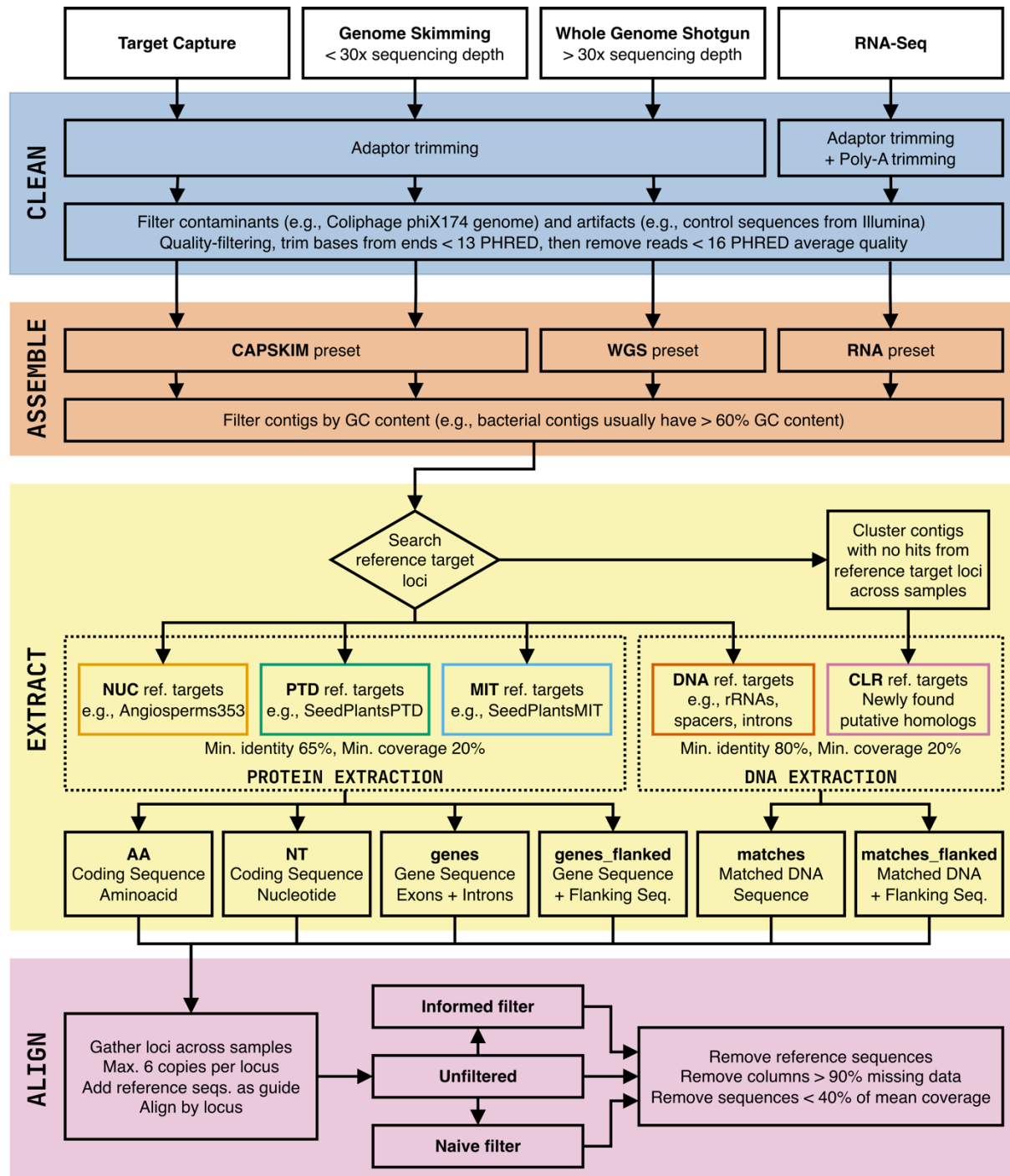
183 Libraries were grouped and pooled according to quality and quantity. Hybridization of  
184 the pooled libraries was done using the Arbor Biosciences myBaits Target Capture Kit,  
185 “Angiosperms353 v1” (Catalog #3081XX), following the manufacturers manual Version 4.01  
186 (myBaits® Kit Manual – Arbor Biosciences 2020). Hybridized libraries were enriched with  
187 the NEBNext Ultra II Q5 Master Mix (New England BioLabs, Ipswich, Massachusetts, USA).  
188 According to their respective quantity and quality, library pools were normalized and pooled  
189 for sequencing on a MiSeq platform (Illumina, San Diego, California, USA) at the Jodrell  
190 Laboratory, Royal Botanic Gardens, Kew or at Macrogen Europe B.V. in Amsterdam  
191 (Macrogen, Inc., Seoul, Korea) with a HiSeq system (Illumina, San Diego, California, USA).

192

### 193 *Sequence Analysis Workflow*

194 CAPTUS was implemented in Python 3, is freely available and maintained through  
195 GitHub (<https://github.com/edgardomortiz/Captus>) and fully documented  
196 (<https://edgardomortiz.github.io/captus.docs/>). For convenience, it can be installed directly  
197 from Bioconda (<https://bioconda.github.io/recipes/captus/README.html>). The workflow of  
198 CAPTUS consists of four steps controlled by their respective modules called `clean`,  
199 `assemble`, `extract`, and `align`, which are typically run in that order (Fig. 1).





200

201 FIGURE 1. Workflow of the analysis pipeline CAPTUS. The default thresholds shown can be  
 202 changed by the user via command options.

203

204 Alternatively, the analysis can be started at different points depending on the sample  
 205 data provided, if raw reads are provided one should start with the `clean` module, if the reads  
 206 have already been cleaned, they can be directly analyzed with the `assemble` module, and if

207 previously assembled data or reference genomes are provided the analysis can start using the  
208 `extract` module. CAPTUS provides flexibility in combining samples that enter the workflow  
209 at different stages. For example, a phylogenomic dataset could be composed of samples  
210 represented by target capture raw sequencing reads, samples with clean RNA-Seq reads, and  
211 genomic assemblies downloaded from GenBank in order to increase taxon sampling.

212

213 *The clean module.*--- CAPTUS uses BBDUK from BBTOOLS (Bushnell 2022) to clean raw  
214 reads. Cleaning is performed in two steps. First, adaptors and poly-A tails (when cleaning  
215 RNA-Seq reads) are trimmed in two consecutive rounds. Then, adaptor-free reads are quality-  
216 trimmed and filtered for common HTS contaminants. In this step, leading and trailing bases  
217 with PHRED scores <13 are trimmed and then trimmed reads with average PHRED score <16  
218 are removed, both thresholds can be altered by the user.

219 When cleaning is completed, FASTQC (Andrews 2019) or FALCO (de Sena Brandine  
220 and Smith 2021) is run both on the raw and the cleaned files to evaluate and compile quality  
221 statistics. FALCO is a faster implementation of FASTQC that produces identical results (de  
222 Sena Brandine and Smith 2021). Finally, CAPTUS summarizes the cleaning statistics of all the  
223 samples before and after cleaning in a single HTML report that allows a quick overlook of all  
224 quality measurements.

225 For the Cucurbitales analysis, default settings were used for all samples in the `clean`  
226 module except RNA-Seq, for which the option `--rna` was added to trim poly-A tails.

227

228 *The assemble module.*--- The clean reads are passed to the assembly module which uses  
229 MEGAHIT (Li et al. 2015) for *de novo* assembly. MEGAHIT was designed to handle  
230 enormous metagenomic datasets and therefore its default settings are tuned to that purpose.  
231 We tested several combinations of MEGAHIT settings in order to optimize the assembly of  
232 other types of data, these are provided in CAPTUS under three presets: (i) CAPSKIM for target

233 sequence capture, genome skimming data, or a combination of both, (ii) RNA for RNA-Seq  
234 data, and (iii) WGS for high-depth whole genome sequencing data.

235 The `assemble` module also provides an option to subsample a fixed number of reads per  
236 sample prior to assembly, which is useful when the sequencing depth is too high for a  
237 particular sample or for speeding up assembly during trial runs.

