American Journal of Botany 89(11): 1809–1817. 2002.

MOLECULAR DATA PLACE HYDNORACEAE WITH ARISTOLOCHIACEAE[1]

DANIEL L. NICKRENT,[2] ALBERT BLARER,[3] YIN-LONG QIU,[4]
DOUGLAS E. SOLTIS,[5] PAMELA S. SOLTIS,[5] AND MICHAEL ZANIS[6]

[2]Department of Plant Biology and Center for Systematic Biology, Southern Illinois University, Carbondale, Illinois 62901-6509 USA;
[3]Institute of Systematic Botany, University of Zurich, Zollikerstrasse 107, 8008 Zurich, Switzerland;
[4]Department of Biology, Morrill Science Center, University of Massachusetts, Amherst, Massachusetts 01003-5810 USA;
[5]Department of Botany, University of Florida, Gainesville, Florida 32611-8526 USA; and
[6]Department of Biology, Washington State University, Pullman, Washington 99164-4236 USA

Utilization of molecular phylogenetic information over the past decade has resulted in clarification of the position of most angiosperms. In contrast, the position of the holoparasitic family Hydnoraceae has remained controversial. To address the question of phylogenetic position of Hydnoraceae among angiosperms, nuclear SSU and LSU rDNA and mitochondrial *atp1* and *matR* sequences were obtained for *Hydnora* and *Prosopanche*. These sequences were used in combined analyses that included the above four genes as well as chloroplast *rbcL* and *atpB* (these plastid genes are missing in Hydnoraceae and were hence coded as missing). Three data sets were analyzed using maximum parsimony: (1) three genes with 461 taxa; (2) five genes with 77 taxa; and (3) six genes with 38 taxa. Analyses of separate and combined data partitions support the monophyly of Hydnoraceae and the association of that clade with Aristolochiaceae sensu lato (s.l.) (including Lactoridaceae). The latter clade is sister to Piperaceae and Saururaceae. Despite over 11 kilobases (kb) of sequence data, relationships within Aristolochiaceae s.l. remain unresolved, thus it cannot yet be determined whether Aristolochiaceae, Hydnoraceae, and Lactoridaceae should be classified as distinct families. In contrast to most traditional classifications, molecular phylogenetic analyses do not suggest a close relationship between Hydnoraceae and Rafflesiaceae. A number of morphological features is shared by Hydnoraceae and Aristolochiaceae; however, a more resolved phylogeny is required to determine whether these represent synapomorphies or independent acquisitions.

Key words: *atp1*; *atpB*; *Hydnora*; Lactoridaceae; matR; *Prosopanche*; *rbcL*; ribosomal DNA.

Molecular phylogenetic methods employing multiple genes in combination have been used to address relationships among all angiosperms (Savolainen, Chase, and Qiu, 2000; Soltis et al., 2000), basal members of this clade (Parkinson, Adams, and Palmer, 1999; Qiu et al., 1999, 2000), or the eudicots (Hoot, Magallón, and Crane, 1999). These data have been instrumental in reshaping current thought about higher-level relationships and indeed have provided the impetus to construct a revised classification of the flowering plants (APG, 1998). In the APG classification, 27 families were listed as ''position uncertain,'' and among these six were holoparasite families whose evolutionary relationships to other angiosperms have long been controversial: Balanophoraceae sensu lato (s.l.), Rafflesiaceae s.l., and Hydnoraceae. The phylogenetic position of the latter family within angiosperms has been the source of much disagreement. Cronquist (1988) placed Hydnoraceae in Rafflesiales, an order thought to be related to Santalales of subclass Rosidae. In the system of Takhtajan (1997), the family was placed in its own order, allied with Rafflesiales in Rafflesianae of subclass Magnoliidae. The latter placement was more in accord with traditional systems that placed these orders with Aristolochiales. Molecular phylogenetic analyses of angiosperms have consistently documented the existence of a eudicot clade with tricolpate pollen and a grade composed of taxa with mainly monosulcate pollen (Chase et al., 1993; Soltis et al., 2000). The presence of monosulcate pollen in *Hydnora* represented an inconsistency in those classifications that placed Hydnoraceae among the eudicots (Cronquist, 1981) as opposed to the magnoliids (Thorne, 1992; Takhtajan, 1997). In this paper we use the results of our molecular phylogenetic analyses to address this long-standing dilemma and explore the reasons for conflict among previous classifications.

