Phylogenetic relationship of Rafflesiales based on two nuclear and four mitochondrial genes

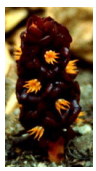
Albert Blarer [1], Daniel L. Nickrent [2], Hans Bänziger [3], Peter K. Endress [1], and Yin-Long Qiu [1,4].

1. Institute of Systematic Botany, University of Zurich, Zollikerstrasse 107, CH-8008 Zürich, Switzerland. ablarer@noachide.com. 2. Department of Plant Biology and Center for Systematic Biology, Southern Illinois University, Carbondale, IL, USA 62901-6509. nickrent@plant.siu.edu. 3. Department of Entomology, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand 50200. 4. Department of Biology, Morrill Science Center, University of Massachusetts, Amherst, MA, 01003-5810. yqiu@bio.unmass.edu (present address)

Sapria himalayana:
A member of Rafflesiaceae, the 'large-flowered clade' (A). (flower diam. c. 10 cm)
Photo: H. Bänziger

Pilostyles thurberi:
A member of Apodanthaceae, the 'small-flowered clade' (B). (flower diam. c. 3 mm)
Photo: K. Robertson

Mitrastema yamamotoi:
A member of Mitrastemonaceae, the 'superior-ovary clade' (C). (flower diam. c. 1 cm)
Photo: S.-C. Hsiao



Bdallophyton americanum:
A member of Cytinaceae, the 'inflorescence clade' (D). (flower diam. c. 1.3 cm)
Photo: J. García-Franco

Introduction

The group of holoparasites that includes the spectacular *Rafflesia* has been variously classified, either as a single family (Rafflesiaceae s.lat.) composed of two subfamilies and four tribes (Bouman & Meijer 1994) or as an order (Rafflesiales) composed of four families (Takhtajan 1997). Following the second treatment, the component taxa are: Apodanthaceae (3 genera, ca. 20 species), Cytinaceae (2 genera, ca. 10 species), Mitrastemonaceae (1 genus, 2 species), and Rafflesiaceae s. str. (3 genera, 19 species).

All members of Rafflesiales are endoparasitic, achlorophyllous herbs. Aerial portions of the plant consist only of solitary flowers or many-flowered inflorescences (Cytinaceae) that burst out of the host's cortex. The vegetative endophyte resides in the host and is often compared to a fungal mycelium. Apodanthaceae is distributed in Western Australia, Iran, East Africa, and the Americas. *Cytinus* of Cytinaceae occurs in the Mediterranean region, South Africa, and Madagascar, whereas *Bdallophyton* is found in Mexico and Central America. Rafflesiaceae s. str. is restricted the Indo-Malayan region. Mitrastemonaceae is monogeneric and composed of two species with disjunct distributions in eastern Asia and Central America to northern South America. Among these families, the rank of Mitrastemonaceae has been most often debated. It was considered a part of Rafflesiaceae s. lat. by Watanabe (1936, 1937), a separate family by Cronquist (1981) and Takhtajan (1997), or as an intermediate taxon in an evolutionary series leading to Hydnoraceae (Coccuci 1983).

Despite over one century of research, the phylogenetic relationship of Rafflesiaceae s. lat. with other angiosperms remains controversial. Traditional classifications have allied Rafflesiaceae s. lat. with Hydnoraceae and placed both within or near Aristolochiales (Melchior 1964; Takhtajan 1997). In contrast to this placement among magnoliids, Cronquist (1981) considered Rafflesiales as closely related to Santalales. Rafflesiaceae s. lat. was classified with Hydnoraceae and placed among an unresolved group of magnoliid families (e.g. Ceratophyllales and Piperales) at the base of the angiosperm phylogenetic tree by the APG (1998). Previous molecular phylogenetic work using nuclear SSU rDNA resulted in a clade composed of Hydnoraceae and Aristolochiaceae (Nickrent & Duff 1996). This result has recently been confirmed using other nuclear and mitochondrial gene sequences (D. Nickrent, A. Blarer, Y.-L. Qiu, D. Soltis, M. Zanis, unpublished). Moreover, these analyses show that Hydnoraceae is not related to Rafflesiales, the latter being a component of the eudicot clade.

Sequences from 13 species representing all nine genera of Rafflesiales were used to elucidate phylogenetic relationships within the order. The molecular phylogenetic analysis of DNA sequences is derived from nuclear SSU rDNA and ITS as well as mitochondrial *atp1*, *matR*, and SSU and LSU rDNA.

Material and Methods

DNA was extracted by using a 2X CTAB method modified from that of Doyle & Doyle (1987) or using extraction columns (Qiagen). The nuclear and mitochondrial genes were PCR amplified using primers reported elsewhere (Nickrent & Starr 1994; Qiu et al. 1999; Qiu, Lee, Dombrovska, unpublished). Sequencing was conducted using manual and automated methods (ABI-Prism 377 DNA sequencer, PE Applied Biosystems) according to manufacturer's protocols. Of the 78 sequences possible for a 13 taxon by 6 gene matrix, 48 (61%) were available for this study.

Each of the six separate gene partitions were aligned by eye using the computer program Se-Al (Rambaut 1996) and then assembled into a multigene data set. Owing to sequence divergence, ITS-1 and ITS-2 could not be unambiguously aligned across all taxa, hence only 5.8S rDNA sequences were included in the nuclear gene partition. The nuclear and mitochondrial gene partitions were analysed separately and in combination using parsimony with all characters equally weighted (PAUP* version 4.0, Swofford 2000). Search parameters employed the branch-and-bound algorithm with all default settings. Furthermore, 500 replicates of resampling were performed to calculate the bootstrap values applying a heuristic search strategy. *Arabidopsis* was choosen as the outgroup given that sequences for all six genes were available from this nonparasitic taxon.