238         Once assembly is completed, CAPTUS can also filter contigs based on their GC  
239 content. For example, a maximum of 60% GC would be appropriate to remove bacterial  
240 contamination in most eukaryotic genomes (Fierst and Murdock 2017). Finally, CAPTUS  
241 computes assembly statistics and produces a single HTML report for all processed samples.

242         Target capture and genome skimming samples of the Cucurbitales were run with the  
243 default assembly preset (`--preset CAPSKIM`), RNA-Seq samples were assembled using `-`  
244 `--preset RNA`, and high-depth whole genome sequencing samples using `--preset WGS`.  
245 To remove potential bacterial contamination, we used the option `--max_contig_gc 60`  
246 for all assemblies.

247

248 *The extract module.*--- Once assemblies are completed, they can be searched for particular  
249 loci of interest (i.e., reference target loci). CAPTUS allows the simultaneous search and  
250 extraction of five types of markers (i) nuclear proteins (NUC), (ii) plastid proteins (PTD), (iii)  
251 mitochondrial proteins (MIT), (iv) miscellaneous DNA regions (DNA), and (v) new  
252 clustering-derived putative homologs (CLR).

253         Protein extractions (NUC, PTD, and MIT markers) use reference target loci provided as  
254 amino acid sequences or coding sequences in nucleotides (CDS). CAPTUS uses the program  
255 SCIPPIO (Hatje et al. 2011) to perform protein extractions. SCIPPIO is able to automatically  
256 correct frameshifts that could result from sequencing or assembly error and it can recover  
257 proteins that are spread across several contigs (Hatje et al. 2011), an important advantage

258 given that most assemblies resulting from target capture data are highly fragmented. CAPTUS  
259 comes bundled with four reference target sets, two NUC references (the Angiosperms353  
260 (Johnson et al. 2019) target file and a curated version of the taxonomically expanded  
261 Mega353 (McLay et al. 2021) target file), as well as a plastid PTD reference  
262 (SeedPlantsPTD), and a mitochondrial MIT reference (SeedPlantsMIT).  
263 Our curation of the Mega353 reference target file consisted in removing contaminated  
264 sequences (e.g., fungal, algal, organellar sequences) and then clustering at 78% identity to  
265 reduce sequence redundancy. Thus, CAPTUS still takes advantage of the expanded taxonomic  
266 representation of the Mega353 reference targets (McLay et al. 2021) while bypassing the  
267 additional step of filtering by taxon suggested by the instructions  
268 (<https://github.com/chrisjackson-pellicle/NewTargets>). The PTD and MIT references  
269 represent carefully curated sets of proteins found in all organellar genomes of seed plants  
270 originally downloaded from GenBank. Reference target sets for organism groups other than  
271 seed plants are being developed.

272       Miscellaneous DNA extractions are aimed at recovering other types of DNA regions  
273 (e.g., complete genes with introns, non-coding regions, ribosomal genes, individual exons,  
274 RAD markers, etc.). In this case, CAPTUS uses the program BLAT (Kent 2002) to search the  
275 assembly and the hits that are found across contigs are greedily assembled and concatenated  
276 using our own code.

277       Additionally, CAPTUS can be used to find new putative homologs by clustering across  
278 samples the contigs that had no hits from the reference target loci. In this case, the program  
279 MMSEQS2 (Steinegger and Söding 2018) is used, and several of its settings are available  
280 through CAPTUS. Once the sequence clusters have been found, they are filtered by the number  
281 of samples in the cluster and sequence length, and only the most represented sequences per  
282 cluster are selected as new reference targets to perform another extraction of the

283 miscellaneous DNA type in CAPTUS. The output from this kind of extraction is indicated by  
284 the prefix CLR.

285       The output files from protein extractions (NUC, PTD, MIT) are provided in four  
286 possible formats (Fig. S1a): the protein sequence in amino acids (AA), the coding sequence in  
287 nucleotides or CDS (NT), the complete gene sequence including introns (*genes*), and the  
288 complete gene sequence flanked by a fixed number of nucleotides (*genes\_flanked*).  
289 Similarly, the output from a miscellaneous DNA extraction and clustering-derived markers  
290 (DNA, CLR), have two possible formats (Fig. S1b): the segment of sequence that was matched  
291 to the reference (*matches*), and the matched segments flanked by a fixed number of  
292 nucleotides (*matches\_flanked*).

293       Once extractions are finished, CAPTUS collects extraction statistics (e.g., recovered  
294 marker length, similarity to the reference sequence, number of paralogs, number of contigs  
295 used in the gene assembly, etc.) from all the processed samples and produces a single HTML  
296 report that provides a quick look at the marker recovery across markers and samples using a  
297 dynamic heatmap.

298       For our Cucurbitales dataset, the minimum recovery percentage was set at 20%, the  
299 default in CAPTUS. We used the option `-n Angiosperms353` to extract nuclear markers  
300 using the original Angiosperms353 reference targets and `-n Mega353` to extract nuclear  
301 markers using our curated version of the taxonomically expanded Mega353. Additionally,  
302 organellar proteins were extracted with options `-p SeedPlantsPTD` and `-m`  
303 `SeedPlantsMIT`. Finally, we created a custom miscellaneous DNA reference by  
304 segmenting the Cucurbitales plastomes available in GenBank in 38 pieces ranging from ~3  
305 kbp to ~5 kbp which we stored in a FASTA file (`Plastome38.fasta`, Appendix S1) and  
306 extracted in CAPTUS using the option `-d`.

307           Additionally, we created a set of 5,435 of reference genes derived from publicly  
308 available transcriptomic data. We took the 240 transcriptomic assemblies from CAPTUS and  
309 removed the transcripts that had hits to organellar proteins, thereby retaining only putatively  
310 nuclear transcripts. We ran CODAN (Nachtigall et al. 2021) on the nuclear transcripts in order  
311 to find coding regions within them, searching both strands and using the `PLANTS_full`  
312 model which only emits a CDS when a complete protein, from start to stop codon, is  
313 identified within the transcript. Then we clustered the coding sequences across samples using  
314 the `extract` module of CAPTUS, with the following options:

```
315 -c --mmseqs2_method easy-cluster --cluster_mode 2 --  
316 cl_min_identity 70 --cl_seq_id_mode 1 --cl_min_coverage 66 --  
317 cl_rep_min_len 540 --cl_min_samples 144 --cl_max_copies 4. These
```

318 CAPTUS options retained 5,435 clusters that were at least 540 bp in length, grouped at least  
319 144 transcriptomic samples (60%), and contained at most an average of four gene copies.  
320 After removing within-cluster redundant sequences, the resulting reference contained 13,492  
321 sequences representing these 5,435 CDS clusters, this reference is called from this point  
322 onwards `RNA5435 (RNA5435.fasta, Appendix S2)`. The new reference targets were used  
323 in the `extract` module of CAPTUS across all 454 samples using the option `-n`  
324 `RNA5435.fasta --nuc_min_score 0.15 --nuc_min_identity 70` to match  
325 the clustering identity threshold used for creating the reference .

326

327 *The align module.*--- The extraction output is then processed by the alignment module.  
328 Individual markers are collected across samples and organized in separate FASTA files per  
329 locus. By default, CAPTUS collects a maximum of five paralogs per sample and per marker to  
330 be aligned. The reference sequences are also added to each locus file to serve as alignment  
331 guides in case the sequences recovered from the samples are fragmentary. Then, the  
332 alignment is performed with MAFFT (Katoh and Standley 2013) or MUSCLE5 (Edgar 2022)

333 using default settings or by selecting one of their specific algorithms through CAPTUS.  
334 However, if protein sequence in amino acid (AA) and their corresponding coding sequence in  
335 nucleotides (NT) are aligned in the same run, CAPTUS aligns the AA with MAFFT and then  
336 uses the AA alignment as template for the NT, thus producing a codon-aware alignment for the  
337 CDS. CAPTUS also allows the user to provide the sample(s) that should be considered as  
338 outgroup, in this case the program will place those samples as the first sequence(s) in the  
339 alignments in the order provided. This feature (`--outgroup`) takes advantage of a common  
340 feature of many phylogenetic estimation programs (e.g., IQ-TREE, RAXML, MRBAYES)  
341 which arbitrarily draw the first sample in the alignment at the root of their output trees.

