



Back together: Over 1000 single-copy nuclear loci and reproductive features support the holoendoparasitic Apodanthaceae and Rafflesiaceae as sister lineages in the order Malpighiales

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ABSTRACT

The systematics of the holoendoparasitic flowering plant families Apodanthaceae and Rafflesiaceae has been discussed for over two centuries. The morphological reduction of roots, shoots and leaves in all members of both families, resulting in a cryptic mycelium-like vegetative body, has been interpreted either as a key common feature, or as a result of convergent evolution due to full dependence upon their hosts. Historically, the two families have been placed together due to similar morphological features, but recent analyses based on few mitochondrial and ribosomal gene markers placed them in the distantly related orders Cucurbitales and Malpighiales. Here we reevaluate the affinities of the Apodanthaceae and the Rafflesiaceae using a phylogenomic approach. We present (1) a historical account on their affinities over the last 200 years; (2) phylogenetic analyses reinstating their sister group relationship as part of the order Malpighiales, based on over 1000 single-copy nuclear protein-coding loci; and (3) a comprehensive list of putative morphoanatomical and developmental synapomorphies in light of the phylogenomic results, with emphasis on shared reproductive traits regardless of dramatic differences in floral size.

1. Introduction

The Apodanthaceae and the Rafflesiaceae are two of the most remarkable holoendoparasitic flowering plant families. All members of the two lineages possess a highly reduced and cryptic endophyte that becomes apparent only when the unisexual flowers emerge from the host stems (Fig. 1). Whereas the Rafflesiaceae (currently placed in the order Malpighiales; see Barkman et al. 2004; Davies and Wurdack, 2004; APG IV, 2016) were established almost two centuries ago by Dumortier (1829), the Apodanthaceae (currently placed in the order Cucurbitales; APG IV, 2016) were formally recognized at the family rank more recently (Takhtajan, 1987, 1997), based on the seminal work by Van Tieghem, 1906.

The family Apodanthaceae consists of 13 species in the genera *Apodanthes* Poit. (one New World species) and *Pilostyles* Guill. (12 spp.

from North-, Central- and South America, Iran, Irak, Syria, subtropical eastern Africa and southwestern Australia; Bellot and Renner, 2014a; González and Pabón-Mora, 2014). All members of the family are achlorophyllous holoendoparasites with a diffuse and histologically reduced endophyte (Dell et al., 1982; Rutherford, 1970; Endriss, 1902; González and Pabón-Mora, 2017; Solms-Laubach, 1874b; Solms-Laubach, 1874a; Solms-Laubach, 1878). The exophyte consists of small gregarious flowers that emerge from woody stems of *Casearia* and *Xylosma* species (Salicaceae), the primary hosts for *Apodanthes*, or various non-closely related members of Fabaceae, which are the hosts for *Pilostyles*. On the other hand, the Rafflesiaceae comprise the genera *Rafflesia* R.Br. ex Gray (41 spp. from Borneo, Java, Malaya, Sumatra and Thailand), *Rhizanthus* Dumort. (4 spp. from Borneo, Java, Malaya, Sumatra and Thailand), and *Sapria* Griff. (4 spp. from Bhutan, southern-central China, and Indo-China), all of them parasitizing lianas of

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Fig. 1. A-E *Apodanthes caseariae*. A. Emerging floral buds of *A. caseariae* through the bark of *Casearia* sp. B. Pre-emerging floral buds of *A. caseariae*, evident through a transverse section of the host stem. C. Anthetic flowers of *A. caseariae*. D-E. Fruits of *A. caseariae* in frontal view (D) and side view (E). F-I *Pilostyles boyacensis*. F. Emerging floral buds of *P. boyacensis* through the bark of its host *Dalea cuatrecasasii*. G. Pre-emerging floral buds of *P. boyacensis* through a transverse section of the host stem. H. Developing, pre-emerging floral buds of *P. boyacensis*. I. Emerged floral buds of *P. boyacensis* after rupturing the bark of its host *D. cuatrecasasii*. J. Compound plate of *Rafflesia arnoldii* taken from [Brown \(1844\)](#); note striking similarities with corresponding developmental stages of *A. caseariae* (A-E) and *P. boyacensis* (F-I). Arrows point to pre-emerging floral buds. Scales were intentionally left out.

Tetrastigma (Vitaceae). Most species of Rafflesiaceae display floral gigantism, associated to a dramatic floral size increase in relatively short divergence times (Davies et al. 2007; Barkman et al. 2008), and non-gregarious flowers, unlike those of Apodanthaceae.

The systematic relationships of the Apodanthaceae and the Rafflesiaceae have been debated for over two centuries, either as closely related taxa (often merged into a single family, namely, the Rafflesiaceae *sensu lato*; cf. de Vattimo-Gil 1955; Apg, 1998; APG, 1998), or as families currently placed in different orders. Barkman et al. (2004) and Davies and Wurdack (2004) confidently placed the Rafflesiaceae *sensu stricto* in the order Malpighiales. This view was soon adopted by the APG III classification (APG III, 2009), which, however, kept apart the Apodanthaceae as one of the families with uncertain phylogenetic affinities. This debate began with Robert Brown's original description of the genus *Rafflesia*: "I am inclined to think that *Rafflesia*, when its structure is completely known, will be found to approach either to *Asarinæ* or *Passifloræ*; and that, from our present imperfect materials, notwithstanding the very slight affinity generally supposed to exist between these two orders, it cannot be absolutely determined to which of them is most nearly allied." (Brown, 1821:223). Brown's concept of *Asarinæ* placed together the genera *Asarum* L. (Asaraceae, a current member of the order Piperales) and *Cytinus* L. (Cytinaceae, a current member of the order Malvales).