Results and Discussion

Branch-and-bound analyses of the 6-gene data set yielded three trees. One of them, with bootstrap values added, is shown in Fig. 1. Four major clades are present: *Rafflesia, Rhizanthes*, and *Sapria* (clade A); *Apodanthes, Berlinianche*, and *Pilostyles* (clade B); *Mitrastema* (clade C); and *Cytinus* and *Bdallophyton* (clade D). Clade A (Rafflesiaceae s. str.) represents taxa with flowers up to several decimeters in diameter. Clade B (Apodanthaceae) includes those taxa with solitary flowers less than a centimeter in diameter. Clade C (Mitrastemonaceae) contains the only member of Rafflesiales with a superior ovary. The taxa of Clade D (Cytinaceae) have several flowers per inflorescence instead of solitary flowers. Within clade A, the relative positions of *Rafflesia*, *Sapria* and *Rhizanthes* are unresolved, mainly owing to conflict between the nuclear and mitochondrial gene partitions (see below). This 'large-flowered clade' (A) is sister to the 'small-flowered clade' (B). Clades A and B are sister to *Mitrastema* (clade C) and the 'inflorescence clade' (D) is sister to clades A, B and C. The other two most parsimonious trees (not shown) differed from Fig. 1 only by slightly different positions of *Mitrastema* and *Rhizanthes*.

Three trees resulted for the nuclear rDNA gene partition. One of them, with bootstrap values added, is shown in Fig. 2. Unlike the 6-gene analyses, *Rhizanthes* was sister to *Rafflesia,* and *Cytinus* and *Bdallophyton* were unresolved and occupied the basal portion of the tree. Additional sequences are required to assess the relevance of these topological differences. Furthermore, analyses required the exclusion of clade B, because nuclear rDNA sequences have yet to be obtained from these taxa. The other two not shown most parsimonious trees differed only in the relative positions of *Sapria* species.

A single most-parsimonious tree was obtained from analysis of the mitochondrial gene partition (Fig. 3). In contrast to the 6-gene and nuclear gene analyses, but in general agreement with floral morphology, *Rafflesia* and *Sapria* form a clade (89% BS) that is sister to *Rhizanthes*. Furthermore, analysis of the mitochondrial genes results in *Mitrastema* occupying the base of the Rafflesiales tree, albeit with weak support (53% BS).

These results are consistent with general affinities ascertained from observations of ovule and seed morphology (Bouman & Meijer 1994). A cladistic analysis of morphological characters (Nickrent, unpublished, modified from Beaman et al. 1992) resulted in a tree that is generally congruent with Fig. 3.

nuclear & mitochondrial genes

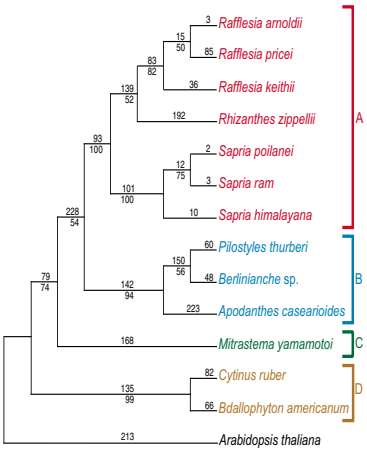


Fig. 1: One of three most parsimonious trees obtained for analyses of the 6-gene data matrix. Branch lengths are indicated above, bootstrap values below the branches. Tree length = 2368, C.I. = 0.8298, R.I. = 0.7071.

nuclear genes

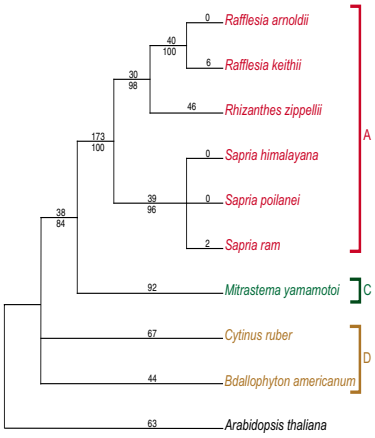


Fig. 2: One of three most parsimonious trees for combined analyses of nuclear 5.8S and SSU rDNA. Branch lengths are indicated above, bootstrap values below the branches. Bootstrap values less than 50% are not shown. Tree length = 663, C.I. = 0.8401, R.I. = 0.8320.

mitochondrial genes

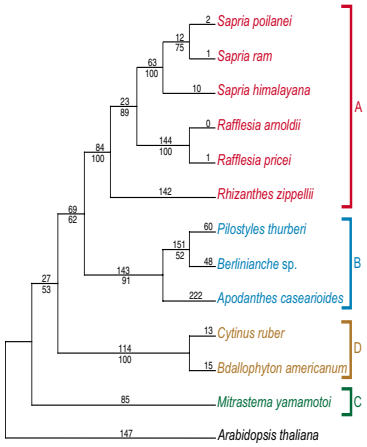


Fig. 3: The single most parsimonious tree obtained from analyses of mitochondrial LSU and SSU rDNA, *atp1*, and *matR*. Branch lengths are indicated above, bootstrap values below the branches. Tree length =1576, C.I. = 0.8794, R.I. = 0.7090.

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