342       Once the FASTA files are aligned, paralogs are filtered using two alternative  
343 algorithms, `naive` and `informed`. In the `naive` filter, only the best hit is kept for each  
344 sample and no further filtering is performed. The `informed` filtering algorithm takes  
345 advantage of reference datasets that contain multiple sequences per locus, such as the  
346 `Angiosperms353` (Johnson et al. 2019) or `Mega353` (McLay et al. 2021) target files as well as  
347 the ones developed for CAPTUS (`SeedPlantsPTD` and `SeedPlantsMIT`). For example, the  
348 `Angiosperms353` reference target set (as well as its expansion `Mega353`) was derived from the  
349 1KP Project (Matasci et al. 2014; One Thousand Plant Transcriptomes Initiative 2019) data,  
350 where each gene could potentially be present in more than 1,000 species. To build a less  
351 redundant sequence collection while still covering the entire phylogenetic diversity of the  
352 angiosperms, each selected locus was clustered at ~70% identity, usually selecting only one  
353 representative sequence per cluster for the final reference dataset. Thanks to this feature, one  
354 could expect that when analyzing a single taxonomic group such as a family or genus, all  
355 samples would have a best match to only one sequence in the reference targets collection for a  
356 given locus (i.e., the closest relative present in the reference targets). However, a few samples  
357 could have better matches to a different reference sequence than the rest which can be  
358 possible when the locus in question has multiple copies (paralogs) in the studied group, and



359 the most common copy found in the group is absent from those few samples. In these cases,  
360 CAPTUS compares all recovered paralogs with the reference sequence that best matches most  
361 of the samples and keeps the copy that is most similar to that reference sequence across  
362 samples. Finally, it could also happen that a sample presents a single copy for a specific locus  
363 but with a sequence that is much more divergent than the average in the alignment (e.g., a  
364 remote paralog found in a contaminated contig). By default, CAPTUS will remove any  
365 sequence with average pairwise identity that is more than 4.0 standard deviations below the  
366 mean pairwise identity of the entire alignment, the number of standard deviations can be  
367 changed with the option `--tolerance`.

368       Recently developed coalescent methods, such as ASTRAL-PRO (Zhang et al. 2020;  
369 Zhang and Mirarab 2022a), are capable of analyzing trees of genes with paralogs (i.e., multi-  
370 copy genes), therefore CAPTUS also provides the alignments with paralogs as well as the file  
371 required by ASTRAL-PRO for mapping the paralog names to the names of the samples for  
372 species tree calculation. The reference target sequences are then removed from the  
373 alignments. Finally, all the produced alignments and their filtered versions are trimmed using  
374 CLIPKIT (Steenwyk et al. 2020), which can remove alignment columns based on criteria like  
375 informativeness or proportion of missing data. By default, CAPTUS removes columns with >  
376 90% missing data and sequences with < 40% mean coverage. Alternatively, a minimum  
377 number of sites per column instead of a percentage can be specified with `--`  
378 `min_data_per_column`. As in previous modules, CAPTUS computes alignment statistics  
379 as well as sample occupancy statistics from all the FASTA files along all filtering and  
380 trimming stages and produces a comprehensive HTML report that can be used to determine  
381 outlier markers or samples that should be removed or curated more carefully before  
382 proceeding to phylogenetic analysis.

383       For the Cucurbitales data, all extracted loci were aligned in CAPTUS using MAFFT's  
384 most accurate algorithm E-INS-i (`--align_method mafft_genafpair`). For coding



385 markers we used the option `-f AA, NT` to take advantage of codon-aware alignments for  
386 CDS, and for Plastome38 we used `-f MA`. For trimming, we used `--`  
387 `min_data_per_column 6`, to keep alignment columns with six or more sequences. For  
388 the Plastome38 we also decreased the tolerance of the `informed` paralog filter to `--`  
389 `tolerance 2.0`.

390

### 391 *Phylogenetic Analyses*

392 We chose the trimmed, codon-aligned coding sequences (format NT) from the  
393 extractions performed with reference targets sets Angiosperms353, Mega353 and RNA5435.  
394 For the reference targets set Plastome38 (the plastome segments) we selected the format  
395 `matches` from the CAPTUS output. We analyzed the unfiltered alignments (i.e., including  
396 paralogs) as well as the alignments resulting from the `naive` and `informed` paralog  
397 filtering strategies for nuclear markers but only the alignments filtered by the `informed`  
398 algorithm for the plastome.

399

400 *Gene tree estimation.*--- For each individual nuclear gene alignment we inferred a phylogeny  
401 using IQ-TREE v. 2.2.2.6 (Minh et al. 2020b). During the run we used MODELFINDER  
402 (Kalyaanamoorthy et al. 2017) to determine the most appropriate nucleotide substitution  
403 model with `-m TEST`. Nodal support was inferred from 1,000 ultrafast bootstrap replicates  
404 with option `-bb 1000` (Hoang et al. 2018). For comparison purposes, we also estimated  
405 nuclear gene tree phylogenies using FASTTREE v. 2.1.11 (Price et al. 2010) with the manual  
406 recommended options to increase the accuracy of the search and using the GTR substitution  
407 model (`-pseudo -spr 6 -mlacc 3 -slownni -gtr`).

408

409 *Species tree estimation.*--- We estimated species trees using two methods, concatenation of  
410 alignments followed by maximum likelihood estimation in IQ-TREE v. 2.2.2.6 (Minh et al.  
411 2020b), and coalescent estimation by summarization of quartet frequencies in gene trees using  
412 ASTRAL-PRO v. 1.15.1.3 (Zhang et al. 2020; Zhang and Mirarab 2022a). For concatenation,  
413 each individual locus alignment must contain a single sequence per sample (no paralogs  
414 allowed), therefore we can only apply this method to the alignments that were filtered for  
415 paralogs, while ASTRAL-PRO was applied to the filtered alignments as well as to the  
416 alignments containing multiple copies. The concatenation analyses in IQ-TREE were run with  
417 the same options as for the individual gene trees, and the loci alignments were provided as  
418 separate files in a single directory with the option `-p`, so IQ-TREE can automatically  
419 concatenate them into a supermatrix prior to analysis. The concatenation method was applied  
420 only to the set 353 nuclear genes (extracted using Angiosperm353 or Mega353) and to the  
421 plastome segments (Plastome38)

422 For the ASTRAL-PRO analysis, the individual gene trees calculated by IQ-TREE or  
423 FASTTREE were provided as well as the required file that maps the paralog names to their  
424 corresponding sample name produced by CAPTUS (`captus-`  
425 `assembly_align.astral-pro.tsv`). We also increased the number of placement and  
426 subsampling rounds to 16 (`-R`), as well as the proportion of taxa subsampled (`--`  
427 `proportion 0.75`) and calculated alternative quartet frequencies (`-u 3`).

428  
429 *Site concordance analyses.*--- In order to measure concordance between alignment sites and  
430 the species tree, we performed a concordance factor analysis for each species tree (Minh et al.  
431 2020a). The analysis was done in IQ-TREE v. 2.2.2.6 by supplying the species tree (`-t`), the  
432 folder containing the loci alignments (`-p`), and averaging the site concordance over 1,000  
433 quartets (`--scf1 1000`).

434

435 *Phylogenetic conflict analysis of selected nodes.*--- Contentious relationships in the  
436 Cucurbitales are centered around the relationships among tribes in the Cucurbitaceae as well  
437 as the relationships among Cucurbitales families. In order to visualize the amount of conflict  
438 at selected nodes in the species tree, we used the branch quartets frequencies analysis in  
439 DISCOVISTA v. 1.0 (Sayyari et al. 2018), after annotating the species belonging to each  
440 Cucurbitaceae tribe, to each of the families in Cucurbitales, and to the clades in the outgroup.

