Mitochondrial DNA sequences reveal the photosynthetic relatives of *Rafflesia*, the world's largest flower

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All parasites are thought to have evolved from free-living ancestors. However, the ancestral conditions facilitating the shift to parasitism are unclear, particularly in plants because the phylogenetic position of many parasites is unknown. This is especially true for *Rafflesia*, an endophytic holoparasite that produces the largest flowers in the world and has defied confident phylogenetic placement since its discovery >180 years ago. Here we present results of a phylogenetic analysis of 95 species of seed plants designed to infer the position of *Rafflesia* in an evolutionary context using the mitochondrial gene *matR* (1,806 aligned base pairs). Overall, the estimated phylogenetic tree is highly congruent with independent analyses and provides a strongly supported placement of *Rafflesia* with the order Malpighiales, which includes poinsettias, violets, and passionflowers. Furthermore, the phylogenetic placement of *Mitrastema*, another enigmatic, holoparasitic angiosperm with the order Ericales (which includes blueberries and persimmons), was obtained with these data. Although traditionally classified together, *Rafflesia* and *Mitrastema* are only distantly related, implying that their endoparasitic habits result from convergent evolution. Our results indicate that the previous significant difficulties associated with phylogenetic placement of holoparasitic plants may be overcome by using mitochondrial DNA so that a broader understanding of the origins and evolution of parasitism may emerge.

Parasitic organisms have evolved independently from free-living ancestors in most of the major lineages of prokaryotes and eukaryotes (1), but identification of the relatives of highly reduced parasites has proven to be a phylogenetic challenge in many cases (2). The evolutionary shift to an advanced parasitic lifestyle is often associated with degeneration of both morphologies and genomes, leaving few reliable characters with which to infer phylogenetic relationships (3). In angiosperms, evolutionary relationships of many hemiparasites (parasitic plants that retain the ability to photosynthesize) have recently been clarified (4), largely because they retain vegetative and floral characteristics linking them to their nonparasitic relatives and because their genomes evolve similarly to nonparasites in rate and pattern (5). In contrast, inferring the phylogenetic relationships of many holoparasitic plants (parasites that can no longer photosynthesize) has been particularly problematic because of the reduced vegetative features of holoparasites and genes that may be missing or evolve at extremely high rates under relaxed constraint (3–8). Thus, despite the fact that a comprehensive framework within which to study angiosperm phylogeny exists, provided by combined plastid and nuclear sequences from >500 species (9–11), the photosynthetic relatives of most holoparasites are currently unknown.

Particularly problematic is the holoparasite *Rafflesia* (Rafflesiaceae), whose phylogenetic affinities have remained obscure since its description in 1822 (12). *Rafflesia*, a genus of 20 species, is notable for producing the largest flowers in the world, measuring up to 1 m in diameter and weighing up to 7 kg (13). These massive blooms smell of rotting flesh and attract carrion flies for pollination (14). As endophytes growing completely embedded within their hosts, *Rafflesia* and its close parasitic relatives *Rhizanthes* and *Sapria* are hardly plant-like because they lack leaves, stems, and roots and emerge only for sexual reproduction when they produce flowers (3). These Southeast Asian endemic holoparasites rely entirely on their host plants (exclusively species of *Tetrastigma* in the grapevine family, Vitaceae) for all nutrients, including carbohydrates and water (13). Circumscriptions of Rafflesiaceae have varied to include, in the strict sense, only *Rafflesia*, *Rhizanthes,* and *Sapria*; however, in the broad sense, the family includes the divergent endophytic holoparasites *Apodanthes, Pilostyles*, *Cytinus*, *Bdallophyton,* and *Mitrastema* as well (15, 16). Regardless of strict or broad interpretations of the family, all of these divergent parasitic plants remain unclassified within the recent ordinal treatment of angiosperms (11). Molecular phylogenetic placement of *Rafflesia* using plastid DNA sequences has not been achieved because *Rafflesia* seems to lack the commonly studied gene *rbcL* (4) and may not have a plastid genome at all (7). Furthermore, molecular studies of the nuclear 18S gene from *Rafflesia* indicate that it evolves at least 3.5 times faster than in photosynthetic plants (17). This rate acceleration has frustrated previous attempts to infer its phylogenetic position (4, 18). Because no strongly supported phylogenetic placement of *Rafflesia* has been proposed, the understanding of the ancestral conditions facilitating the evolution of its massive flowers and endoparasitic habit has been hampered.

