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Floral Gigantism in Rafflesiaceae

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The Rafflesiaceae are leafless, stemless, and rootless nonphotosynthetic parasites that live embedded in host plants (*1*). With flowers measuring up to a meter in diameter and weighing up to 7 kg, Rafflesiaceae *sensu stricto* [Supporting Online Material (SOM) text] possess the largest flowers of all angiosperms. Like other holoparasitic angiosperms, the phylogenetic affinities of Rafflesiaceae have proved difficult to resolve because of their reduced vegetative morphology, highly modified reproductive structures (*1*), and anomalous and often accelerated molecular evolution, particularly in plastid (cp) DNA (*2*–*6*).

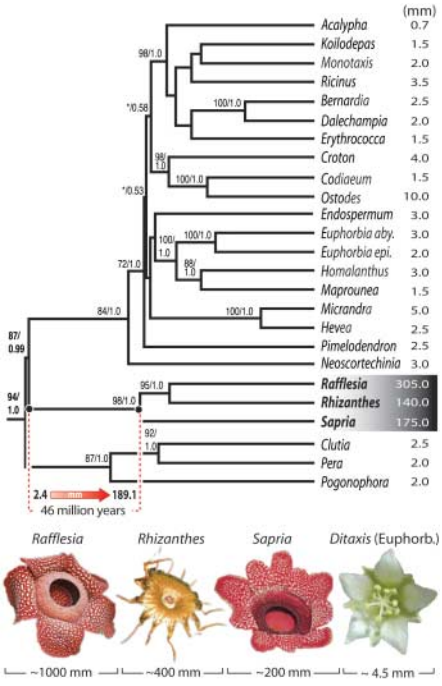

Fig. 1. Phylogeny of Euphorbiaceae [including Rafflesiaceae (bold)] based on a temporally calibrated ML tree (see SOM text for details and fig. S1 for full tree). ML BS and BPP, respectively, are provided. Support values ≤ 50%/0.50 are designated with asterisks. Flower size diameters (in mm) are provided (right), and ancestral flower size estimates are indicated at the stem and crown nodes of Rafflesiaceae. Reconstructions indicate a 79-fold increase in floral diameter for stem lineage Rafflesiaceae (with a 95% confidence interval of 74- to 83-fold). For additional ancestral size estimates see SOM text. Color images with scale bars illustrate the approximate sizes of flowers representative of the three genera of Rafflesiaceae (*Rafflesia arnoldii*, *Rhizanthes lowii*, and *Sapria himalayana*), plus a representative of Euphorbiaceae (*Ditaxis neomexicana*), the latter being similar in size to the inferred ancestral flowers at the stem node of Rafflesiaceae.

By analysis of slowly evolving genes, especially from mitochondrial (mt) DNA, Rafflesiaceae were shown to be members of the Malpighiales (*4*–*6*), a diverse group of circa (ca.) 16,000 species, with 29 major subclades [mostly recognized as families (*7*)]. However, the position of Rafflesiaceae within the order was unclear because of either insufficient taxon sampling (*4*, *6*) or a lack of phylogenetic signal (*5*). We used maximum likelihood (ML) and Bayesian inference (BI) to estimate the phylogeny of Malpighiales from ca. 11,500 base pairs of sequence data (see SOM text for detailed information). Five mt genes (*ccmB*, *cob*, *matR*, *nad6* and *rps3*) and one cp gene [*matK* (*8*)] were sampled from 111 accessions representing all families of Malpighiales (*7*) and 22 outgroup species, including Rafflesiaceae's obligate host, *Tetrastigma* (Vitaceae). Nuclear (nr) small- and large-subunit ribosomal DNA regions were also included for a subset of 40 taxa. Examination of ML bootstrap scores (BS) and Bayesian posterior probabilities (BPP) in the individual analyses of the eight gene regions revealed no significant topological discord, and thus the data were concatenated and analyzed in combination.