441  
442 *Phylogenetic network estimation.*--- We concatenated the Angiosperms353 alignments  
443 filtered by the `informed` method in CAPTUS keeping only the Cucurbitales families. This  
444 concatenated alignment was used as input for SPLITTREE v. 4.18.2 (Huson and Bryant 2006)  
445 in order to estimate a phylogenetic network using the Neighbor Net algorithm on a matrix of  
446 uncorrected P distances.

447

#### 448 *Pipeline Comparison*

449 To compare the locus recovery efficiency between CAPTUS and HYBPIPER (Johnson et  
450 al. 2016) on different data types, we extracted the Angiosperms353 loci (Johnson et al. 2019)  
451 from a selection of 80 samples (20 from each data type) representing all families of  
452 Cucurbitales and all tribes of Cucurbitaceae (Table S3). Since the HYBPIPER workflow does  
453 not include a read cleaning step, we used reads cleaned by CAPTUS as a common input for  
454 both pipelines. CAPTUS was run for the `assemble` and `extract` modules with the  
455 aforementioned presets optimized for each data type. HYBPIPER v2.1.2 was run using either  
456 BLASTx (Camacho et al. 2009) or DIAMOND (Buchfink et al. 2021), hereafter HYBPIPER-  
457 BLASTx and HYBPIPER-DIAMOND respectively, with an identity threshold of 65% (`--`  
458 `thresh 65`) to equalize with that of CAPTUS. A protein target file downloaded from  
459 <https://github.com/mossmatters/Angiosperms353> was used as a reference for both pipelines.  
460 All commands were run on a MacOS X system equipped with a 2.7 GHz Intel Xeon E5

461 processor with 24 threads and 64 GB RAM, allocating 6 threads per sample. Running time,  
462 number of loci recovered, and total CDS length recovered for each sample were compared  
463 across pipelines, considering a locus to be "recovered" when at least 20% of the reference  
464 sequence length was retrieved.

465 To assess how the differences in locus recovery efficiency among pipelines affect  
466 phylogenetic inference, we estimated coalescent species trees from the CDS recovered by  
467 each pipeline and compared their topologies and nodal supports. For CAPTUS, trimmed CDS  
468 alignments with paralogs removed by the `informed` filtering were generated using the  
469 `align` module with default settings. For HYBPIPER-BLASTx and HYBPIPER-DIAMOND,  
470 trimmed CDS alignments were generated using MAFFT v7.520 (Katoh and Standley 2013)  
471 with the automatic strategy selection mode (`--auto`) and CLIPKIT v1.4.1 (Steenwyk et al.  
472 2020) with default settings. A maximum likelihood tree was estimated for each locus using  
473 IQ-TREE v2.2.2.3 (Minh et al. 2020b) under the best-fit substitution model determined by  
474 MODELFINDER (Kalyaanamoorthy et al. 2017) with nodal support inferred from 1,000  
475 ultrafast bootstrap replicates (Hoang et al. 2018). Coalescent species trees were estimated  
476 from the set of gene trees using WASTRAL-HYBRID v1.15.2.3 (Zhang and Mirarab 2022b)  
477 with 16 rounds each for placements and subsampling (`-R`), and then visualized using  
478 TOY TREE v2.0.5 (Eaton 2020).

479

## 480 RESULTS

### 481 *Sequencing*

482 From our 118 library preparations for target capture, 92 (78%) worked at the first  
483 attempt. Only eight of these successful libraries needed post-processing steps such as library  
484 concentration or increased number of PCR cycles to improve their quality prior to sequencing.  
485 For the remaining 26 libraries (22%) that had to be repeated, we used material of the same  
486 herbarium voucher for 14, while we had to select a different specimen for 12. Among the

487 repeated libraries, only two needed post-processing. Nonetheless, once the sequencing was  
488 completed, three samples had to be discarded due to insufficient data. Two of the discarded  
489 samples corresponded to replicated libraries of *Octomeles sumatrana* for which we had a  
490 replacement within the target capture batch. Only one of them, *Bambekea racemosa*, needed  
491 to be replaced by additional high-depth whole genome sequencing, which was performed for  
492 a total of seven additional samples. In the end, a total of 122 samples (115 target capture and  
493 7 high-depth whole genome sequencing) yielded enough high-quality data to be used for the  
494 analyses (Table S4).

495

#### 496 *Read Cleaning and Assembly*

497 RNA-Seq data had the largest average percentage of reads and percentage of base  
498 pairs removed with 4.7% and 9.2% respectively. The data type that had the largest average  
499 percentage of raw reads with adaptors was target capture with 14.6%, adaptors were fully  
500 removed from all data types after cleaning (Table 1: Cleaning). Average assembly size after  
501 removing contigs with GC content > 60% was similar for target capture and RNA-Seq data  
502 (c. 44 Mbp), followed by genome skimming (289.3 Mbp) and high-depth whole genome  
503 sequencing (591.1 Mbp). Despite their similar assembly sizes, target capture data produced  
504 more fragmented assemblies than RNA-Seq as indicated by their average number of contigs  
505 (88.3 k vs. 62.7 k respectively) and their average N50 (513.3 bp vs. 1084 bp respectively),  
506 while the largest average N50 corresponded to high-depth whole genome sequencing data  
507 with 5023.7 bp. The GC content across data types was similar and ranged between 34.8% to  
508 42.6% (Table 1: Assembly).

509 The largest average percentage of contigs removed because of exceeding the threshold of  
510 60% GC content belongs to target capture data with 4%. The average GC content of the  
511 filtered contigs ranged from 59.8% to 65.4% across data types (Table 1: Assembly).