The first mention of *Apodanthes* and *Pilostyles* as closely related to *Rafflesia* was discussed by Guillemain (1834) and Brown's public conference in 1834 (published ten years later, 1844), and soon adopted by Lindley (1836) and Endlicher (1841). Thus, Endlicher's Rafflesiaceae comprised *Apodanthes*, *Pilostyles*, *Brugmansia* Blume (a synonym of *Rhizanthus*), *Frostia* Bertero ex Guill. (a synonym of *Pilostyles*), *Zippelia* Rchb. (a synonym of *Rhizanthus*) and *Rafflesia*. A close affinity between *Apodanthes* and *Cytinus* had already been proposed in the original description of the former by Poiteau (1824). Brown (1844) redefined the family Rafflesiaceae, comprising the Rafflesiaceae (with the genera *Rafflesia*, *Sapria* and *Brugmansia*), the Hydnoaceae (with the sole genus *Hydnora*), the Cytineae (with the sole genus *Cytinus*), and the Apodantheae (with the genera *Apodanthes* and *Pilostyles*). The placement of these disparate taxa as part of the Rafflesiaceae (e.g. Endlicher, 1841; Brown, 1844; Solms-Laubach, 1878; Eichler, 1878; Engler, 1912; Harms, 1935; Melchior, 1964; Meijer, 1993; Cronquist, 1988) or as closely related families in the order Rafflesiaceae (Takhtajan, 1997) is mostly based on the parasitic lifestyle and several floral morphological traits. However, floral differences between Rafflesiaceae s.str. and Apodanthaceae, on one side, and the Cytinaceae and Hydnoaceae, on the other, were previously dismissed.

More recently, the Apodanthaceae have been linked either with members of the order Malvales, based largely on the presence of a nectarial disk, the androecial morphology, and the parietal placentation (Blarer et al. 2004; Nickrent et al. 2004; Heide-Jørgensen, 2008); or placed in the order Cucurbitales, based on *matR* and 18S sequence data (Nickrent et al. 2004; Filipowicz and Renner, 2010; Schaefer and Renner, 2011). The latest comprehensive analysis based on the Angiosperm 353 nuclear gene dataset placed the Apodanthaceae in its own order Apodanthales, as sister to the clade Hualales + Malpighiales, and the Rafflesiaceae nested in the order Malpighiales as sister to the clade (Linaceae (Elatinaceae + Malpighiaceae)) (Zuntini et al. 2024).

The aims of our study are: (1) to test the (still elusive) relationships between the currently distant families Apodanthaceae and Rafflesiaceae, by using a comprehensive dataset of 1011 single-copy nuclear genes gathered from *de novo* Apodanthaceae transcriptomes (*Apodanthes caseariae* and *Pilostyles boyacensis*) generated by us, plus 52 core eudicot genomes available in public repositories, significantly increasing the loci number used so far (Zuntini et al. 2024); and (2) to revisit the similarities of the lifestyle, endophyte anatomy, and flower, fruit and seed development and morphology in both families.

2. Material and Methods

2.1. Sampling

For this study, we covered 12 plant orders. Genome references for most of these orders were available in the NCBI database. Specifically, for *Sapria*, publicly available RNA-seq raw data was downloaded from the SRA-NCBI database (Guo et al., 2023). For *Pilostyles* and *Apodanthes*, new RNA-seq data was generated as explained below.

2.2. Reference genomes

For our phylogenomic analysis, we utilized the genomes of 52 species, which were downloaded from the NCBI Datasets website (Suppl. Table 1).

2.3. Reference transcriptomes

The reference transcriptome of *Rafflesia cantleyi* was assembled *de novo* using a combination of RNA-seq data from bud and flower tissues obtained from the Sequence Read Archive (SRA) under the following accession codes: SRR5499430 (Bud Stage 1), SRR5499429 (Bud Stage 2), SRR5499428 (Bud Stage 3), and SRR3599593 (Flower) (Lee et al., 2016). Each library comprised paired-end reads that were filtered using CUTADAPT v2.10 (<https://doi.org/10.14806/ej.17.1.200>) with a quality threshold of Q30, a minimum read length of 70 bases, and excluding reads carrying ambiguous bases ("Ns"). The mixed transcriptome was then assembled using the program Trinity v2.13.2 (<https://doi.org/10.1038/nbt.1883>) with default parameters.

The transcriptomes of *Pilostyles* correspond to those first described in González et al. (2020), while transcriptomes of *Apodanthes caseariae* were newly generated for this work. Three samples spanning the full life cycle of the endoparasitic *A. caseariae* were collected: (1) endophytic tissue of *A. caseariae* growing inside the *Casearia* sp. host stem (hereafter called AcE + C); (2) individual preanthetic flowers of *A. caseariae* already emerged from the host (hereafter called AcFl); and (3) young and fully formed fruits and seeds of *A. caseariae* (hereafter called AcFr). These samples were collected in wet tropical forests around Villavieja (Department of Meta, Colombia) for total RNA extraction (Voucher NP 495 deposited at HUA). The tissues were flash-frozen in the field and stored in liquid nitrogen until RNA extraction was performed.

The Spectrum Plant Total RNA Kit (Sigma-Aldrich TM) was used for RNA extractions. Total RNA was resuspended in 1 ml of EtOH 100 % and sent out to the sequencing facility (Macrogen, South Korea). The RNA-seq experiment was conducted using the TruSeq Stranded mRNA library construction kit (Illumina) and sequenced on a Illumina Novaseq 6000 instrument in Macrogen. Read cleaning was performed with a quality threshold of Q30, and a minimum read length of 70 bp, singletons were excluded. Contig assembly was computed using the Trinity V2.5.1 software (Grabherr et al., 2011) with default settings with TRIMMOMATIC adapter removal. Assemblage metrics are summarized in Table 1.

Table 1

Statistics of the *Apodanthes caseariae* transcriptomes. See abbreviations in the text.

	AcE + C	AcFl	AcFr
Total length of sequences	3884446	113576032	74650757
Number of contigs	9303	114317	73410
Average contig length	417	993	1016
Largest contig	6415	13736	9528
Shortest contig	190	178	177
N50	429	1867	1735
GC%	45.70	39.92	41.07

2.4. Annotation of conserved genes in available genomes and reference transcriptomes

Genomes and transcriptomes were annotated using the Benchmarking Universal Single-Copy Orthologs (BUSCO) pipeline (<https://doi.org/10.1093/bioinformatics/btv351>). The annotation utilized the eudicot database (ODB10) and the AUGUSTUS software (<https://doi.org/10.1002/cpbi.57>) with *Arabidopsis* reference gene models. For genome annotation, the BUSCO pipeline was run with the “-m genome” mode. For transcriptome annotation, the “-m transcriptome” mode was employed. The single-copy peptides annotated by BUSCO were used for subsequent ortholog search analysis.

