Supporting Online Material for

**Floral Gigantism in Rafflesiaceae**

Charles C. Davis,* Maribeth Latvis, Daniel L. Nickrent, Kenneth J. Wurdack, David A. Baum

*To whom correspondence should be addressed. E-mail: cdavis@oeb.harvard.edu

Published 11 January 2007 on *Science Express*
DOI: 10.1126/science.1135260

This PDF file includes:

- SOM Text
- Fig. S1
- Table S1
- References
Supporting Online Material for **Floral gigantism in Rafflesiaceae** Davis et al.

**Materials and Methods**

Fig. S1

Table S1

Alignment S1 (mt+cp data, 133 taxon sampling)

Alignment S2 (nrDNA, 40 taxon sampling)

**Supplementary References**

**Data matrix assembly.** All families of Malpighiales sensu Davis et al. (S1) were sampled, including all major lineages of Euphorbiaceae (S2) and all three genera of Rafflesiaceae (S3). Recent molecular evidence (S3-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 (S6, S7) 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, Fabaceae, Huaceae, Magnoliaceae, Oxalidales, and Vitaceae (following S8). 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 (S1, S4). 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* GTCGAGCCCTGCTTGGTCTCT, *nad6-r* GTGCCTCTCCTCATTAGTC) by CCD and KJW, and for *rps3* (*rps3-F1* GTTCGATACGTCCACCTAC, *rps3-F12* GTCTTCGYCTCGGTAGGTG, *rps3-F3* CGKGGCCTWCAAGCATCC, *rps3-R1* GTACGTTCGGATATRCC, *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 invariant 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 (S11) 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 Euphorbiaceae 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 (S12, S13) 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 (S14-S16). 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 (S4). It is possible that Rafflesiaceae have formerly been parasites on members of Euphorbiaceae and have acquired mt DNA via horizontal gene transfer. However, in instances of reported gene transfer (S17-S19) 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 horizontal gene transfer (similar to S4). 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 (S20) 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 (S1). Additionally, we used a well-characterized Euphorbiaceae fruit belonging to
tribe Hippomaneae (S21) to assign a minimum age of 40 million years (stratigraphic age from S22) 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 (S23-S27) 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 (S28, S29) 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 (S30) of the Mesquite software package ver. 1.1 (S31) for log-transformed flower diameter data using weighted squared-change parsimony (WSP). To obtain 95% confidence intervals we used the approach of ref. (S32). In order to correct for different rates of floral evolution, we modified the tree by elongating the stem
lineage of Rafflesiaeeae 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 (S33).

Supplementary References

S1. C. C. Davis, C. O. Webb, K. J. Wurdack, C. A. Jaramillo, M. J. Donoghue,
S7. A. Takhtajan, Diversity and Classification of Flowering Plants (Columbia
S12. S. Steinhauer, S. Beckert, I. Capesius, O. Malek, V. Knoop, J. Mol. Evol. 48,
303 (1999).
S14. M. C. Thomson, J. L. Macfarlane, C. T. Beagley, D. R. Wolstenholme,
S17. U. Bergthorsson, A. O. Richardson, G. J. Young, L. R. Goertzen, J. D.
2237 (2005).
S22. F. W. Potter, Jr., D. L. Dilcher, in Biostratigraphy of Fossil Plants D. L.
Dilcher, T. N. Taylor, Eds. (Dowden, Hutchinson & Ross, Stroudsburg,
S23. J. Nais, Rafflesia of the World (Sabah Parks, Kota Kinabalu, 2001), pp. xiii,
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.

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