Both the ML and BI analyses showed that Rafflesiaceae are nested within Euphorbiaceae (Fig. 1). Strong support was found for both the Rafflesiaceae plus Euphorbiaceae clade (BS = 94% and BPP = 1.0) and the clade that includes Rafflesiaceae and all Euphorbiaceae except *Pera*, *Clutia*, and *Pogonophora* (BS = 87% and BPP = 0.99).

The phylogenetic association of Rafflesiaceae and Euphorbiaceae is robust and not attributable to phylogenetic artifacts (*9*). Although the morphology of Rafflesiaceae prevents identification of unambiguous synapomorphies, some reproductive traits (*10*) are consistent with a placement of Rafflesiaceae within Euphorbiaceae.

We conducted a quantitative analysis of floral size evolution in the context of the estimated phylogeny (SOM text). Flower sizes were determined from the literature and herbarium data. A likelihood ratio test (*11*) rejects the hypothesis that there was a single rate of flower size evolution in the entire Euphorbiaceae-plus-Rafflesiaceae clade. Instead, the optimal model assigns one rate to all Euphorbiaceae lineages and crown-group Rafflesiaceae but a different, higher rate to the stem lineage of Rafflesiaceae. This demonstrates that floral gigantism evolved principally along the stem lineage of Rafflesiaceae, whereas subsequent flower-size evolution within crown group Rafflesiaceae reverted to the original euphorbiaceous rate. Flower size evolved about 91 times faster along the stem lineage than in the rest of the phylogeny. By using Brownie (*12*), we estimated flower diameter to have increased from 2.4 [confidence interval (CI) of 1.1 to 5.3 mm] to 189.1 mm (CI of 91.2 to 392.2 mm) along the stem lineage of Rafflesiaceae (Fig. 1 and SOM text): a ca. 79-fold increase in size in a period of ca. 46 million years (Fig. 1). Thus, a placement of giant-flowered Rafflesiaceae within Euphorbiaceae, whose flowers are nearly all tiny, only increases the evolutionary enigma of "the greatest prodigy of the vegetable world" (*13*).

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SOM Text
Fig. S1
Table S1
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Supporting Online Material for

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Alignment S2 (nrDNA, 40 taxon sampling)

Data matrix assembly. All families of Malpighiales sensu Davis *et al.* (S*1*) were sampled, including all major lineages of Euphorbiaceae (S*2*) and all three genera of Rafflesiaceae (S*3*). Recent molecular evidence (S*3-S5*) indicate that Rafflesiaceae *sensu stricto* includes only the large-flowered southeast Asian genera *Rafflesia*, *Rhizanthes* and *Sapria*, whose close relationship is supported by morphological data (S*6,* S*7*) and the fact that all three rely exclusively on host plants of the genus *Tetrastigma* (Vitaceae). Primary analyses included 111 Malpighiales and 22 outgroups sequenced for five mitochondrial (mt) regions, one plastid (cp) gene, and two nuclear (nr) ribosomal genes (Table S1). Outgroup species were included from Amaranthaceae, Brassicaceae, Celastrales, Dilleniaceae, Fabacaeae, Huaceae, Magnoliaceae, Oxalidales, and Vitaceae (following S*8*). Nuclear ribosomal (nr) small-subunit SSU and large subunit LSU were sampled across a subset of these taxa, including most major subclades of Malpighiales, a broad representation of Euphorbiaceae, and all genera of Rafflesiaceae. The nr data were used to test agreement with our estimate of the phylogenetic placement of Rafflesiaceae from concatenated mt and cp data, and to potentially improve resolution when analyzed with these data.