2.5. Orthologous protein analysis

The SonicParanoid tool (<https://doi.org/10.1093/bioinformatics/bty631>) was employed to identify groups of orthologous proteins among the 55 reference species analyzed in this study. For the subsequent phylogenomic analysis, only single-copy orthologous protein groups were considered. Additionally, only orthologous groups present in at least 90 % of the analyzed species were included in the analysis. Ultimately, 1,011 groups of orthologous proteins met the criteria and were used to construct the alignment protein supermatrix for the next step of the phylogenomic analysis. The seemingly large set of single-copy nuclear genes identified here may be due to the total number of species sampled (only 55) and their close phylogenetic proximity, as they are all core-eudicots. It is expected that the number of orthogroups increases across core-eudicots when compared to evaluations across all angiosperms. This is true at least for all nuclear genes that have undergone large-scale duplication events independently in eudicots and monocots, while retained predominantly as single copy in ANA, Magnoliids, and basal eudicots.

2.6. Phylogenomic analysis based on single-copy nuclear genes

Single-copy orthologous groups of proteins were individually aligned with MAFFT version 7.490 using default parameters (<https://doi.org/10.1093/molbev/mst010>). The individual protein alignments were then concatenated using the program CATSEQUENCES (<https://github.com/ChrisCreevey/catsequences>). The reference *Vitis vinifera* Pinot Noir (GCF_030704535) was used as the outgroup.

The concatenated matrix included 55 sequences with a total of 1,193,628 amino acid sites. The matrix contained 551,064 constant sites (46.1671 % of all sites), 551,064 invariant sites (46.1671 % of all sites), 430,786 parsimony-informative sites, and 673,893 distinct site patterns.

Maximum-likelihood (ML) trees were computed using IQ-TREE2 v. 2.1.32 (<https://doi.org/10.1093/molbev/msaa015>). Three different trees were generated. The first tree was computed using the LG4M substitution model (<https://doi.org/10.1093/molbev/mss112>) with 5,000 ultra-fast bootstrap (UFB) pseudoreplicates (<https://doi.org/10.5281/zenodo.854445>). A second tree was calculated using a partition strategy, and we used the option “-rcluster 10” to avoid overparameterization, reducing the number of substitution models to be taken into account in the analysis. Additionally, 5,000 UFB pseudoreplicates were performed. The best substitution models were selected according to the Bayesian Information Criterion (BIC) (10.1186/147112148-14182).

The third tree was generated by calculating Gene Concordance Factor (GCF) (<https://doi.org/10.1093/molbev/msaa106>) and Site Concordance Factor (SCF) with the option “-scf 100” based on the best partition scheme generated for the second tree. The consensus trees generated were edited in FigTree v1.4.4 (<https://tree.bio.ed.ac.uk/software/figtree/>) or ITOL (<https://itol.embl.de/>).

To test whether the resulting topology was affected by regions difficult to align, we performed an additional analysis where the aligned protein supermatrix was processed using TrimAl. This program

automatically identifies and removes problematic alignment regions, retaining only the most consistent portions of the matrix. Applying TrimAl reduced the matrix to 33 % of its original size. With this reduced matrix, we generated a new tree using UFBoot and aLRT (Guindon et al., 2010), which showed the same topology (Suppl. Fig. 1).

In addition, we used a threshold of three times the median branch length to identify long branches (Suppl. Fig. 2). Importantly, the use of partition schemes in IQ-TREE 2 can help mitigate long branch attraction (LBA) artifacts to some extent.

2.7. Phylogenetic analysis based on small and large ribosomal subunits

An additional analysis using the entire nuclear rDNA cistron was performed. The small and large ribosomal subunits were isolated by BLASTN in all 55 reference species analyzed in this study. Regions were aligned and analyzed as described above for the single-copy nuclear genes. In this analysis the ribosomal subunits of *Dalea cuatrecasii*, the host of *Pilotyles boyacensis* was also included to test for putative horizontal gene transfer. Values of UFBS (Ultra Fast Bootstrap)/aLRT (approximate likelihood-ratio test) are generated in this ML analysis (Guindon et al., 2010).

2.8. Morphological traits compared

Primary references that describe in detail each of the morphoanatomical and developmental traits occurring in Apodanthaceae and Rafflesiaceae are given throughout the Discussion. Specialized terminology for floral structures follows González & Pabón-Mora (2017) and Nikolov et al., 2013; Nikolov et al., 2014a; Nikolov et al., 2014b.

3. Results

Two phylogenetic reconstructions based on 1,011 single-copy genes from 55 core eudicot species (Suppl. Table 1) yielded the same overall topology. The first one used the LG4M evolutionary model, a total of 1,193,628 amino-acid sites, 46.1671 % constant sites, without partitions, and calculated 5000 Ultra Fast Bootstrap (UFBS). The second analysis used 59 partitions, for a total of 1,193,628 total sites, with only 0.260777 % missing data, and same UFBS parameters (Log-likelihood of consensus tree = -21138686.181496). A complementary analysis, in addition to UFBS, calculated values of Gene Concordance Factor (GCF) and Site Concordance Factor (SCF) (Fig. 2). GCF measures the proportion of gene trees supporting a clade, while SCF measures the proportion of alignment sites supporting a clade. Within Rosids, we recover the clade ((Rafflesiaceae + Apodanthaceae) + Malpighiales) with UFBS/GCF/SCF support values of 100/73.6/48.5. The monophyly of the Apodanthaceae was supported with 100/95.6/61.9, and that of the Rafflesiaceae was supported with 100/95.5/86.9. While high GCF values (>60) support Apodanthaceae and Rafflesiaceae, a moderate SCF (48.5) supports them as sister to each other, suggesting that there are still some conflicting signals in the sequence data (Fig. 2). The same topology was obtained when regions with uncertain alignments were removed (Suppl. Fig. 1). Finally, long branches were detected in the Apodanthaceae + Rafflesiaceae clade, but were also found in various lineages including Cucurbitales, various Malpighiales (*Euphorbia*, *Hypericum* and *Linum*), Brassicales and Santalales (Suppl. Fig. 2).