Hydnoraceae contains only two genera: *Hydnora*, with approximately five species from Africa, the Arabian Peninsula, and Madagascar (Musselman and Visser, 1989), and *Prosopanche*, with two species from South and Central America (Cocucci, 1965). Hydnoraceae are quite distinctive; indeed *Hydnora* has been called the ''strangest plant in the world'' (Musselman and Visser, 1986). This epithet is deserved given the highly modified vegetative and floral morphology of these plants. Hydnoraceae are the only angiosperms known that lack leaves (or modifications such as scales). Two types of roots exist in *Hydnora*: horizontal rhizome-like ''pilot roots'' that are hexagonal in cross section and vermiform outgrowths from the ridges of the pilot roots called ''haustorial roots,'' whose function is to attach to the host (Fig. 1).

In *Hydnora*, flower buds often arise from the roots at the point where a haustorial connection to the host has been established. The epigynous flowers are composed of three or four fleshy, valvate tepals that fuse with the staminal filaments to form a tepalostemon (perianth tube). Some species, such as *H. africana* and *H. johannis*, produce their flowers at or above ground, whereas *H. triceps* flowers are subterranean. The inner

[1] Manuscript received 12 March 2002; revision accepted 4 June 2002.

The authors thank those individuals who have contributed plant material that was the source for DNA used in this study, in particular, S. Carlquist for donating samples of *Hydnora africana* and L. Musselman for providing samples of *Prosopanche americana*. Help in translating German to English was provided by Larry Walker and Vanessa Ashworth. The illustration in Fig. 1 was prepared by John Myers. Financial support for this research was obtained from the National Science Foundation (MCB-9808752 and DEB-9407984 to DLN).