Total cellular DNA extractions, PCR amplification, cloning, and sequencing protocols were performed as described (S*1,* S*4*). New amplification and sequencing primers were developed for *ccmB* (**ccmB-f** ATGAGACGACTYTTTCTTGAAC, **ccmB-r** AACTAATCGAGACCGAAATTGGA), *cob* (**cob-f** ATGACTATAAGGAACCAACGA, **cob-r** CATCGGATTAGCAGGTATATAATTG), and *nad6 (***nad6-f** GTCGAGCCCTGCTTTGGTCTCT, **nad6-r** GTCGTCCTCCTCATTATAGTC) by CCD and KJW, and for *rps3 (***rps3-F1** GTTCGATACGTCCACCTAC, **rps3-F12** GCTTTCGYCTCGGTAGGTG, **rps3-F3** CGKGGCCTWCAAGCATCC, **rps3-R1** GTACGTTTCGGATATRGCA, **rps3-R12** GTTTCGGATATRGCACGT) by Y-L. Qiu (University of Michigan, Ann Arbor).

Sequences were aligned by eye; the ends of sequences and ambiguous indel regions were trimmed to maintain complementary data between taxa. Our sequences (GenBank EF135073-EF135618) and associated statistics analyzed in conjunction with existing GenBank data are shown in Table S1. GenBank contains all taxon and voucher information and alignments are included with this manuscript.

Phylogenetic analysis. The optimal model of molecular evolution was determined by the Akaike Information Criterion (AIC) using Modeltest ver. 3.7 (*9, 10*). In each case the optimal model was the General Time Reversible model, with rate heterogeneity modeled by assuming that some proportion of sites are invariable and that the rate of evolution at other sites is modeled using a discrete approximation to a gamma distribution [GTR+I+Γ]). Maximum likelihood (ML) analyses of the individual and combined matrices were implemented in GARLi ver. 0.94 (distributed by D. Zwickl at http://www.zo.utexas.edu/faculty/antisense/Garli.html) starting from random trees and using 10,000,000 generations per search. ML bootstrap support (BS) values were estimated from 100 bootstrap replicates. Bayesian analyses were implemented in the parallelized version of MrBayes ver. 3.1.2 (S*11*) following Davis *et al. (1)*. Bayesian posterior probabilities (BPP) were calculated five times with a burn-in period of 150,000 generations (BPPs varied little).

Whereas *matR* supported a placement of Rafflesiaceae with Euphorbiaceae with over 75% BS and 0.75 BPP (embedded in Euphorbiaceae at over 55% BS and 0.65 BPP), the other six mt and nr gene regions produced gene trees with support values generally less than < 50% BS/0.50 BPP for all relevant branches. *matK*, which is most likely absent in Rafflesiaceae, was included to increase resolution among clades of autotrophic plants. Its inclusion or exclusion in our analyses did not change the placement of Rafflesiaceae. No strongly supported (≥ 75% BS/0.75 BPP) clades conflicted among the independent analyses, suggesting little discordance.

The data were concatenated and analyzed in five different ways: i) a combined six-gene mt and cp data set for all 133 taxa (ca. 17% missing cells; alignment included with SOM; Fig. S1); ii) a combined SSU and LSU nr data set of 40 taxa (16% missing cells; alignment included with SOM); iii) a combined mt, cp, and nr data set with the same taxon sampling as the first analysis, and with missing data included for taxa not sampled for nr data (43% missing cells); iv) an eight-gene data set limited to taxa for which nr data were available (i.e., 35 taxa; 15% missing cells), and v) an eight-gene data set limited to taxa sampled for most mt regions across a comprehensive set of Euphorbiaceae, but with some missing nr data (i.e., 55 taxa; 31% missing cells).

The combined six-gene mt and cp data (i) supported the placement of Rafflesiaceae with Euphorbaiceae at 94% and 1.0 BPP, and as nested members of Euphorbiaceae at 75% and 0.93 BPP or greater. No nodes were supported at greater than 75% BS/0.75 BPP in the combined nr data (ii). However, the addition of nr data to all combined analyses always increased support for the placement of Rafflesiaceae in Euphorbiaceae. Our global analyses (iii-v) supported the placement of Rafflesiaceae with Euphorbiaceae at 92% BS and 1.0 BPP or greater, and as nested members of Euphorbiaceae at 83% BS and 0.90 BPP or greater, with the nested placement supported at 87% BS and 0.99 BPP for the most comprehensive taxon and character analysis (v).