In addition, we performed a phylogenetic analysis using the small and large ribosomal subunits of 55 core eudicot species. This analysis recovers the same topology ((Rafflesiaceae + Apodanthaceae) + Malpighiales), as that of the nuclear dataset with an UFBS/aLRT = 99.6/94. The monophyly of the Apodanthaceae was supported with 100/100, and that of the Rafflesiaceae was supported with 100/100. In this analysis, the sister group relationship of Apodanthaceae and Rafflesiaceae is supported with 88.3/81. Similar to the analyses reconstructed from nuclear single-copy loci, the rDNA tree shows long branches for Rafflesiaceae and Apodanthaceae (Fig. 3).

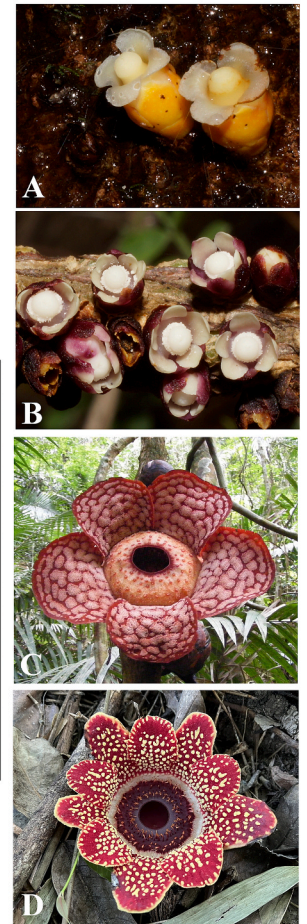
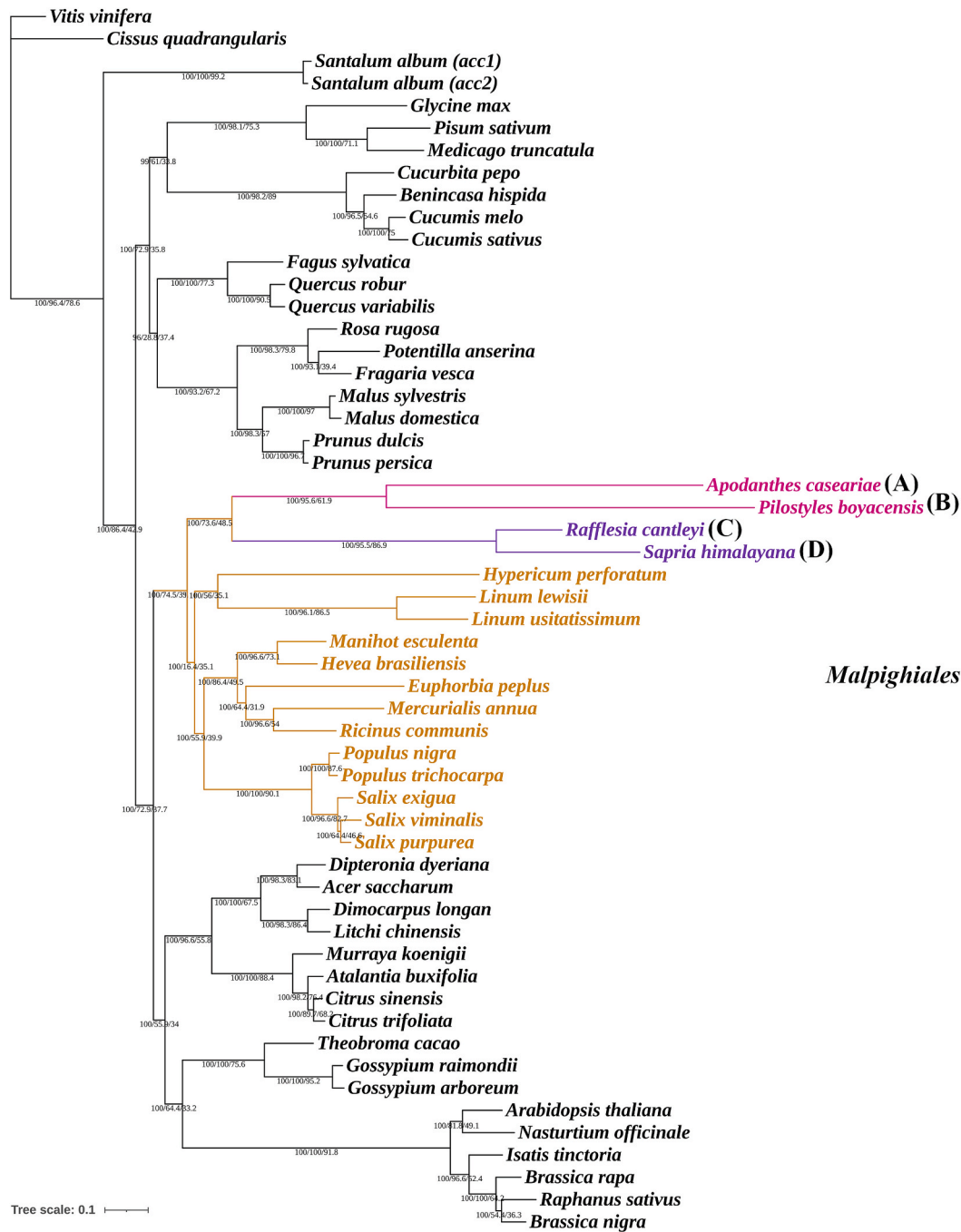


Fig. 2. Tree resulting from the phylogenomic analysis based on 1,011 single-copy nuclear genes from 52 complete core eudicot genomes and three additional transcriptomes from *Apodanthes caseariae*, *Pilostyles boyacensis* and *Rafflesia cantleyi*. Note the sister group relationship between Apodanthaceae and Rafflesiaceae. Nodes display values for UFBS (Ultra Fast Bootstrap)/GCF (Gene Concordance Factor) /SFC (Site Concordance Factor). A. *Apodanthes caseariae*, photo by Favio González; B. *Pilostyles boyacensis*, photo by Favio González; C. *Rafflesia cantleyi*, photo by Fabio Cianferoni © taken from *inaturalist*. D. *Sapria himalayana*, photo by Chanon Chirachitmicchai © taken from *inaturalist*.