512

513 TABLE 1. Summary statistics by data type.

|                                 | CAP          |       | GSK           |        | WGS           |        | RNA           |        |
|---------------------------------|--------------|-------|---------------|--------|---------------|--------|---------------|--------|
|                                 | N = 126      |       | N = 48        |        | N = 38        |        | N = 240       |        |
|                                 | Mean         | SD    | Mean          | SD     | Mean          | SD     | Mean          | SD     |
| Raw reads (Millions)            | <b>4.7</b>   | 2.6   | <b>17.3</b>   | 12.1   | <b>113.5</b>  | 99.4   | <b>44.1</b>   | 33.2   |
| Raw bases (Gbp)                 | <b>0.7</b>   | 0.4   | <b>2.5</b>    | 1.8    | <b>15.9</b>   | 12.2   | <b>5.9</b>    | 4.4    |
| <b>Cleaning</b>                 |              |       |               |        |               |        |               |        |
| Removed reads (%)               | <b>2.3</b>   | 2.7   | <b>1.3</b>    | 2.3    | <b>2.0</b>    | 3.7    | <b>4.7</b>    | 10.3   |
| Removed bases (%)               | <b>8.4</b>   | 8.9   | <b>4.2</b>    | 6.7    | <b>4.3</b>    | 8.7    | <b>9.2</b>    | 13.1   |
| Raw reads with adaptors (%)     | <b>14.6</b>  | 22.0  | <b>5.6</b>    | 12.6   | <b>1.8</b>    | 6.8    | <b>8.5</b>    | 11.3   |
| Clean reads with adaptors (%)   | <b>0.0</b>   | 0.0   | <b>0.0</b>    | 0.0    | <b>0.0</b>    | 0.0    | <b>0.0</b>    | 0.0    |
| <b>Assembly</b>                 |              |       |               |        |               |        |               |        |
| Contigs (Thousands)             | <b>88.3</b>  | 61.1  | <b>489.0</b>  | 261.4  | <b>823.7</b>  | 654.3  | <b>62.7</b>   | 37.9   |
| Assembly size (Mbp)             | <b>44.4</b>  | 31.7  | <b>289.3</b>  | 162.2  | <b>591.1</b>  | 256.3  | <b>44.7</b>   | 23.8   |
| N50 (bp)                        | <b>513.3</b> | 116.1 | <b>923.3</b>  | 964.8  | <b>5023.7</b> | 4384.0 | <b>1084.0</b> | 289.5  |
| GC content (%)                  | <b>40.8</b>  | 3.6   | <b>37.5</b>   | 2.7    | <b>34.8</b>   | 1.5    | <b>42.6</b>   | 2.1    |
| Filtered contigs (Thousands)    | <b>1.3</b>   | 3.1   | <b>6.2</b>    | 20.1   | <b>16.2</b>   | 49.1   | <b>1.8</b>    | 6.1    |
| Filtered contigs (%)            | <b>4.0</b>   | 21.0  | <b>1.8</b>    | 7.5    | <b>1.5</b>    | 3.6    | <b>1.9</b>    | 3.4    |
| Filtered contigs GC content (%) | <b>59.8</b>  | 14.6  | <b>64.1</b>   | 1.9    | <b>65.4</b>   | 2.4    | <b>62.9</b>   | 1.0    |
| <b>Extraction</b>               |              |       |               |        |               |        |               |        |
| <i>Angiosperms353</i>           |              |       |               |        |               |        |               |        |
| Percentage of loci recov. (%)   | <b>87.9</b>  | 19.3  | <b>70.9</b>   | 30.7   | <b>98.8</b>   | 4.1    | <b>92.6</b>   | 14.0   |
| Total CDS length recov. (kbp)   | <b>183.8</b> | 57.5  | <b>137.2</b>  | 89.0   | <b>255.9</b>  | 28.5   | <b>229.2</b>  | 53.3   |
| <i>Mega353</i>                  |              |       |               |        |               |        |               |        |
| Percentage of loci recov. (%)   | <b>88.0</b>  | 19.3  | <b>71.3</b>   | 30.6   | <b>98.9</b>   | 3.9    | <b>92.7</b>   | 14.0   |
| Total CDS length recov. (kbp)   | <b>190.2</b> | 60.6  | <b>145.6</b>  | 95.1   | <b>271.3</b>  | 30.1   | <b>241.8</b>  | 57.1   |
| <i>RNA5435</i>                  |              |       |               |        |               |        |               |        |
| Percentage of loci recov. (%)   | <b>20.8</b>  | 14.9  | <b>61.2</b>   | 30.5   | <b>97.4</b>   | 8.4    | <b>90.5</b>   | 14.8   |
| Total CDS length recov. (kbp)   | <b>808.6</b> | 713.6 | <b>3964.6</b> | 2757.1 | <b>8207.7</b> | 1136.5 | <b>6968.8</b> | 1832.1 |
| <i>SeedPlantsPTD</i>            |              |       |               |        |               |        |               |        |
| Percentage of loci recov. (%)   | <b>78.6</b>  | 24.9  | <b>94.9</b>   | 5.3    | <b>96.3</b>   | 0.7    | <b>76.2</b>   | 20.6   |
| Total CDS length recov. (kbp)   | <b>45.9</b>  | 20.8  | <b>64.8</b>   | 6.7    | <b>67.0</b>   | 1.2    | <b>39.9</b>   | 16.9   |
| <i>SeedPlantsMIT</i>            |              |       |               |        |               |        |               |        |
| Percentage of loci recov. (%)   | <b>73.9</b>  | 26.5  | <b>92.4</b>   | 10.9   | <b>93.7</b>   | 3.9    | <b>63.8</b>   | 21.9   |
| Total CDS length recov. (kbp)   | <b>22.0</b>  | 10.6  | <b>31.8</b>   | 4.1    | <b>32.2</b>   | 0.9    | <b>18.0</b>   | 8.2    |
| <i>Plastome38</i>               |              |       |               |        |               |        |               |        |
| Percentage of loci recov. (%)   | <b>88.6</b>  | 25.6  | <b>99.0</b>   | 5.3    | <b>100.0</b>  | 0.0    | <b>81.3</b>   | 20.6   |
| Total length recov. (kbp)       | <b>145.3</b> | 63.4  | <b>208.3</b>  | 30.6   | <b>211.7</b>  | 7.3    | <b>106.6</b>  | 45.5   |

514

515

516

517 *Extraction and Alignment*

518 Since the Angiosperms353 and the Mega353 reference targets aim to recover the same  
519 set of 353 genes, gene recovery in terms of number of loci and total CDS length was very  
520 similar for both. The average percentage of genes recovered across data types varied from c.  
521 71% for genome skimming to c. 99% for high-depth whole genome sequencing data while the  
522 data type with the longest total CDS lengths is high-depth whole genome sequencing with an  
523 average of 271.3 kbp and the shortest is genome skimming with an average of 145.6 kbp.  
524 However, the Mega353 reference targets recovered at most 0.4% more loci than  
525 Angiosperms353 for genome skimming and at most 0.1% more for other data types.  
526 Similarly, the Mega353 reference targets produced total CDS lengths only c. 10 kbp longer  
527 than the Angiosperms353 reference targets in average across data types.

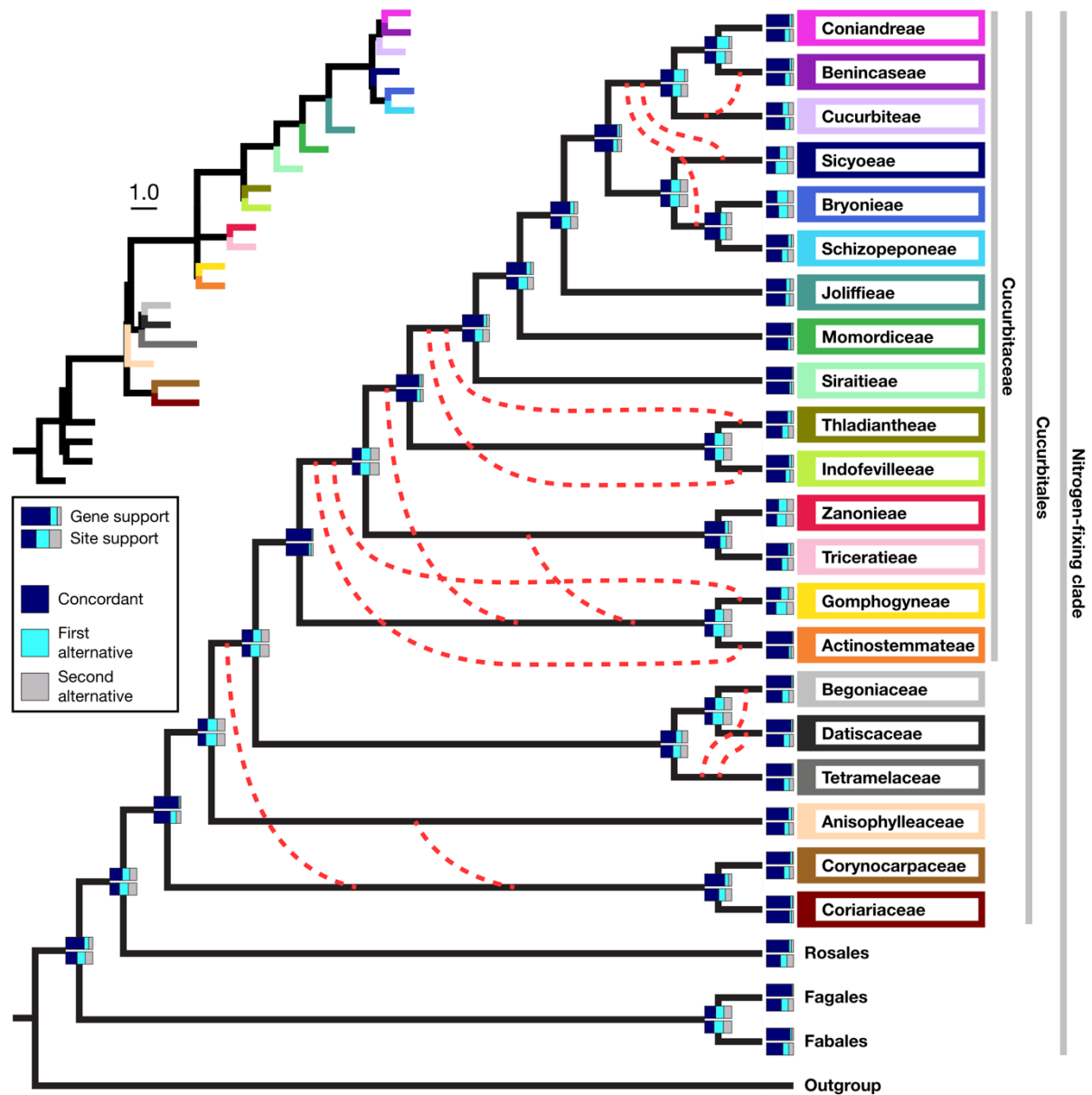
528 For the RNA5435 reference targets, target capture data had the lowest average  
529 percentage of recovered loci (20.8%, SD 14.9%) as well as the shortest average total CDS  
530 length (808.6 kbp, SD 713.6 kbp). For the rest of data types, the percentage of RNA5435  
531 genes recovered ranges from 61.2% to 97.4% while the average total CDS length ranges from  
532 3.96 Mbp to 8.2 Mbp. Recovery of organellar proteins was also high across data types,  
533 exceeding 73% except for mitochondrial proteins from RNA-Seq data where only 63.8% of  
534 the genes were recovered. The 38 plastome segments were successfully recovered across data  
535 types, where the minimum was 81.3% for RNA-Seq data (Table 1: Extraction). The total  
536 aligned ungapped length across samples was similar for Angiosperms353 (Fig. S2a) and  
537 Mega353 alignments (Fig. S2b) with c. 85% of samples with lengths between c. 200 kbps and  
538 c. 290 kbps and only c. 2.5% of samples showing less than 50 kbps aligned. As for RNA5435  
539 (Fig. S2c) around 55% of samples had total ungapped aligned lengths between 6 and 8.2 Mbp,  
540 while around 20% of the samples had less than 1 Mbp aligned, corresponding mostly to target  
541 capture samples.