To determine the position of *Rafflesia* within the context of angiosperm phylogeny we have used mitochondrial (mt) DNA sequences, which have recently been used to study plant evolutionary relationships alone or in combination with data from other genomic compartments (19–24). One reason for the utility of plant mtDNA may be the lower substitution rates that characterize this genome (25), which seem to provide characters with low levels of homoplasy (20, 24). The low mutation rates of mtDNA may facilitate the phylogenetic study of plant parasites as well, because holoparasitic plant 19S mt sequences show only modestly elevated divergences (≈2 times higher) relative to photosynthetic plants (26). Here we present the results of a phylogenetic analysis, based on mtDNA sequence variation from 95 species of seed plants, that strongly places the enigmatic holoparasite *Rafflesia* within angiosperm phylogeny.

Methods

Using the recent ordinal classification of angiosperms (11) as a guide, we explicitly sampled at least one family from 43 of 45

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Abbreviations: mt, mitochondrial; BS, bootstrap support; JS, jackknife support; PP, posterior probabilities; NJ, neighbor joining; P-BS, parsimony BS; P-JS, parsimony JS.

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monophyletic orders to produce a broad mtDNA phylogenetic framework. Sampling included 92 species from 80 angiosperm families. Of primary interest for this study was the inclusion of *Rafflesia keithii* and its close parasitic relative *Rhizanthes zippelii*. Both of these species traditionally have been placed in Rafflesiaceae *sensu stricto*. *Mitrastema yamamotoi* (Mitrastemonaceae), another extreme holoparasite that has long been placed in Rafflesiaceae *sensu lato*, also was sampled to determine its phylogenetic affinities. Like *Rafflesia*, this parasite grows as an endophyte within its host (various members of the oak family, Fagaceae), emerging only during flower production (3). Because the morphology of *Mitrastema* is so divergent from that of photosynthetic plants, its affinities have remained obscure, with no relatives ever proposed aside from other endophytic parasites like *Rafflesia* (27). Three gymnosperms, *Pinus*, *Ginkgo*, and *Zamia*, were included as outgroups to root phylogenetic estimates. Table 1, which is published as supporting information on the PNAS web site, lists all of the species included in this study, the GenBank accession numbers for all of the sequences analyzed, and the voucher numbers for the newly generated sequences. Molecular methods, including DNA extraction, and DNA sequencing were performed as previously described (19). Details of the PCR methodology, including the primer sequences used, are available as *Supporting Text*, which is published as supporting information on the PNAS web site. In total, 55 *matR* mtDNA sequences were generated for this study.

CLUSTALX (28) was used to produce a preliminary alignment of the *matR* sequences that was followed by minor manual adjustments. The aligned data matrix is available from TreeBASE (www.treebase.org/treebase) (S981–M1631). Regions of uncertain alignment were excluded before analysis; however, their inclusion did not alter the fundamental conclusions of this study. After exclusion, there were 1,499 included characters and 636 parsimony-informative characters. Unweighted parsimony analyses were conducted by using PAUP* V. 4.0b10 (29) with 100 random addition sequences, and tree bisection–reconnection swapping. Bootstrap support (BS) (30) and jackknife support (JS) (with 33% character deletion) values were obtained from 2,000 replicates by using "fast" stepwise addition. Although the fast stepwise addition analyses are expected to provide estimates of support that are less than those obtained when comprehensive branch-swapping analyses are performed (31, 32), such analyses were not computationally feasible with this data set. MODELTEST V. 3.06 (33) was used to determine the best-fit model of nucleotide substitution for the data set, and this best-fit model [K81 (34), assuming unequal nucleotide frequencies and a Γ parameter allowing for rate heterogeneity] then was implemented during Bayesian analyses performed by using MRBAYES V. 3.0b4 (35). Four chains were run simultaneously for one million generations, and these were sampled every 100 generations. The first 10,000 generations were discarded as the "burn-in" period, and posterior probabilities (PP) for individual clades then were obtained from the remaining samples. Neighbor-joining (NJ) analyses were performed by assuming the optimal model of nucleotide substitution chosen with MODELTEST, and BS was obtained from 2,000 replicates by using PAUP* V. 4.0b10.