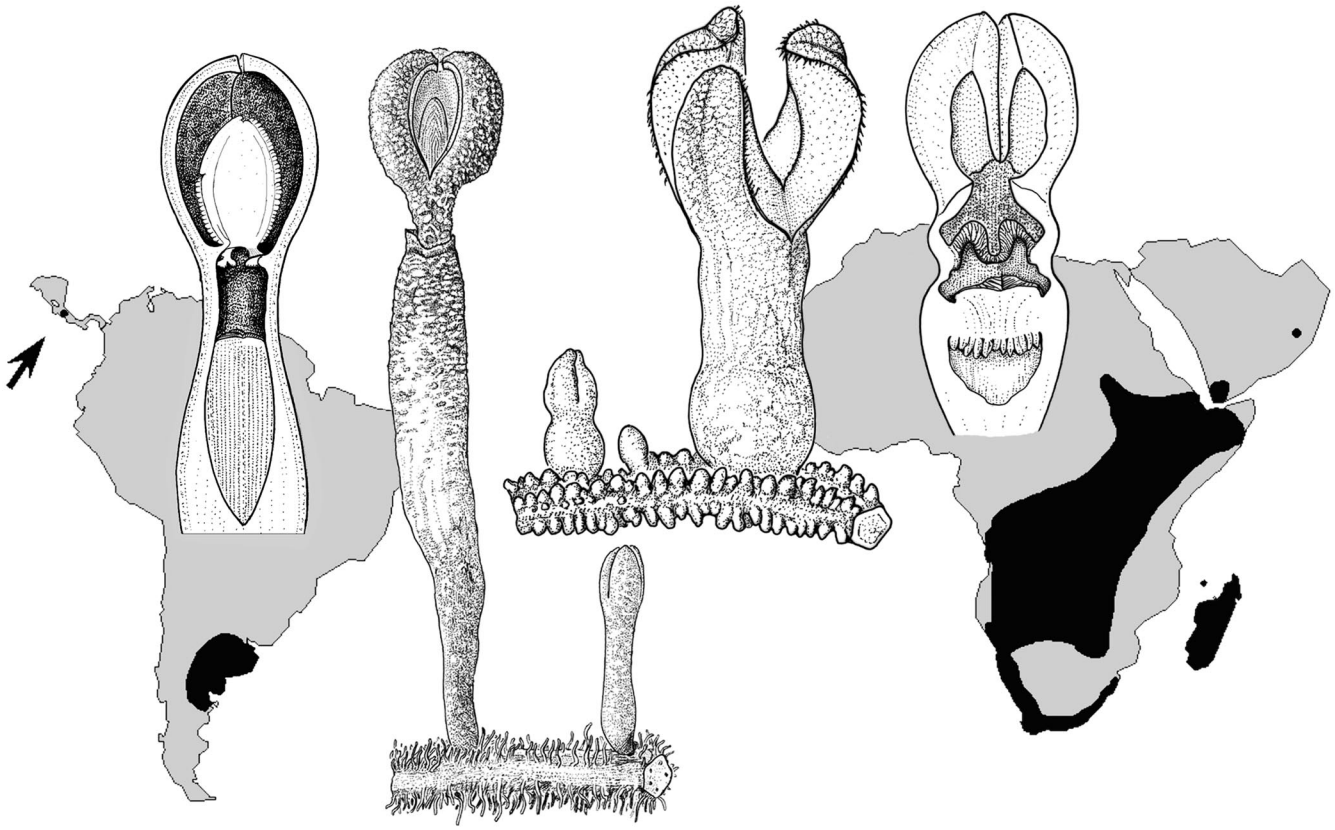

Fig. 1. General morphology of *Prosopanche americana* (left) and *Hydnora africana* (right) with flower longitudinal sections for each. See text for discussion of the floral features. The distributions of the genera are shown in black. Note that the disjunct taxon in Costa Rica was originally named *P. costaricensis* but has since been placed in synonymy with *P. americana* (Coccuci and Coccuci, 1996).

margins of the perianth lobes in some species (e.g., *H. africana*) have ''bait bodies'' (Köderkörper) that emit a strong smell and attract pollinators, such as dermestid beetles or carrion flies. In other species (e.g., *H. johannis*) the perianth is tipped by a ''cucullus region'' that is osmophoric. The ovary has intrusive parietal placentation and develops into a berry.

Vegetative and floral features of *Prosopanche* are unmistakenly similar to *Hydnora*; however, some differences exist. The androecium is fused into a dome-like structure, and staminodes are present as the second (innermost) whorl (Cocucci, 1975). Only the upper flower parts (those above the ovary) emerge above the soil where they are then visited by a number of pollinators including small nitidulid beetles that are attracted by a pineapple-like smell. These beetles mate within the protogynous flower, which has a temperature elevated over ambient. The fruit begins development underground and with further development splits irregularly and circumscissally, exposing the pleasant-smelling endocarp. The fruit and numerous seeds are likely dispersed by nocturnal mammals (Cocucci and Cocucci, 1996).

The biogeographic pattern of Hydnoraceae strongly suggests a Gondwanan distribution, although it might also be explained by long-distance dispersal. Given the holoparasitic nature of the plants and their host requirements, vicariance is a more likely explanation. *Prosopanche americana* frequently parasitizes legumes (principally *Prosopis*), whereas *P. bonacinae* occurs on host plants representing at least nine other families. *Hydnora* species occur mainly on hosts in Euphorbiaceae (*H. africana* and *H. triceps*) and *Acacia* (*H. esculenta* and *H. johannis*).