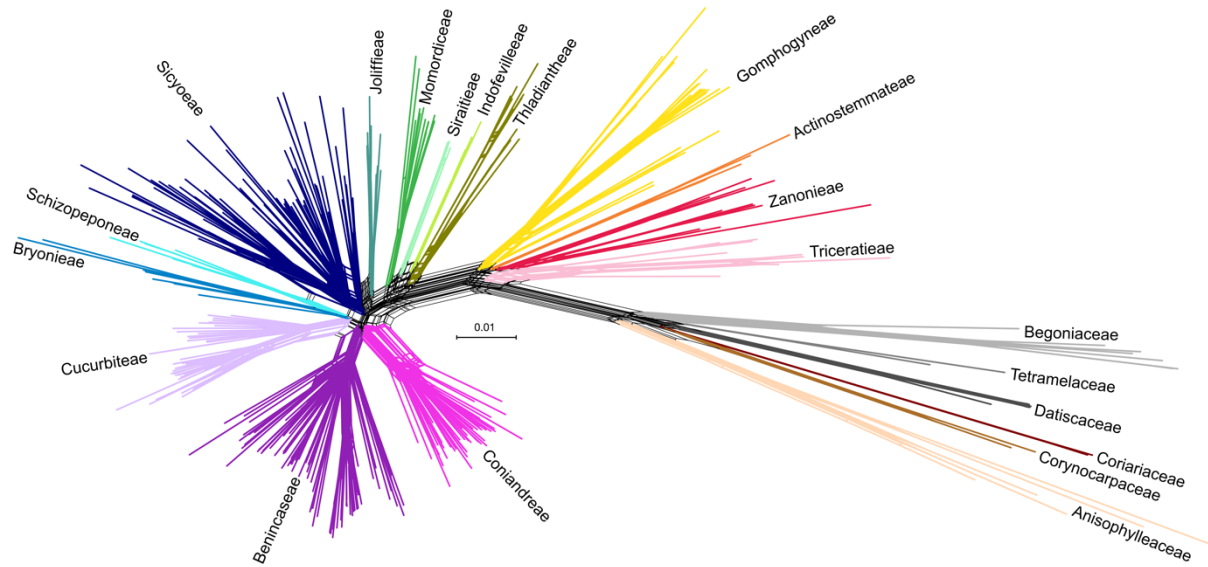
568 species tree configurations observed coincide with the alternative quartet configurations  
569 suggested for the nodes in conflict (Fig. S4). The best supported alternative shows a grade,  
570 where the clade Coriariaceae + Corynocarpaceae is followed by Anisophylleaceae and by the  
571 remaining families, but there is also considerable gene tree and site support for a clade in  
572 which Anisophylleaceae is sister to a clade comprising Corynocarpaceae + Coriariaceae and  
573 the remainder of the order (Fig. 2). The woody Tetramelaceae is resolved as sister to the two  
574 herbaceous families Begoniaceae and Datisceae but again there is also considerable gene  
575 support for the two alternative combinations within the triplet (Fig. 2). Within Cucurbitaceae,  
576 there is also gene support for different relationships between the tribes, but the best supported  
577 topology is a clade comprising Actinostemmateae and Gomphogyneae, found as sister to the  
578 remaining tribes. Among the genera with multiple samples, only *Kedrostis* is found to be  
579 polyphyletic (Fig. S3, Appendix S3, Fig. S5). The extinct Cambodian species *Khmeriosicyos*  
580 *harmandii*, known only from the type collection, is confidently placed in Benincaseae as  
581 sister to *Borneosicyos* from Mount Kinabalu in the nuclear species trees (Fig. S3, Appendix  
582 S3) and in a clade with *Borneosicyos* and the southeast Asian *Solena* in the plastome tree  
583 (Fig. S5). The network analysis revealed a deep reticulation within and between Benincaseae  
584 and Cucurbiteae and between Schizopeponeae and Sicyoeae. There is also evidence for more  
585 recent reticulation within Coniandreae and very recent within Thladiantheae (Fig. 3). The  
586 species trees resulting from the concatenated analysis of the complete plastomes (Fig. S5)  
587 agree with the reticulated representation of the Cucurbitales topology (Fig. 2, Fig. 3).



588

589 FIGURE 2. Coalescent cladogram of the Cucurbitales inferred with ASTRAL-PRO based on the  
 590 RNA5435 nuclear gene trees (Fig. S4a) with families and Cucurbitaceae tribes collapsed into  
 591 a single branch each. Gene support calculated with ASTRAL-PRO and site support calculated  
 592 using IQ-TREE is indicated at each branch, the most important alternative topologies are  
 593 shown with red broken lines. Branch lengths in the inset phylogram are in coalescent units.

594



595

596 FIGURE 3. Phylogenetic network estimate for the Cucurbitales calculated with SPLITSTREE  
597 using the Neighbor Net algorithm on the concatenated alignment of the Angiosperms353  
598 alignments which were filtered using the informed filter of CAPTUS.