Results

Parsimony analyses of *matR* from 95 species of seed plants resulted in 61,911 equal-length trees of 2,464 steps [consistency index = 0.5657]. The strict consensus tree (Fig. 1) represents an mtDNA-only estimate of broad, angiospermwide phylogeny, and it is largely congruent with estimates based on independent plastid and nuclear DNA sequences (9–11, 36). Levels of parsimony BS (P-BS) and Bayesian PP are shown on all nodes receiving estimates higher than 50 or 0.5, respectively. The complete majority-rule consensus tree obtained from the Bayesian analysis is available as Fig. 3, which is published as supporting information on the PNAS web site. Parsimony JS (P-JS) values and NJ-BS values could not be shown in Fig. 1 but are presented below. The complete set of JS values and the NJ tree with BS values are available in Figs. 4 and 5, respectively, which are published as supporting information on the PNAS web site. In general, the P-BS for all nodes obtained with fast stepwise addition was lower than the JS, NJ-BS, or PP and may be conservative. The P-BS values and PP shown for each node may therefore be interpreted as the lower and upper bounds of node reliability, respectively (37). The strict consensus tree suggests that the members of the ANITA (*Amborella*, Nymphaeales, and Austrobaileyales) clade (19, 22, 23) are the basal-most angiosperms, followed by an unresolved set of relationships among magnoliid orders, including all monocots. Expected major eudicot relationships include monophyletic rosids and asterids. All recently recognized orders are monophyletic when represented by more than one family, and, in most cases, BS and PP are high at the ordinal level (Fig. 1).

Within the context of this global angiosperm mtDNA phylogeny, the placement of *Rafflesia* as sister to the Malpighiales is supported (P-BS = 93, P-JS = 97, NJ-BS = 98, PP = 1.0). Parsimony analyses of amino acid sequences also suggested the same relationship (P-BS = 77, P-JS = 89) (Fig. 6, which is published as supporting information on the PNAS web site). The nested position of *Rafflesia* within the monophyletic Oxalidales + Malpighiales (P-BS $<$ 50, P-JS = 56, NJ-BS = 57, PP = 1.0) increases our confidence in its placement because these orders are expected to be sisters (10, 11). Not supported by these data were traditional placements of *Rafflesia* + *Rhizanthes* with either Piperales (38) or Santalales (27).

Placement of the enigmatic Southeast Asian holoparasitic plant *Mitrastema* (Mitrastemonaceae) with the Ericales (Fig. 1) was obtained by using the *matR* mtDNA phylogenetic framework produced here (P-BS = 70, P-JS = 82, NJ-BS = 91, PP = 1.0). This phylogenetic placement of *Mitrastema* with Ericales was surprising but is supported by parsimony analyses of amino acid data as well (P-BS = 76, P-JS = 91) (Fig. 6). Whereas *Mitrastema* has long been classified with *Rafflesia* + *Rhizanthes,* or close to them, these taxa are only distantly related.

Discussion

Holoparasite Phylogenetic Placement. The positions of the holoparasites, *Rafflesia* + *Rhizanthes* and *Mitrastema*, inferred with *matR* sequence data, are robust for several reasons. First, our results are not method-dependent, because the same strongly supported placements were obtained with parsimony, NJ, and Bayesian analysis. Second, long-branch attraction (39) does not seem to have misled these analyses, because the inclusion of random or misaligned sequences (40) did not influence the placement of *Rafflesia* + *Rhizanthes* or *Mitrastema* (Figs. 7 and 8, which are published as supporting information on the PNAS web site). Furthermore, it is clear that these lineages have not been artifactually placed with any of the other longest branches in this data set (Fig. 2*A*). Third, RNA editing and the potential for processed paralogy in plant mt genomes can confound phylogenetic estimates (41); however, it is unlikely that the placement of these holoparasites has been affected, because the removal of known *matR* RNA edit sites from the data set (42–44) did not change the level of support for the inferred relationships (Fig. 9, which is published as supporting information on the PNAS web site). Finally, because we have sampled the host plants of the parasites (or their close relatives), contamination cannot be invoked as an explanation for our results.