As discussed in Nickrent et al. (1998), holoparasitic plants present unique problems for those interested in inferring their phylogeny. As typically accompanies the holoparasitic habit, Hydnoraceae have highly modified or reduced vegetative and floral features. These modifications and losses prevent comparison to characters present in more conventional (photosynthetic) plants, thus impeding phylogenetic analysis based on morphology. A similar phenomenon may also occur at the molecular level where gene losses and rate accelerations have been documented for all three subcellular genomes in some holoparasites (Nickrent and Starr, 1994; Nickrent et al., 1998). When such divergent sequences are analyzed along with less divergent ones, long-branch artifacts (Felsenstein, 1978) can occur, thus casting doubt upon the inferred phylogeny. Despite such difficulties, substitution rates among such holoparasites are heterogeneous, thus those groups with relatively lower rates are less likely to result in artifactual relationships. Relative rates tests involving *Prosopanche* were not significantly different (less than two standard errors) than comparisons using nonparasitic plants (Nickrent and Starr, 1994). Preliminary analyses using nuclear small-subunit (18S) rDNA sequences for over 200 angiosperms placed *Hydnora* and *Prosopanche* near Aristolochiaceae (Nickrent and Duff, 1996; Nickrent et al., 1998), thus supporting traditional classifications. Because this analysis did not go to completion, and because only a single gene was used, we wished to confirm this result with

additional (independent) data. We reasoned that if the results obtained from genes derived from separate subcellular compartments were congruent, it would be more likely that the actual organismal phylogeny is being detected.

We generated sequence data from nuclear (SSU and LSU rDNA) and mitochondrial (*atp1* and *matR*) genes for *Hydnora* and *Prosopanche*. Two chloroplast genes (*rbcL* and *atpB*) were also included from photosynthetic angiosperms but were not included for Hydnoraceae because these genes appear to be absent in these plants. This was inferred from negative results of numerous polymerase chain reaction (PCR)-based experiments (D. L. Nickrent, personal observation). Three different data sets were constructed with differing taxon density and gene sampling. From these data sets, our major objective was to determine the position of Hydnoraceae within the global angiosperm phylogeny.

MATERIALS AND METHODS

Taxon sampling—The majority of taxa sampled for this study was derived from the five-gene analysis reported by Qiu et al. (1999, 2000) and the three-gene analysis of Soltis et al. (2000). Large subunit rDNA sequences on nonparasites are reported in Zanis et al. (2002). Previously unpublished sequences include *Hydnora africana* large-subunit rDNA (GBAN-AF503353 and GBAN-AF503354), *atp1* (GBAN-AF503356), *matR* (GBAN-AF503358), and *Prosopanche americana* large-subunit rDNA (GBAN-AF503355), *atp1* (GBAN-AF503357) and *matR* (GBAN-AF503359). (The prefix GBAN- has been added to link the online version of the American Journal of Botany to GenBank but is not part of the actual accession number.) Placeholders were used in five instances such that no missing data were incorporated into the matrix other than *rbcL* and *atpB* for *Hydnora* and *Prosopanche*. The generic placeholders were *Menispermum* + *Cissampelos* (Menispermaceae), *Brassica* + *Arabidopsis* (Brassicaceae), *Pisum* + *Vicia* (Fabaceae), *Placospermum* + *Persoonia* (Proteaceae), and *Oryza* + *Triticum* (Poaceae). For the 77 taxon data set, 77 genera in 48 familes were included. Accession information including GenBank numbers have been archived on the *American Journal of Botany* Supplementary Data website, http://www.ajbsupp.botany.org/v89/.

Molecular and analytical methods—The protocols used here for extracting genomic DNA, PCR amplification, cloning, and sequencing have been reported (Qiu et al., 1993; Nickrent, 1994; Kuzoff et al., 1998; Soltis et al., 2000). Both manual and automated DNA sequencing methods were used. All alignments were conducted by eye. For chloroplast protein-coding genes (*rbcL* and *atpB*), alignment was straightforward given the lack of length variation, hence all positions were included. Alignment of the mitochondrial protein-coding genes (*atp1* and *matR*) was guided by the use of translated sequences. For *atp1*, 19 sites in two regions and for *matR* 278 sites in 19 regions were removed owing to ambiguous alignment using the exclude characters command in PAUP* (Swofford, 2001). In most cases the removed regions represented sequence that was autapomorphic for the outgroup taxa. For SSU rDNA, two regions (positions 190–199 and 1340–1342) were removed and for LSU rDNA 13 regions totalling 156 positions were removed. These alignments are available on the *American Journal of Botany* Supplementary Data website.