4. Discussion

The inclusion of the Apodanthaceae in the order Cucurbitales, based on mitochondrial genes (Nickrent et al. 2004; Filipowicz and Renner, 2010; Schaefer and Renner, 2011) was likely an outcome of the low number of loci used, and the fact that the mitogenomes of many parasitic plants, including the Apodanthaceae, are particularly prone to horizontal gene transfer. We have previously observed that most of the critical developmental transcription factors analysed to date in Apodanthaceae (e.g. González et al., 2020; González et al., 2024) exhibit

high similarities with homologs of members of the order Malpighiales, in most cases with high support values. For instance, 12 (*AGO1*, *AGO10*, *ANT*, *BAM*, *LFY*, *HDG11/12*, *PIN11*, *PLT/BBM*, *REV*, *RPK2*, *WOX4*, and *WR1*) out of the 18 *Pilostyles* homologs assessed by González et al. (2020; 2024) cluster with orthologs from Malpighiales lineages, rather than to homologs from members of the Cucurbitales. These results challenge the inclusion of the Apodanthaceae in the order Cucurbitales (Filipowicz and Renner, 2010) and are in line with the present research on comparative phylogenomics in the Apodanthaceae, which recovers the family as sister to Rafflesiaceae in the order Malpighiales. The sister

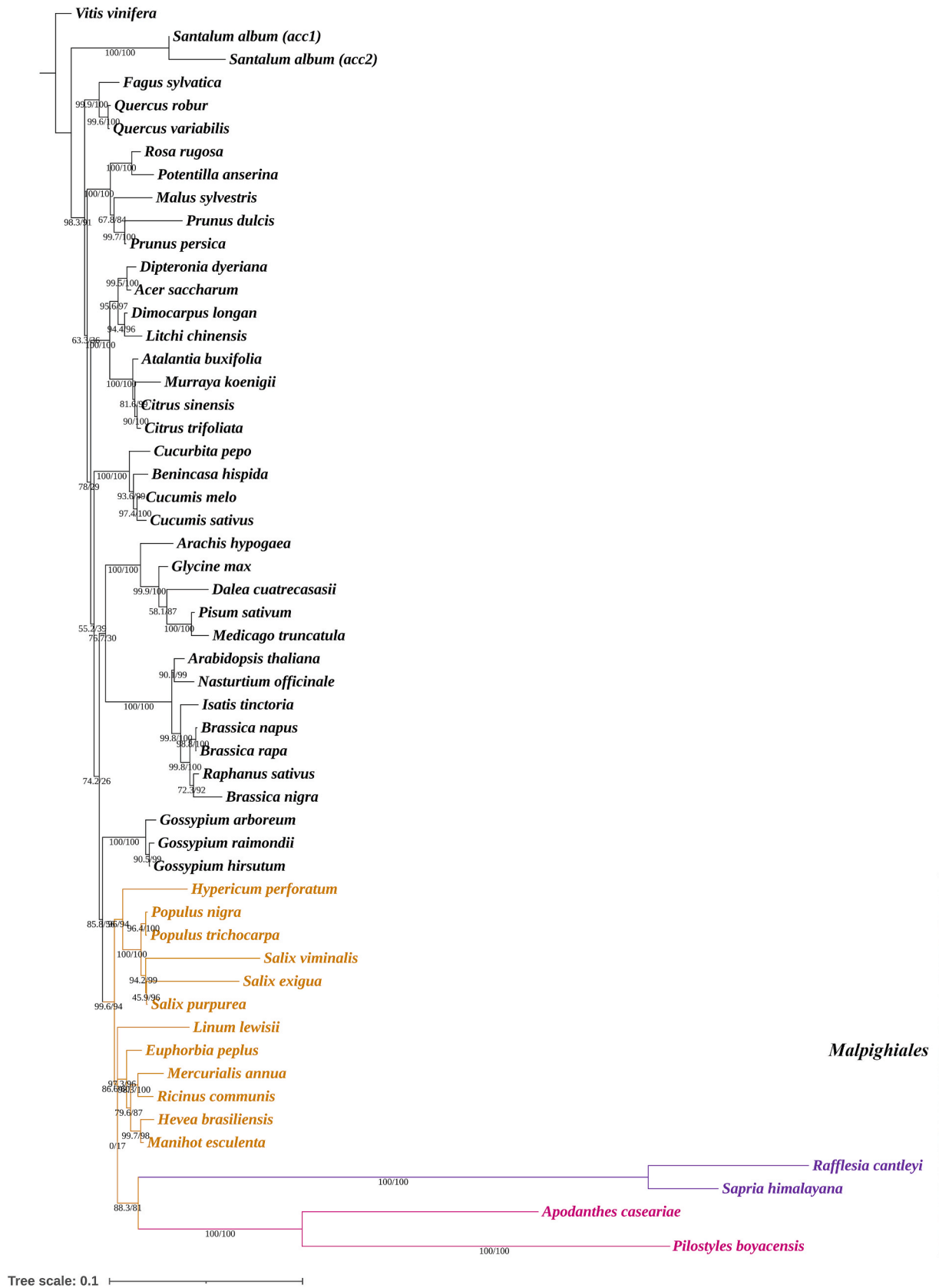


Fig. 3. Tree resulting from the phylogenetic analysis based on rDNA from 52 complete core eudicot genomes and four additional transcriptomes from *Apodanthes caseariae*, *Pilostyles boyacensis*, its host *Dalea cuatrecasii*, and *Rafflesia cantleyi*. This dataset also recovers the Apodanthaceae as sister to the Rafflesiaceae, and rules out horizontal gene transfer in rDNA. Nodes display values for UFBS (Ultra Fast Bootstrap)/aLRT (approximate likelihood-ratio test).

group relationship between Apodanthaceae and Rafflesiaceae revives the pioneering views by Guillemin (1834) and Brown (1844), who stated that similar lifestyles and floral morphologies (disregarding the obvious disparity in floral size) unite two of the most remarkable holoendoparasitic lineages of flowering plants. Thus, the Rafflesiaceae + Apodanthaceae relationship is here reinstated and highly supported.