599

600

### *Pipeline Comparison*

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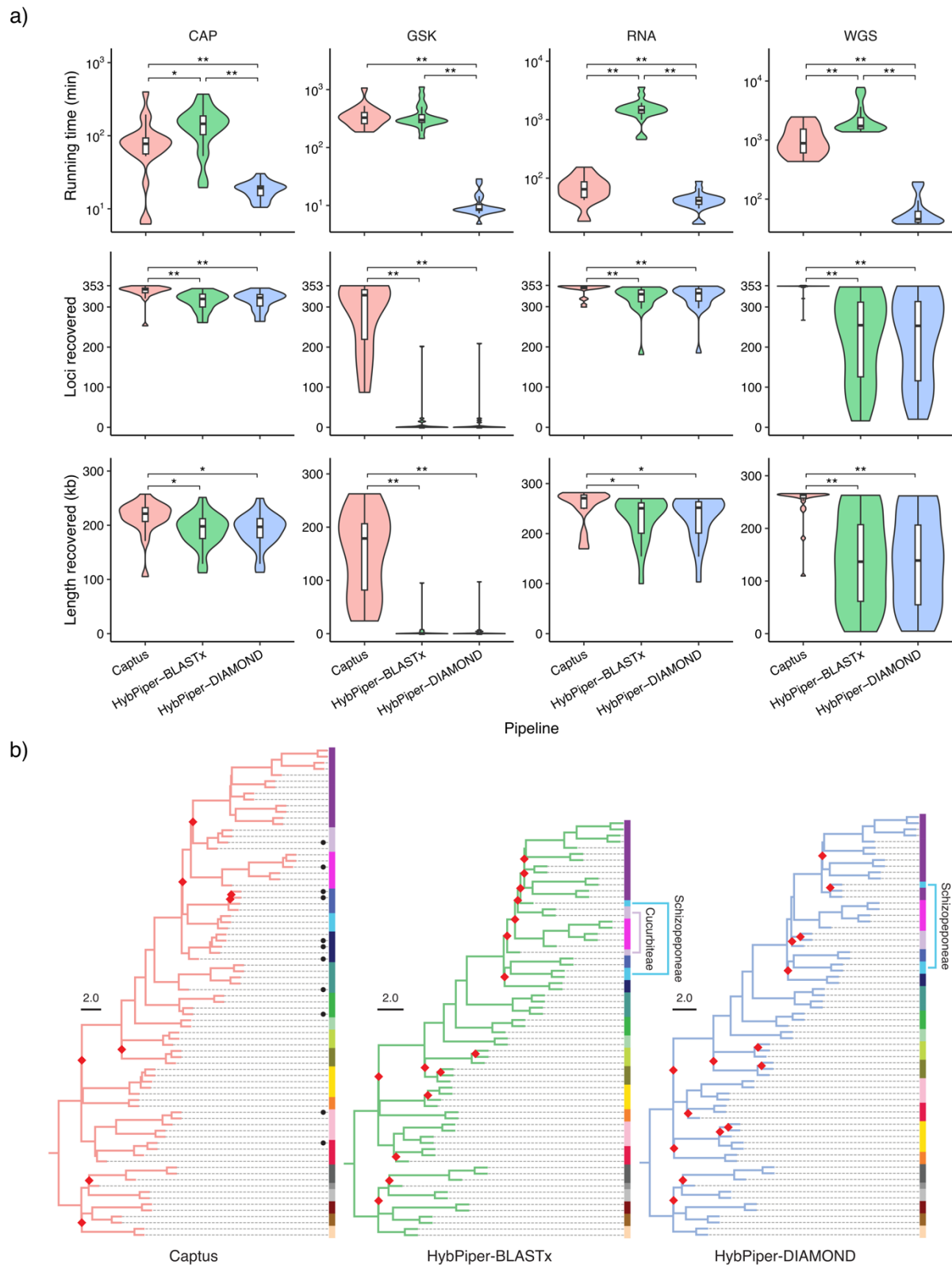
610

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612

In comparison to the two methods offered by HYBPIPER, CAPTUS consistently recovers a larger number of more complete genes regardless of data type, showing significant to highly significant differences in each case (Fig. 4a). Gene recovery is more similar for target capture data, however HYBPIPER could only recover a few gene fragments from genome skimming data. Regarding processing times, HYBPIPER-DIAMOND was faster than CAPTUS for every data type while keeping its gene recovery statistics essentially identical to HYBPIPER-BLASTx which was the slowest of the methods compared (Fig. 4a). HYBPIPER was not able to produce useful data for several genome skimming samples, which therefore could not be included in their respective species trees (Fig. 4b). The few genome skimming samples for which HYBPIPER produced data resulted in two polyphyletic groups, despite being consistently recovered as the monophyletic tribes Schizopeponaceae and Cucurbitaceae with CAPTUS for the reduced (Fig. 4b) and the full Cucurbitales species trees (Fig. 2, Fig. S3,

613 Appendix S3, Fig. S5). The number of nodes with low support is also greater in the  
 614 phylogenies derived from HYBPIPER data than in the CAPTUS tree (Fig. 4b).



615  
 616 **FIGURE 4. Benchmarking of Captus against HybPiper-BLASTx and HybPiper-DIAMOND**  
 617 using a selection of 80 samples belonging to four different data types. **a)** Comparison of locus

618 recovery efficiency between pipelines for each data type, evaluating the running time required  
619 for the assembly and extraction of the Angiosperms353 loci (top), the number of loci  
620 recovered (middle), and the total length of coding sequences recovered (bottom). Statistical  
621 testing was performed using a Wilcoxon rank-sum test with Bonferroni correction: \*  $p < 0.05$ ,  
622 \*\*  $p < 0.01$ . **b)** Coalescent-based species trees resulting from each pipeline. Vertical bar along  
623 each tree color-codes the different tribes/families in the Cucurbitales. Red diamonds indicate  
624 conflicting or poorly supported nodes with a local posterior probability below 0.9. Samples  
625 recovered only by Captus are marked with a black dot. Two tribes inferred as polyphyletic  
626 groups in the HybPiper analyses are indicated with name and position. Tree files in Newick  
627 format are provided in Appendix S4.

628

## 629 DISCUSSION

630 The pipeline comparison shows that CAPTUS is slower than HYBPIPER-DIAMOND but  
631 consistently recovers a higher number of more complete genes across a larger number of  
632 species than either HYBPIPER-BLASTX or HYBPIPER-DIAMOND (Fig 4a). This can be  
633 explained by a combination of factors. First, the efficient and thorough *de novo* assembly by  
634 MEGAHIT allows the assembly of *all* reads in the sample in contrast to HYBPIPER which  
635 only assembles prefiltered reads that match the reference loci, which leads to a more restricted  
636 assembly potentially missing intronic regions and small exons. Second, SCIPPO's capacity to  
637 reconstruct gene models across several contigs is essential to deal with fragmentary  
638 assemblies such as the ones resulting from target capture data and particularly genome  
639 skimming (Hatje et al. 2011). Finally, SCIPPO also outperforms (Hatje et al. 2011)  
640 EXONERATE's (Slater and Birney 2005) in gene reconstruction, which is the method used by  
641 HYBPIPER.

642 The ability of CAPTUS to successfully combine different DNA data types is confirmed  
643 by the topological results where data available from diverse sources for the same species,

644 consistently formed well-supported and stable clades (Supplement Trees). Also, by  
645 assembling the entire set of reads in a sample, CAPTUS uses off-target reads efficiently instead  
646 of excluding them like HYBPIPER. Even though our target capture data was supposed to  
647 comprise only 353 genes, CAPTUS was able to find almost three times as many nuclear genes  
648 (c. 1130 genes on average) using our RNA5435 reference targets, as well as every organellar  
649 protein and mostly complete plastomes. This indicates that CAPTUS takes better advantage of  
650 the imperfect process of DNA hybridization that will usually carry over many other genomic  
651 regions that would remain unused otherwise.

652         Regarding the phylogenetic estimate, our results show a well-supported nitrogen-  
653 fixing clade where Cucurbitales + Rosales are sister to Fabales + Fagales (Fig. S3, Appendix  
654 S3, Fig. S4). This topology has also been recovered by recent nuclear phylogenomic analyses  
655 (Guo et al. 2021; Zuntini et al. in review) but differs from phylogenetic estimates mostly  
656 based on chloroplast data (Soltis et al. 1995; The Angiosperm Phylogeny Group 2016; Li et  
657 al. 2021) where the topology (Fabales (Rosales (Cucurbitales, Fagales) is recovered.  
658 Assuming the gain of nitrogen-fixing capacity evolved in the common ancestor of this clade,  
659 the net number of independent losses inside the clade (e.g., Griesmann et al. 2018) should not  
660 be affected by this new topology, however future studies on the subject could benefit by also  
661 interpreting gains and losses according to this new topology. Within the Cucurbitales, all the  
662 currently accepted families are recovered as monophyletic groups in our analyses (Fig. 2, Fig.  
663 S3, Appendix S3, Fig. S4, Fig. S5). The relationships among families agree well with  
664 previous phylogenetic and phylogenomic studies of Cucurbitales, except for the position of  
665 the holoparasitic Apodanthaceae, where we find strong support for a position outside  
666 Cucurbitales, in contrast to the earlier results of Filipowicz and Renner (2010). In our dataset,  
667 Apodanthaceae groups with Malpighiales in agreement with the recent result of Zuntini et al.  
668 (in review) based on a 58% sampling of the 13,600 genera of angiosperms. However, our  
669 analyses indicate a highly supported sister group relationship between the Apodanthaceae and