Horizontal gene transfer (45, 46) of *matR* between *Rafflesia* and/or *Rhizanthes* and a member of the Malpighiales, or between *Mitrastema* and a member of the Ericales, is also an unlikely explanation for these results for several reasons. First, although host-to-parasite transfer of macromolecules is possible

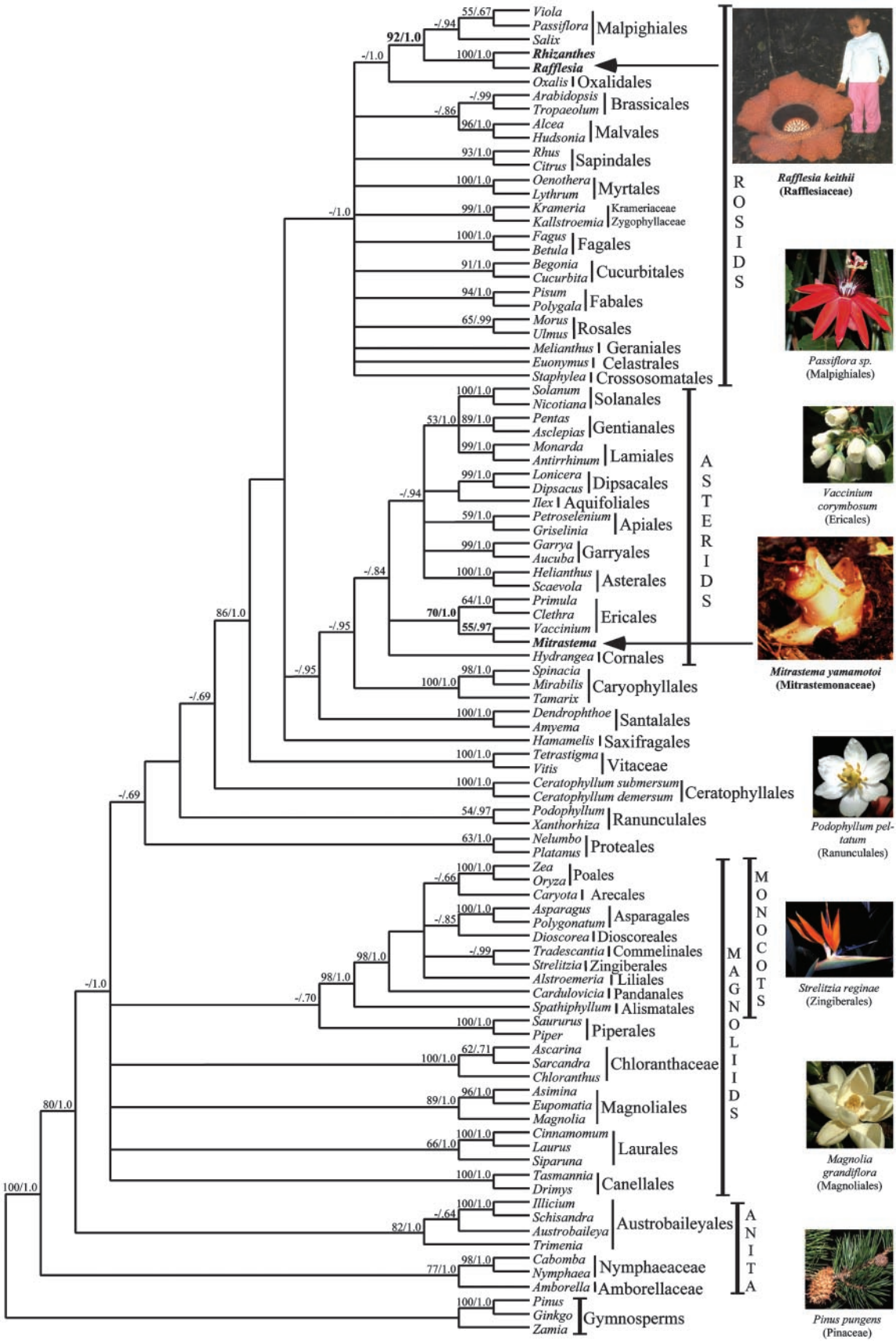

Fig. 1. Strict consensus tree from an unweighted parsimony analysis of *matR* mtDNA sequences (number of trees = 61,911, tree length = 2,464, consistency index = 0.5657). BS from the parsimony analysis is listed before the slash, and PP from the Bayesian analysis are listed after the slash for all nodes receiving support of ≥50 (0.5). When present, a dash represents BS < 50. Images of representative species are shown for heuristic purposes.