Considering the strong support recovered from phylogenomic datasets analyzed here that link the Apodanthaceae and the Rafflesiaceae, the following morphoanatomical features are potential synapomorphies that support these two families as sister taxa:

- Holoendoparasites with mycelium-like and completely cryptic endophyte that lacks typical (i.e. epidermal, parenchymatic, cambial, and vascular) histological organization; thus, root and shoot apical meristems lacking and resulting in the absence of roots, stems and leaves. Data for Apodanthaceae taken from Dell et al., 1982; González and Pabón-Mora, 2014; Rutherford, 1970; Brown, 1821; Endriss, 1902; González and Pabón-Mora, 2017; Solms-Laubach, 1874b; Solms-Laubach, 1874a; Solms-Laubach, 1878, and González et al. (2020, 2024); data for Rafflesiaceae taken from Griffith (1845), Solms-Laubach (1878), Heinricher (1906), Ernst and Schmid (1913), Hunziker (1920), Bänziger (1996, 2004), Nikolov et al. (2014a), and Mursidawati et al. (2019).
- Multicellular massive haustoria lacking, and replaced by individual, few-celled tapered extensors (sinkers) directly connecting the parasitic endophyte and the host vasculature. Data for Apodanthaceae taken from Dell et al., 1982; González and Pabón-Mora, 2014; Rutherford, 1970; González and Pabón-Mora, 2017; Solms-Laubach, 1874b; Solms-Laubach, 1874a; Solms-Laubach, 1878, and González et al., 2020; González et al., 2024); data for Rafflesiaceae taken from Griffith (1845), Solms-Laubach (1878), Peirce (1893), Heinricher (1906), Hunziker (1920), Nikolov et al. (2014a), and Mursidawati et al. (2019).
- Gravitropism of the extensors and the exophyte (flowers and fruits) null. Data for Apodanthaceae taken from Solms-Laubach, 1874b; Solms-Laubach, 1874a; Solms-Laubach, 1878, and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Peirce (1893), Heinricher (1906), Ernst and Schmid (1913), Hunziker (1920), Bänziger (1995, 2004), Barcelona et al. (2009), and Nikolov et al., 2014a; Nikolov et al., 2014b). The lack of gravity-sensing, largely overlooked in both families, is likely the result of the complete suppression of typical roots, including the root-cap and the statocytes.
- Cells with exceedingly large nuclei, small vacuoles (if any), and dense cytoplasm. Data for Apodanthaceae taken from Dell et al. (1982), and González and Pabón-Mora, 2017); data for Rafflesiaceae taken from Ernst and Schmid (1913), Hunziker (1920), and Nikolov et al. (2014a).
- Chloroplasts, chlorophylls and photosynthesis suppressed. Data for Apodanthaceae taken from Dell et al. (1982; however, residual plastids lacking starch reported), González & Pabón-Mora (2017), and Arias-Agudelo et al. (2019); data for Rafflesiaceae taken from Molina et al. (2014).
- Protective cap-like structure developed surrounding each floral primordium (Fig. 1). Data for Apodanthaceae taken from Rutherford, 1970; Solms-Laubach, 1876; Solms-Laubach, 1874b; Solms-Laubach, 1874a; Solms-Laubach, 1878, and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Griffith (1845), Solms-Laubach (1891), Peirce (1893), Heinricher (1906), Ernst and Schmid (1913), Hunziker (1920), Bänziger (2004), Bänziger and Hansen (1997, 2000), Heinricher (1906), Barcelona et al. (2009), Nikolov et al. (2014a), Susatya (2020), and Tolod et al. (2021). This structure likely plays a mechanical and/or biochemical role to facilitate floral emergence of the parasite through the host cortex.
- Flowers developed directly from the mycelium-like endophyte (Fig. 1). Data for Apodanthaceae taken from Solms-Laubach, 1876; Endriss, 1902; González and Pabón-Mora, 2017; Solms-Laubach, 1874b; Solms-Laubach, 1874a; Solms-Laubach, 1878, and González et al. (2020, 2024); data for Rafflesiaceae taken from Ernst and Schmid, 1913; Griffith, 1845; Hunziker, 1920; Solms-Laubach, 1876; Brown, 1821; Solms-Laubach, 1874b; Solms-Laubach, 1874a; Solms-Laubach, 1878, and Nikolov et al., 2013; Nikolov et al., 2014a; Nikolov et al., 2014b).
- Floral development occurring entirely in the parenchymatic zone of the host cortex (Fig. 1). Data for Apodanthaceae taken from Dell et al., 1982; Solms-Laubach, 1876; González and Pabón-Mora, 2017; Solms-Laubach, 1874b; Solms-Laubach, 1874a; Solms-Laubach, 1878, and González et al., 2020; González et al. 2024); data for Rafflesiaceae taken from Griffith, 1845; Solms-Laubach, 1876; Solms-Laubach, 1874b; Solms-Laubach, 1874a; Solms-Laubach, 1878, and Nikolov et al., 2013; Nikolov et al., 2014a; Nikolov et al., 2014b).
- Floral phyllotaxis imbricate (Fig. 1), except for the innermost organs that are valvate (fused in *Rafflesia* forming the diaphragm). Data for Apodanthaceae taken from Bellot and Renner, 2013; Blarer et al., 2004; Guillemin, 1834; Rutherford, 1970; Solms-Laubach, 1876; Baillon, 1888; Solms-Laubach, 1874b; Solms-Laubach, 1874a; Solms-Laubach, 1878, and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Brown (1821, 1844), Griffith (1845), Miquel (1853), Beccari (1875), Solms-Laubach (1876, 1878, 1891), Baillon (1888), Heinricher (1906), Ernst and Schmid (1913), Hunziker (1920), Bänziger (1995, 2004), Bänziger and Hansen (1997, 2000), Barcelona et al. (2009), Nikolov et al., 2013, Nikolov et al. (2014a), and Tolod et al. (2021).
- Perianth organs with stomata. Data for Apodanthaceae taken from Endriss (1902), Rutherford (1970), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Heinricher (1906; but see Hunziker, 1920).
- Anthocyanin-rich flowers; anthocyanin synthesis occurring in the dark (Fig. 1; Elejalde-Baena et al., 2024).
- Flowers unisexual, scented. Data for Apodanthaceae taken from Guillemin (1834), Solms-Laubach (1878), Baillon (1888), Endriss (1902), Rutherford (1970), Blarer et al. (2004), Bellot and Renner (2013), Sipes et al. (2014), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Brown (1821, 1844), Griffith (1845), Miquel (1853), Beccari (1875), Solms-Laubach (1876, 1878, 1891), Baillon (1888), Heinricher (1906), Ernst and Schmid (1913), Beaman et al. (1988), Bänziger (1995, 1996, 2004), Bänziger and Hansen (1997, 2000), Hidayati et al. (2000), Barcelona et al. (2009), Nikolov et al., 2013; Nikolov et al., 2014a; Nikolov et al., 2014b), and Tolod et al. (2021). Some reports of bisexual flowers in *Rafflesia baletii*, *R. verrucosa* and *Rhizanthes* spp. likely correspond to functionally unisexual flowers (Balet et al. 2010). The exceptional development of chimeric, half-male, half-female flowers in *Pilostyles*, is likely caused by the early fusion of two contiguous floral meristems into a single flower, due to the tight proximity of the floral meristems in the gregarious flowers inside the host stems (González and Pabón-Mora 2017). This rare process does not result in an outer staminate whorl and an inner carpellate whorl, which rules out any hypothesis of primary homology with typical bisexual flowers. Note that vestigial pollen sacs in female flowers were reported by Beaman et al. (1988).
- Nectarial disk present. Data for Apodanthaceae taken from Solms-Laubach (1878), Endriss (1902), Rutherford (1970), Blarer et al. (2004), Bellot and Renner (2013), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Brown (1844), Beccari (1875), Solms-Laubach (1891), Heinricher (1906), Ernst and Schmid (1913), Hunziker (1920), Beaman et al. (1988), Bänziger (1995, 2004), Bänziger and Hansen (1997, 2000), Nikolov et al., 2013; Nikolov et al., 2014b), and Tolod et al. (2021).
- Column disk highly reflective (Fig. 1). Data for Apodanthaceae taken from González and Pabón-Mora (2017); data for Rafflesiaceae taken