670 the Rafflesiaceae (Fig. S3, Appendix S3, Fig. S4) while Zuntini et al. (in review) recover the  
671 Rafflesiaceae well-nested within Malpighiales but with low nodal support. A deeper  
672 phylogenomic analysis of Malpighiales that takes advantage of the increased taxon sampling  
673 in Zuntini et al. (in review) and our increased gene sampling could prove useful to better  
674 resolve these relationships. For the autotrophic families we find most support for a grade  
675 where the clade Coriariaceae + Corynocarpaceae is followed by Anisophylleaceae, a clade  
676 with the triplet Tetramelaceae plus Begoniaceae and Datisceae, and by Cucurbitaceae (Fig.  
677 2, Fig. S3a, Fig. S4). Earlier studies placed Anisophylleaceae as sister to all other except  
678 Apodanthaceae (Zhang et al. 2006; Schaefer and Renner 2011b; Zuntini et al. in review).  
679 Even though our analyses show the highest support for the clade Coriariaceae +  
680 Corynocarpaceae as sister to the rest of Cucurbitales, there is almost equal support among  
681 gene trees for Anisophylleaceae as sister to the rest (as in previous studies) as well as for a  
682 clade (Anisophylleaceae (Coriariaceae, Corynocarpaceae) as sister to the rest of families in  
683 the order (Fig. 2, Fig. S3a, Fig. S4). For Datisceae, Zhang et al. (2006) and Schaefer and  
684 Renner (2011b) also found a sister group relationship to Begoniaceae, albeit with low support.  
685 Here, we find that such a clade has the highest gene tree support but the alternatives  
686 Datisceae sister to Tetramelaceae [also recovered by Zuntini et al. (in review)] and  
687 Datisceae sister to Begoniaceae + Tetramelaceae also receive almost equal support (Fig. 2,  
688 Fig. S4). In the network (Fig. 3), the two dioecious families Datisceae + Tetramelaceae  
689 form a clade, which is sister to the monoecious Begoniaceae.

690 Tribal relationships within Cucurbitaceae match well the phylotranscriptomics results  
691 of Guo et al. (2020). The four conflicting nodes identified in Bellot et al. (2020) concerning  
692 the position of *Luffa*, *Hodgsonia*, *Bryonia*, and *Indofevillea* are stable when coalescent and  
693 concatenated phylogeny estimates are compared (Fig. S3, Appendix S3): *Indofevillea* is  
694 placed as sister to the Southeast Asian Thladiantheae; the sponge gourds (*Luffa*) are sister to  
695 all other Sicyoeae; the Asian *Hodgsonia* with the Neotropical Sicyoeae are sister to



696 *Trichosanthes*; and Bryoniae plus Schizopeponeae are sister to Sicyoeae. Looking at the  
697 gene tree analyses, however, the considerable number of trees supporting alternative positions  
698 of those lineages is evident (Fig. 2, indicated in red), indicating frequent hybridization events  
699 in the evolutionary history of Cucurbitaceae.

700 CAPTUS allows the disentanglement of the complex pattern of deep reticulated  
701 evolution in Cucurbitales, which seems to be prevalent across the angiosperms (Stull et al.  
702 2023). The comparison of the gene tree frequencies obtained from the 353 captured regions  
703 (Angiosperms353, or the taxonomically expanded Mega353) with the frequencies obtained  
704 from the RNA5435 regions shows stable 1/3:1/3:1/3 ratio for nodes showing incomplete  
705 lineage sorting (e.g., nodes 14, 24, 29, 32, and 41 in Fig. S4a) or 1/2:1/2:0 ratio for nodes of  
706 hybrid origin (e.g. node 7 in Fig. S4a). This indicates that adding more genomic regions is  
707 unlikely to change the family and tribal level relationships in Cucurbitales found in our study.  
708 Future work should focus on the more recent evolutionary history of the clade and  
709 phylogenetic patterns within Cucurbitaceae genera.

710 In conclusion, we show that our new pipeline can handle a complex phylogenomic  
711 analysis in a very efficient way. The clustering capability of CAPTUS enables its application  
712 not only to seed plants but to any taxonomic group, even those where a reference set of  
713 orthologous loci has not yet been developed. Thus, CAPTUS can be used as a universal tool for  
714 the assembly of phylogenomic datasets, even with mixed data of different origins, with  
715 degraded and contaminated samples, and even in taxonomic groups with a very complex  
716 evolutionary history.

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718

#### ACKNOWLEDGEMENTS

719 We are grateful to the curators of the herbarium of Royal Botanic Gardens, Kew (K),  
720 the herbarium of Muséum National d'Histoire Naturelle in Paris (P), and the herbarium of the  
721 Botanische Staatssammlung München (M) for permission to extract DNA from selected



722 specimens. We also thank A. Tellier (TUM) for access to the Population Genetics HPC. The  
723 study was funded by the German Science Foundation DFG - SCHA 1875/4-2 within SPP  
724 1991 Taxon-Omics (to HS), and by grants from the Calleva Foundation to the Plant and  
725 Fungal Trees of Life Project (PAFTOL) at the Royal Botanic Gardens, Kew.

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906 Manuscript submitted for publication.
- 907

908 **Supplementary Material**

909

910 TABLE S1. List of samples sequenced for this study.

911 TABLE S2. List of samples with public data used in this study.

912 FIGURE S1. The CAPTUS output formats, **a)** outputs available when a protein extraction (NUC,

913 PTD, MIT) is performed, **b)** outputs available when a miscellaneous DNA extraction

914 (DNA, CLR) is performed.

915 APPENDIX S1. Plastome segments used as reference targets provided as a file in FASTA

916 format `Plastome38.fasta`

917 APPENDIX S2. Newly found nuclear putative homologs used as reference targets provided as a

918 file in FASTA format `RNA5435.fasta`

919 TABLE S3. Per-sample comparison of running time, number of loci recovered, and total CDS

920 length recovered among CAPTUS, HYBPIPER-BLASTX, and HYBPIPER-DIAMOND.

921 TABLE S4. Summary statistics per sample at each analysis step.

922 FIGURE S2. Total aligned ungapped length per sample using different nuclear reference targets

923 sets, **a)** Angiosperms353, **b)** Mega353, **c)** RNA5435.

924 SUPPLEMENTARY METHOD. Decontamination process for target capture sample *Lemurosicyos*

925 *variegata*.

926 TABLE S5. Samples lacking sufficient chloroplast data for phylogenetic estimation or with

927 chloroplast contamination.

928 FIGURE S3. Coalescent species trees estimated with ASTRAL-PRO using gene trees estimated

929 by IQ-TREE on alignments derived from different reference targets sets (RNA5435,

930 Angiosperms353, Mega353) and paralog filtering strategies (unfiltered,

931 informed, naive).

932 APPENDIX S3. Estimated species trees in NEWICK format.



933 FIGURE S4. DISCOVISTA analyses of relative quartet frequencies imposed on the coalescent  
934 ASTRAL-PRO topology shown in Fig. S3a using sets of gene trees derived from the  
935 different reference target sets (RNA5435, Angiosperms353, Mega353) calculated by  
936 different programs (IQ-TREE, FASTTREE) and filtered for paralogs (informed,  
937 naive).

938 FIGURE S5. Species tree estimated by IQ-TREE on the concatenated alignments derived from  
939 the Plastome38 reference targets set.

940 APPENDIX S4. Species trees in NEWICK format estimated for the pipeline comparison.

941 APPENDIX S5. Reference targets sets used in the decontamination of *Lemurosicyos variegata*  
942 (Supplementary Method).

943