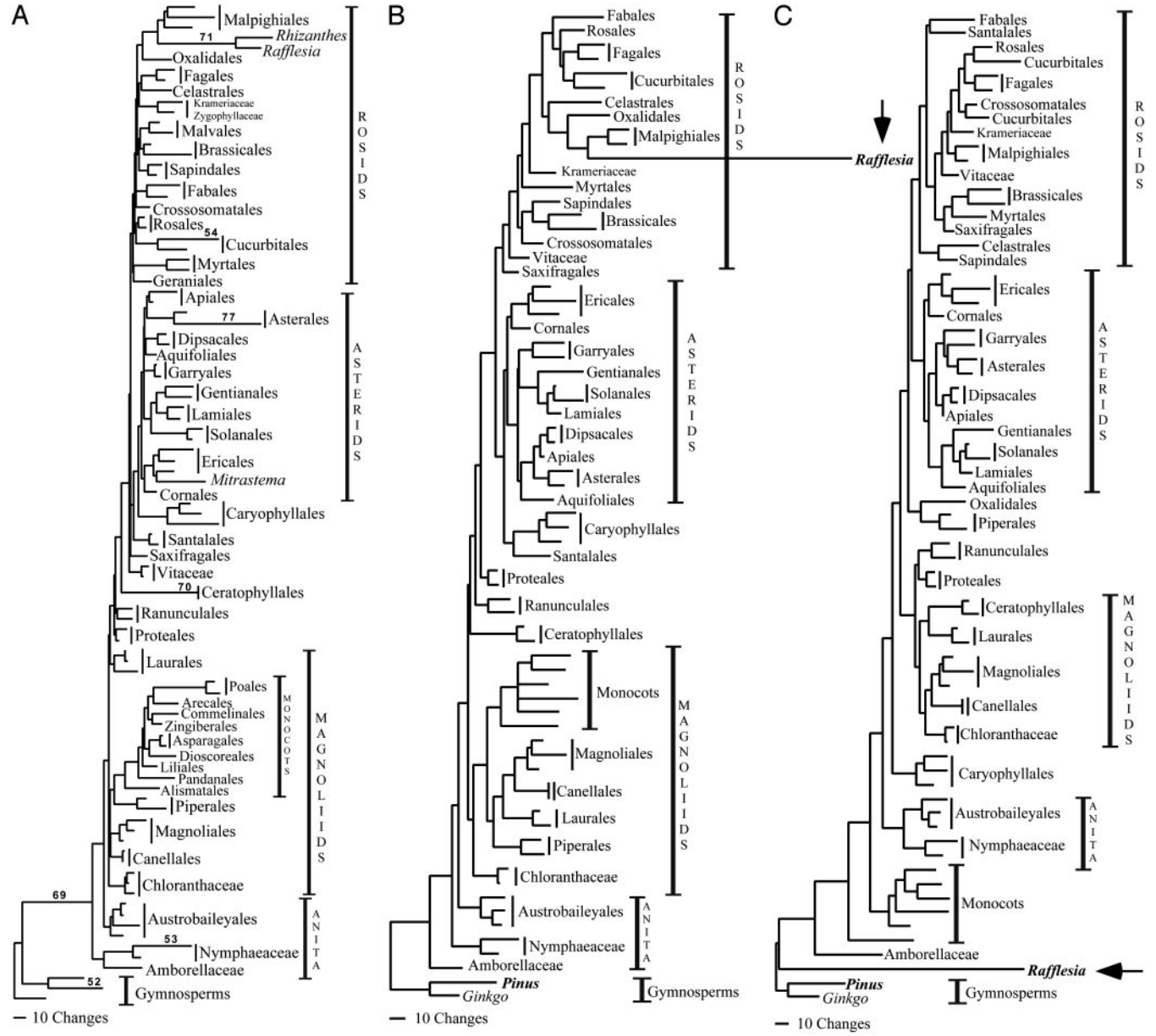

Fig. 2. (*A*) A randomly chosen phylogram of one of the equal-length trees from the *matR* parsimony analysis in Fig. 1. Branch lengths are shown only above lineages that had lengths of >50 steps. (*B*) Phylogram showing estimated 18S branch lengths on a topology that is constrained to represent currently accepted angiosperm phylogenetic relationships for 70 of the 95 taxa represented in Fig. 1. *Rafflesia* was constrained as sister to Malpighiales based on the *matR* results shown in Fig. 1. The *Rafflesia* branch is 4 times longer than the next longest lineage in the tree (the branch separating the angiosperms from gymnosperms). (*C*) A randomly chosen phylogram from 1 of 14 equally parsimonious trees (tree length = 2,032, consistency index = 0.3701), estimated from an unconstrained analysis of 18S sequence data of the same taxa shown in *B*. The position of the highly divergent *Rafflesia* sequence as the basal-most angiosperm is likely artifactual and the result of long-branch attraction (18, 39).