- from Solms-Laubach (1891), Beaman et al. (1988), Bänziger (1995, 1996, 2004), Bänziger and Hansen (1997, 2000), Barcelona et al. (2009), and Tolod et al. (2021).
- Disk in male flowers with more elaborated papillae than that of the female flower. Data for Apodanthaceae taken from Rutherford (1970), Baillon (1888), and González and Pabón-Mora (2017). Data for Rafflesiaceae taken from Brown (1821, 1844), Griffith (1845), Baillon (1888), Solms-Laubach (1891), Heinricher (1906), Ernst and Schmid (1913), Hunziker (1920), Bänziger (1995, 1996, 2004), and Bänziger and Hansen (1997, 2000).
 - Stamens (10-)20–70, athecal (no filament/anther differentiation), sessile, and reduced to individual, globose to ovoid pollen sacs arranged in a 1–3 whorled synandrium, fused to the persistent, non-functional style. Data for Apodanthaceae taken from Guillemin (1834), Solms-Laubach (1878), Baillon (1888), Endriss (1902), Rutherford (1970), Blarer et al. (2004), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Brown (1821), Griffith (1845), Miquel (1853), Beccari (1875), Solms-Laubach (1878, 1891), Baillon (1888), Heinricher (1906), Ernst and Schmid (1913), Hunziker (1920), Bänziger (1995), Bänziger and Hansen (1997, 2000), Barcelona et al. (2009), and Nikolov et al., 2013; Nikolov et al., 2014b).
 - Pollen sacs dehiscent through individual transverse slits, and lacking fibrous endothecium. Data for Apodanthaceae taken from Guillemin (1834), Solms-Laubach (1878), Endriss (1902), Rutherford (1970), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Brown (1821), Griffith (1845), Miquel (1853), Solms-Laubach (1878, 1891), Ernst and Schmid (1913), and Hunziker (1920).
 - Large vesicular papillae formed in the central column. Data for Apodanthaceae taken from Endriss (1902), Rutherford (1970), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Miquel (1853), Ernst and Schmid (1913), Hunziker (1920), Bänziger (1995, 1996, 2004), and Bänziger and Hansen (1997, 2000).
 - Stigmas well developed in staminate flowers. Data for Apodanthaceae taken from Guillemin (1834), Solms-Laubach (1878), Baillon (1888), Endriss (1902), Rutherford (1970), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Brown (1821), Griffith (1845), Miquel (1853), Solms-Laubach (1876, 1878, 1891), Baillon (1888), Ernst and Schmid (1913), Hunziker (1920), Bänziger (1995, 1996, 2004), and Bänziger and Hansen (1997, 2000).
 - Gynoecium completely syncarpous, formed by four carpels (probably more in Rafflesiaceae). Data for Apodanthaceae taken from Solms-Laubach (1878), Baillon (1888), Endriss (1902), Rutherford (1970), Blarer et al. (2004), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Brown (1844), Griffith (1845), Miquel (1853), Solms-Laubach (1878, 1891), and Baillon (1888).
 - Ovary inferior to half-inferior, unilocular, with parietal placentation; placentae often proliferative and intrusive (Fig. 1). Data for Apodanthaceae taken from Solms-Laubach (1878), Baillon (1888), Endriss (1902), Rutherford (1970), Blarer et al. (2004), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Brown (1844), Griffith (1845), Weddell (1850), Solms-Laubach (1878, 1891), Baillon (1888), Ernst and Schmid (1913), Hunziker (1920), and Nikolov et al., 2013; Nikolov et al., 2014b).
 - Funicles or placental extensions schizogenous. Data for Apodanthaceae taken from Solms-Laubach (1874b), Endriss (1902), Bouman and Meyer (1994), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Weddell (1850), Solms-Laubach, 1874b; Solms-Laubach, 1898, Baillon (1888), Hunziker (1920), and Bouman and Meyer (1994).
 - Ovules anatropous, tenuinucellate, bitegmic (outer integument reduced in Rafflesiaceae). Data for Apodanthaceae taken from Solms-Laubach, 1874b; Solms-Laubach, 1898; Baillon (1888), Endriss (1902), Rutherford (1970), Bouman and Meyer (1994), Blarer et al. (2004), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Brown (1844), Griffith (1845), Weddell (1850), Solms-Laubach, 1874b; Solms-Laubach, 1878; Solms-Laubach, 1898; Baillon (1888), Heinricher (1906), Ernst and Schmid (1913), Bouman and Meyer (1994), and Sofiyanti and Yen (2012).
 - Fruits fleshy, polyspermous (Fig. 1). Data for Apodanthaceae taken from Solms-Laubach (1878), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Brown (1844), Griffith (1845), Weddell (1850), Baillon (1888), Solms-Laubach (1878), Heinricher (1906), Ernst and Schmid (1913), Bänziger (1995, 1996, 2004), Bänziger and Hansen (1997, 2000), and Hidayati et al. (2000).
 - Seeds numerous, small, exotestal, endo- or exozoochorous, with elaiosomes (doubtful in Rafflesiaceae). Data for Apodanthaceae taken from Solms-Laubach 1874b; Solms-Laubach, 1878), Endriss (1902), Rutherford (1970), Bouman and Meyer (1994), and González and Pabón-Mora (2017); data for Rafflesiaceae taken from Brown (1844), Weddell (1850), Solms-Laubach 1874b; Solms-Laubach, 1878; Solms-Laubach, 1898), Ernst and Schmid (1913), Bouman and Meyer (1994), Bänziger (1995, 1996, 2004), Bänziger and Hansen (1997, 2000), and Hidayati et al. (2000).
 - Embryos small; differentiation of cotyledons, root and shoot apical meristems, and dermatogen initials lacking. Data for Apodanthaceae taken from Solms-Laubach 1874b; Solms-Laubach, 1878, Rutherford (1970), Bouman and Meyer (1994), González and Pabón-Mora (2017), and González et al. (2024); data for Rafflesiaceae taken from Brown (1844), Weddell (1850), Griffith (1845), Solms-Laubach 1874b; Solms-Laubach, 1878), Ernst and Schmid (1913), and Bouman and Meyer (1994).
- Does floral size matter?** Our results raise new questions regarding the evolution rate of floral size when comparing large-flowered Rafflesiaceae (which ranges from c 15 cm in diameter in *R. verrucosa* to over 1 m in *R. arnoldi*) versus the minute flowers of Apodanthaceae (always less than 7 mm in diameter at full anthesis) (Fig. 1). Thus, previous estimations of accelerated floral size in Rafflesiaceae with respect to other Malpighiales (Davies et al. 2007; Barkman et al. 2008) may actually be higher than previously thought and should be reassessed, as new tiny-flowered players, i.e. the Apodanthaceae, emerge here as its sister group.
- A comment on coevolution and biogeography.** The extremely disparate speciation rates of Apodanthaceae and Rafflesiaceae with respect to their hosts (*Apodanthes* (1 sp.) versus *Casearia* (c 200 spp.) and *Xylosma* (c 80 spp.); *Pilostyles* (13 spp.) versus Fabaceae (c. 19,000 spp.); and Rafflesiaceae (49 spp.) versus *Tetrastigma* (c 140 spp.) point to a significantly low speciation response by both holoendoparasitic lineages. The initial diversification for Rafflesiaceae has been dated back to the mid- to late Cretaceous (Bendiksby et al., 2010; but see Pelser et al., 2019 for different age estimates). A similar time-scale is inferred for the Apodanthaceae (Naumann et al., 2013; Bellot and Renner 2014b), taking into account that the presence of the legume-obligate *Pilostyles* in the Americas, Africa, the Near East, and Australia rules out long distance dispersal events (Arias-Agudelo et al., 2019). This is suggestive of an extensive broad distribution of the Rafflesiaceae + Apodanthaceae ancestor that predated the Gondwana split. Then, two vicariant events would have occurred, one resulting in the first Rafflesiaceae-Apodanthaceae divergence along Southeast Asia, and a second one resulting in the *Apodanthes-Pilostyles* divergence in South America.