The Rafflesiaceae and the clusioid clade (i.e., Bonnetiaceae, Clusiaceae, Hypericaceae, Podostemaceae; Fig. S1) had the longest-branches of the Malpighiales. Thus, if long-branch attraction was confounding the phylogenetic results, one would expect Rafflesiaceae to associate with the clusioids rather than the relatively slowly-evolving Euphorbiaceae.

Convergent RNA editing is known to occur in plant mitochondrial genomes, and can complicate phylogenetic inference (S*12,* but cf. S*13*) but was not a problem here. We conservatively removed four synapomorphic sites (two in *matR*, one in *ccmB*, and one in *rps3)* that are potentially prone to RNA editing (i.e., C to U changes). Removing these sites from our phylogenetic analyses of the six-gene data set did not change the placement of Rafflesiaceae. Moreover, none of the synapomorphic sites that support the affiliation of Rafflesiaceae with Euphorbiaceae have been reported to be prone to RNA editing in other taxa (S*14*-S*16*). Similarly, the guanine-cytosine (GC) content in Rafflesiaceae and in Euphorbiaceae is also unlikely to explain the results. The GC content in Rafflesiaceae and Euphorbiaceae is nearly identical to the mean for Malpighiales. The average GC contents for Rafflesiaceae and Euphorbiaceae are, respectively: five gene mt data set (45%, 46%, mean for all taxa 45%), six-gene mt and cp data set (45%, 42%, mean 43%), and eight-gene mt, cp, and nr data set (48%, 46%, mean 45%).

Previous phylogenetic investigations of Rafflesiaceae have revealed genes acquired via horizontal gene transfer (HGT) from their obligate hosts (S*4*). It is possible that Rafflesiaceae have formerly been parasites on members of Euphorbiaceae and have acquired mt DNA via HGT. However, in instances of reported gene transfer (S*17*-S*19*) one usually finds a vertically inherited copy in addition to one or more horizontally transferred copies. Sequencing twenty clones from Rafflesiaceae for each of the five mt gene regions to screen for putative HGT copies, we recovered only one region, *cob*, where an alternate form of the gene gave a different placement for Rafflesiaceae; however, this second copy (GenBank EF135619) grouped with the current host of Rafflesiaceae, *Tetrastigma* (Vitaceae). It is most likely that this second copy arose through HGT (similar to S4). We also initially considered HGT with *ccmB* based on a preliminary phylogenetic placement of Rafflesiaceae near their hosts, but the removal of a small homoplasious microinversion (aligned positions 1986-1987 in Alignment S1) shared between *Tetrastigma* and *Sapria* (but not *Rafflesia*) resulted in a placement with Euphorbiaceae. Finally, we also directly sequenced PCR products for these gene regions from all Rafflesiaceae, and only *cob* produced chromatograms containing overlapping peaks indicative of multiple copies of this gene region.

Molecular dating. A likelihood ratio test ruled out a global molecular clock ($P < 0.05$). To obtain a chronogram for the combined six-gene data set we used penalized likelihood (S*20*) with an optimal smoothing value of 316.2 estimated by cross-validation (see Fig. 1). A maximum age constraint of 119 million years

was applied to crown group Euphorbiaceae (including Rafflesiaceae; Fig. S1), corresponding to the maximum age estimate for stem-group Euphorbiaceae (S*1*). Additionally, we used a well-characterized Euphorbiaceae fruit belonging to tribe Hippomaneae (S*21*) to assign a minimum age of 40 million years (stratigraphic age from S*22*) to the node represented by the most recent common ancestor of *Euphorbia* and *Maprounea* (Fig. S1). Based on this analysis, the stem lineage of Rafflesiaceae was estimated to have a duration of 46 million years (Fig. 1).