To address the question of phylogenetic position of Hydnoraceae within angiosperms, nuclear SSU rDNA sequences were obtained for *Hydnora* and *Prosopanche* and analyzed using three data sets differing in taxon density and genes. The three-gene data set included nuclear SSU rDNA (1792 sites), plastid *rbcL* (1403 sites), and *atpB* (1440 sites) for 461 taxa totaling 4635 sites. *Hydnora* and *Prosopanche* lack the plastid genes *rbcL* and *atpB*, hence these sites were coded as missing in these two taxa. The intention here is to stabilize the overall tree topology by adding these plastid genes. The position of Hydnoraceae will then be determined by the data that are present (nuclear rDNA). As compared with the next two, this data set has the broadest sampling within angiosperms, particularly eudicots.

The five-gene data set included nuclear SSU rDNA (1737 sites), plastid *rbcL* (1399 sites), *atpB* (1498 sites), *atp1* (1285 sites), and *matR* (2307 sites) for 77 taxa totaling 8226 sites. The strategy here is to include more sequence data to further stabilize angiosperm relationships. Taxon density is lower than the three-gene data set; however, good representation of magnoliids and eudicots was achieved. This data set contained both nuclear and mitochondrial sequences for Hydnoraceae, thus more than one gene was influencing the position of the family in the analysis. The inclusion of mitochondrial data also allowed tests to be made of separate process partitions to determine whether the same signal is being received from gene sequences derived from distinct subcellular compartments.

The six-gene data set included nuclear SSU (18S) rDNA (1661 sites), LSU (26S) rDNA (3469 sites), plastid *rbcL* (1398 sites), *atpB* (1497 sites), *atp1* (1284 sites), and *matR* (2189 sites) for 38 taxa totaling 11 528 sites. Taxon density is lower in this data set because many LSU rDNA sequences were not available (particularly the eudicots). This data set was used to examine the effect on tree topology with increased sequence data. It also allowed tests to be made to determine whether results from the two nuclear partitions are congruent.

All analyses of the above three data sets were conducted using PAUP* 4.0 (Swofford, 2001). Maximum parsimony with heuristic searches, tree bisection-reconnection (TBR) branch swapping, and bootstrap analyses (100 replications) was used. For the 461-taxon three-gene analysis, five searches, each with 100 initial replications of NNI saving five trees per replication, were conducted. The shortest trees were then used as starting trees for subsequent searches with TBR. These TBR searches were allowed to run for 5–7 d. Previous congruence analyses as well as the similar tree topologies obtained for individual genes (reviewed in Soltis et al., 2000) provide justification for combining the six genes used in this study into a single data set.

RESULTS

The results of the maximum parsimony analyses of the three-, five-, and six-gene data sets are presented below.

Three-gene analysis—Each of the five searches of the 461 taxon matrix resulted in more than 4000 trees, with tree lengths ranging from 37 026 to 37 051 (trees not presented here). Four searches placed Hydnoraceae within Aristolochiaceae s.l., and one placed it as sister to Saururaceae/Piperaceae.

Five-gene analysis—Heuristic searches of the five-gene data set recovered 24 shortest trees of length 10 951. These 24 trees occurred in one island found in 693 of 1000 random taxon addition replications. The single other island of six trees one step longer was hit 307 times. The strict consensus of these trees is nearly identical in topology to the bootstrap (BS) consensus, differing only in the position of Chloranthales (sister to monocots in the strict consensus, part of a polytomy in the BS tree). As shown by the BS consensus tree (Fig. 2), 54 of the 66 resolved nodes (five polytomies exist) received BS of 90% or greater. Forty-four percent and 40% of the informative characters derive from the chloroplast and the mitochondrial gene partitions, respectively (Table 1). The consistency index (CI) values are consistently higher for mitochondrial genes than for the nuclear or chloroplast genes. This trend (higher mitochondrial partition) can also be seen in the number of resolved nodes with BS values of 90% or greater. The first branching taxon is *Amborella* followed by Nymphaeales and a clade composed of Illiciales and *Austrobaileya*. Four clades comprising the eumagnoliids emerge next from a polytomy: (1) Chloranthales, (2) monocots plus *Ceratophyllum*, (3) eudicots, and (4) a clade composed of Laurales, Magnoliales, Winterales, and Piperales. Bootstrap support for the latter clade