5. Conclusion

As many as 26 morphoanatomical and developmental traits plus the abundant phylogenomic data here presented support a sister-group relationship between the Apodanthaceae and the Rafflesiaceae. However, it is not yet possible to conclude whether the Apodanthaceae + Rafflesiaceae clade is either an early diverging offspring and sister to the remaining order Malpighiales, or, if it would result nested within the order. A broader sampling in the Malpighiales is required to arrive at a more accurate phylogenetic reconstruction of this exceedingly

diversified and complex order. Thus, the recently (Zuntini et al., 2024) proposed segregation of the Apodanthaceae in its own order Apodanthales, as sister to the clade Huales + Malpighiales, and the placement of the Rafflesiaceae in the order Malpighiales as sister to the clade (Linaceae(Elatinaceae + Malpighiaceae)) is not supported here. In addition, our findings decrease to 11 the number of parasitic lineages of flowering plants, earlier estimated to as 12 (Nickrent, 2020).

Contributions

FG and NPM equally contributed to the conceptualization, field and laboratory work, design, analyses, writing, editing and funding. JFA performed data curation and analyses. All authors wrote and approved the final version of the manuscript.

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CRediT authorship contribution statement

Juan F. Alzate: Writing – review & editing, Resources, Methodology, Formal analysis, Data curation. **Favio A. González:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization. **Natalia Pabón-Mora:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ymp.2024.108217>.

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