(47), *Rafflesia* and *Rhizanthes* are only parasitic on species of *Tetrastigma* (Vitaceae) to which they are clearly unrelated (Fig. 1). Likewise, *Mitrastema* only parasitizes members of the Fagales. Second, horizontal transfer seems to be rare in flowering plants overall (45) and would have to be postulated to have affected only *Rafflesia*, *Rhizanthes*, and *Mitrastema*, because all other nodes with high BS or JS in this analysis are comparably placed by independent estimates of angiosperm phylogeny (11). Finally, phylogenetic analysis of 128 *coxI* mtDNA sequences suggests comparable phylogenetic placements of *Rafflesia* with Malpighiales and *Mitrastema* with Ericales, although with lower support (C. W. dePamphilis, personal communication). Although confident placement of these parasites has been achieved with these data, more studies, including more taxa and characters, are needed to refine the positions of *Rafflesia* + *Rhizanthes* and *Mitrastema* to determine whether they are nested within, or sister to, the Malpighiales and Ericales, respectively. In either case, expanded circumscription of these orders is needed to include these holoparasites.

***Rafflesia* and Its Photosynthetic Relatives.** The relationship between *Rafflesia* + *Rhizanthes* and Malpighiales estimated with *matR* mtDNA has not been suggested in any angiosperm classification or phylogenetic analysis. Not since 1822, when Robert Brown noted similarities in floral structure between *Rafflesia* and the Passifloraceae (12), has a potential relationship to the members

of the currently circumscribed Malpighiales been suggested. Within the order, *Rafflesia* is morphologically most similar to Passifloraceae, with which it shares a hypanthium (perigone tube of *Rafflesia*), an androgynophore (central column of *Rafflesia*), and an annular corona (diaphragm of *Rafflesia*), a suite of characteristics that are otherwise somewhat rare in angiosperms (27). Some of these morphological features are found in other members of Malpighiales; therefore, the potential homology of these features may be ascertained only when the placement of *Rafflesia*, relative to a broader sampling of the order, is determined. The phylogenetic placement suggested by our data implies that a massive increase in flower size has occurred since the origin of *Rafflesia*, because the largest Malpighialian flowers are only 10 cm in diameter. Future comparative studies of MADS box and other floral developmental genes from *Rafflesia* and Malpighiales may reveal the genetic basis for this evolutionary floral size increase.

The placement of *Rafflesia* with Malpighiales also makes particularly tantalizing the 100-year-old observation that an individual of the tropical vine, *Passiflora caerulea*, was parasitic on another angiosperm (48). Although this may have been an aberrant observation of *Passiflora*, it is interesting to note that because *Rafflesia* exists as a vine-like endophyte within its host, the association of the viney growth form with parasitism is found in at least three separate orders: Laurales (49), Solanales (50), and Malpighiales. Although there may be different ancestral conditions giving rise to parasitism, the close physical association between vines and the plants that support them suggests that this interaction may foster the transition to a parasitic lifestyle.

The Asterid Holoparasite *Mitrastema*. The general placement of *Mitrastema* within the asterids has not been suggested previously, but its gamopetalous corolla supports this position. The specific placement of *Mitrastema* with the Ericales is further supported by a suite of morphological characteristics, including opposite and decussate leaves, parietal placentation, and circumscissile fruit dehiscence, that are also found in various members of the order but which collectively do not link it with any family in particular. The traditionally suggested close relationship between *Rafflesia* and *Mitrastema* is not supported by our data and probably reflects a taxonomic emphasis on their similar endoparasitic lifestyle. In fact, the floral morphology of *Rafflesia* is very different from *Mitrastema*, which has small (≈2.54 cm in diameter), white, bisexual flowers, a dehiscent staminal tube, and a superior ovary (Fig. 1). Because *Mitrastema* is no more divergent in floral morphology from *Rafflesia* than are any of the other traditional Rafflesiaceae genera, *Cytinus*, *Bdallophyton*, *Apodanthes*, and *Pilostyles,* it is possible that they represent independent parasitic lineages as well and should be targeted for future studies using mtDNA sequences.