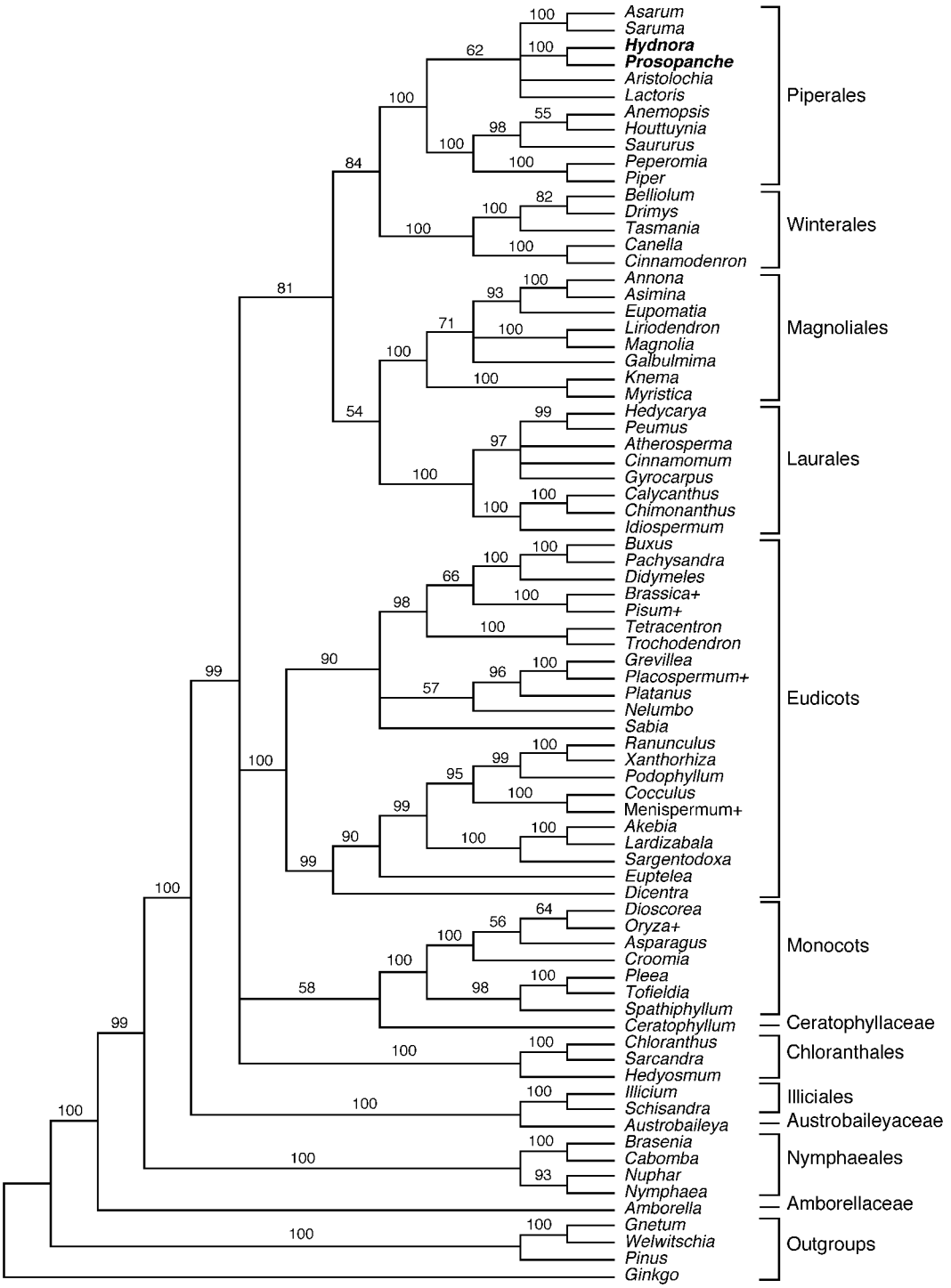

Fig. 2. Bootstrap consensus tree obtained from maximum parsimony analysis of the five-gene, 77-taxon data matrix containing 2166 informative sites. This tree is essentially identical to the strict consensus of 24 equally parsimonious trees (length of 10 951, consistency index minus uninformative sites 0.3555). Taxa with "+" contain placeholders (see MATERIALS AND METHODS).

was 81% and was 100% for the eudicots. Piperales and Winterales were resolved as sister (84% BS) and within Piperales, two clades occur: Piperaceae plus Saururaceae and Aristolochiaceae s.l. The latter family includes genera traditionally placed in Aristolochiaceae (*Aristolochia*, *Asarum*, and *Saruma*) as well as *Lactoris* (Lactoridaceae) and *Hydnora* plus *Prosopanche* (Hydnoraceae). Relationships within Aristolochiaceae s.l. are not fully resolved in this analysis. *Hydnora* is strongly supported as sister to *Prosopanche*, as is *Saruma* with *Asarum*; however, relationships within Aristolochiaceae s.l. are poorly resolved.

When any of the 24 shortest trees from the MP analysis of

TABLE 1. Comparison of 77-taxon and 39-taxon maximum parsimony tree statistics.

	Total characters	Constant	Variable uninformative	Variable informative (percentage of gene)	Variable informative (percentage of all genes)	CI	No. nodes >90%/ no. resolved nodes
77 taxa							
Nuclear only (SSU)	1730	1230	174	326 (18.5)	15.0	0.316	20/66
Mitochondrial only	3231	1752	604	875 (27.0)	40.4	0.477	34/66
Chloroplast only[a]	2897	1561	371	965 (33.3)	44.6	0.307	26/66
All genes	7858	4543	1149	2166 (27.5)	100.0	0.355	40/68
39 taxa							
Nuclear only	4998	3409	553	1036 (20.7)	43.4	0.350	11/35
Mitochondrial only	3186	1921	650	615 (19.3)	25.7	0.507	14/35