Flower size evolution. Floral diameters of the sampled species of Euphorbiaceae and Rafflesiaceae were determined from the literature (S*23*-S*27*) and from herbarium collections, and are available in the main body of the text (Fig. 1). For dioecious taxa, carpellate flowers were chosen because they are generally larger in Euphorbiaceae, which would tend to bias the analyses towards less extreme floral gigantism in Rafflesiaceae. Additionally, because the sampled species may not be typical of the lineages for which they serve as placeholders, we determined the range of flower diameters for each of the major lineages (Fig. S1). Subsequent studies of flower size evolution used three scorings of flower diameter: i) average sizes of the sampled species (the best estimate), ii) largest sizes for each clade of Euphorbiaceae and smallest sizes for each Rafflesiaceae genus (maximally conservative scoring), and iii) smallest sizes for each clade of Euphorbiaceae and largest sizes for each Rafflesiaceae genus (maximally liberal scoring).

We explored whether the data suggest one or multiple rates of flower size evolution in the Euphorbiaceae plus Rafflesiaceae clade, as modeled by Brownian motion. Using Brownie ver. 2.06b (S*28,* S*29*) we compared five models on the ultrametric topology: i) one rate for the entire tree, ii) one rate for crown group Rafflesiaceae and one for Euphorbiaceae plus stem Rafflesiaceae, iii) one rate for crown and stem group Rafflesiaceae and one for everything else, iv) one rate for crown group Rafflesiaceae, a second for stem Rafflesiaceae, and a third for all Euphorbiaceae, and v) one rate for stem Rafflesiaceae, and a second for everything else. The best-fitting model for the observed data (under all three scoring schemes), as determined by the AIC, was model v. Using the best estimate scoring, the rate of evolution on the stem lineage of Rafflesiaceae is 91 times faster than the rate elsewhere on the tree. The estimated rate change drops to 47 under the maximally conservative scoring [flower diameter increased from 3.2 (CI = 1.3–7.9 mm) to 117.7 mm (CI = 51.0–271.5 mm)] and increases to 126 under the maximally liberal scoring [flower diameter increased from 1.3 (CI = 0.6–3.2 mm) to 403.5 mm (CI = 179.1–908.9 mm)]. Thus, regardless of scoring scheme, a major change in floral evolution is indicated for the branch leading to crown group Rafflesiaceae.

To estimate the ancestral flower diameters we used the PDAP module (S*30*) of the Mesquite software package ver. 1.1 (S*31*) for log-transformed flower diameter data using weighted squared-change parsimony (WSP). To obtain 95%

confidence intervals we used the approach of ref. (S*32*). In order to correct for different rates of floral evolution, we modified the tree by elongating the stem lineage of Rafflesiaceae proportionally to its estimated degree of accelerated evolution, which should bring the tree in-line with the single-rate Brownian motion assumption that underlies WSP (S*33*).

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Figure S1. Fifty-percent majority maximum likelihood bootstrap consensus tree from the combined mt and cp data (133 accession sampling). Bootstrap percentage support values and Bayesian posterior probabilities indicated near nodes, respectively. Increased support for the nested placement of Rafflesiaceae shown from the combined eight gene 55 taxon analysis (v). Euphorbiaceae in blue; Rafflesiaceae in red; clusioids in green. Maximum likelihood branch lengths shown in inset, scale bar equals 0.01 substitutions per site. Time constraints to estimate clade ages shown with stars, and indicated as minimum (min) and maximum (max) age constraints (see text). Smallest and largest floral diameters used for maximally conservative and maximally liberal ancestral size estimates indicated in parentheses following sampled taxa.

Table S1. Statistics for newly collected gene regions.

Gene regions	**Length (base pairs)**	**Total Accessions**	**New sequences**
ccmB (mt)	568	111	108
cob (mt)	766	113	108
matR (mt)	1892	128	22
nad6 (mt)	534	107	102
rps3 (mt)	1600	89	85
matK (cp)	1188	117	105
SSU rDNA (nr)	1655	42	2
LSU rDNA (nr)	3234	42	14

Fig. S1
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