Holoparasite mtDNA Evolution. The use of mt sequences to study parasitic plant phylogeny seems promising because the holoparasitic angiosperm family Hydnoraceae was recently placed by using a combination of mtDNA with plastid and nuclear sequences (51). One reason for the utility of mtDNA for studying the phylogeny of holoparasites is that the evolutionary dynamics governing these sequences seem to be similar in parasitic plants and their free-living relatives. Fig. 2*A* shows reconstructed branch lengths from one of the equal-length trees obtained in the parsimony analysis of *matR*. It is clear that branch lengths are heterogeneous across angiosperms in general and that numerous photosynthetic plants are as divergent as *Rafflesia* or nearly so, yet all are confidently placed [except *Ceratophyllum*, which has defied confident placement to date (11)]. Furthermore, a likelihood ratio test (52) was performed to compare the level of selective constraint (estimated by the nonsynonymous/synonymous rate ratio, d_N/d_S) on *matR* in the holoparasitic lineages (*Rafflesia*, *Rhizanthes*, and *Mitrastema*) and photosynthetic lineages. Although the estimated d_N/d_S was higher for the holoparasitic lineages (1.0646) than for the photosynthetic lineages (0.7010), this difference was not statistically significant ($P > 0.05$). Thus, although some holoparasitic plants show elevated substitution rates and significant decreases in selective constraint for chloroplast genes such as *rbcL* and *rps2* compared with their photosynthetic relatives (5, 6), *matR* has likely been maintained for efficient mt functioning even in parasitic plants.

Problems with Holoparasite 18S Nuclear DNA. In addition to mtDNA, nuclear sequence data could provide an important independent genomic estimate of holoparasitic relationships as well. However, the 18S nuclear ribosomal DNA and other loci involved in protein translation have been problematic in studying other extreme parasites like microsporidia (2), and these genes are unlikely to provide useful data for *Rafflesia* or most other plant holoparasites. To illustrate this point for *Rafflesia* (*Mitrastema* has not yet been sequenced), we have obtained 70 nuclear 18S DNA sequences from GenBank for taxa that are also represented in our *matR* data set. Table 2, which is published as supporting information on the PNAS web site, lists all species names and GenBank accession numbers. Fig. 2*B* shows branch lengths for the 18S sequence data on a tree that was constrained to represent the currently accepted estimate of angiosperm relationships (10, 11). Assuming a placement of *Rafflesia* as sister to the Malpighiales, it is clear that this holoparasite has a highly divergent 18S sequence that is 4 times longer (197 steps) than the next longest sequence in the data set (the branch separating angiosperms and gymnosperms; 49 steps), and it is 6–8 times longer than most other branches. As expected with long-branch attraction, *Rafflesia* is placed with the other longest branch in the data set in an unconstrained parsimony analysis (Fig. 2*C*). This result corroborates an earlier study of 18S that found *Rafflesia* artifactually placed as sister to angiosperms and indicates that this gene is of limited utility for studying this divergent holoparasite (18). Even phylogenetic analyses of the slowly evolving 16S plastid ribosomal DNA clearly show extreme divergences in holoparasites (4), indicating that this gene will also be of limited utility in studying parasitic plants like *Rafflesia*. Classes of nuclear gene sequences other than those involved in translation, such as loci involved in primary metabolism, may instead provide better candidates for studying holoparasite relationships (2). Future studies of nuclear genes from *Rafflesia* and other extreme holoparasitic plants are needed because no other loci besides the 18S ribosomal DNA have been sequenced.

Implications. The results of this study have clear implications for studies of angiosperm phylogeny and parasite evolution. First, we show that mtDNA sequences provide a third independent genomic estimate of angiosperm relationships useful for resolving a variety of phylogenetic questions among both anciently and more recently evolving lineages. Second, placement of several other parasitic plants [composing nearly half of the taxa that remain of uncertain positions at the ordinal level (11)] may now be possible using the approach adopted here. Third, the eventual phylogenetic placement of all parasitic plants within flowering plant phylogeny will enable detailed comparative studies aimed at inferring ancestral states that may have facilitated the repeated evolution of parasitism in angiosperms.

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