Chloroplast only*	2895	1768	389	738 (25.5)	30.9	0.368	12/28
All genes	11 079	7098	1592	2389 (21.5)	100.0	0.378	22/34

[a] No *Hydnora* or *Prosopanche* sequences.

the five-gene data are examined, branches leading to holoparasites are not significantly longer than those of nonparasite clades such as Brassicaceae, Fabaceae, *Peperomia*, and Poaceae. Thus, it does not appear that long-branch attraction is affecting the topology of the tree, in agreement with previous analyses that quantified relative rates of substitution in Hydnoraceae and outgroups (Nickrent and Starr, 1994). In contrast, distance analyses (using any of the substitution models available in PAUP* with neighbor-joining or unweighted pair group methods with arithmetic averaging) *do not* provide strong support for the inclusion of Hydnoraceae within Aristolochiaceae. Here, Hydnoraceae most frequently associate with Fabaceae and Brassicaceae, both of which exhibit long branches.

Six-gene analysis—Maximum parsimony heuristic searches of the six-gene data set resulted in four most-parsimonious trees of length 11 640. These four trees occurred in one island found in 850 of 1000 random taxon addition replications. Four other islands (seven trees) of longer trees were hit a total of 150 times. The strict consensus of the four shortest trees is nearly identical to the BS consensus tree that is shown in Fig. 3 (the main difference between them being the position of Chloranthaceae). Although taxon sampling is more limited compared with the 77-taxon data set, representatives of all the orders are present, and the topology of this tree is similar to the consensus of five-gene analysis. Of the 33 resolved nodes present on this tree, 21 received BS support of 90% or greater. The nodes that have lower BS support are clustered mainly

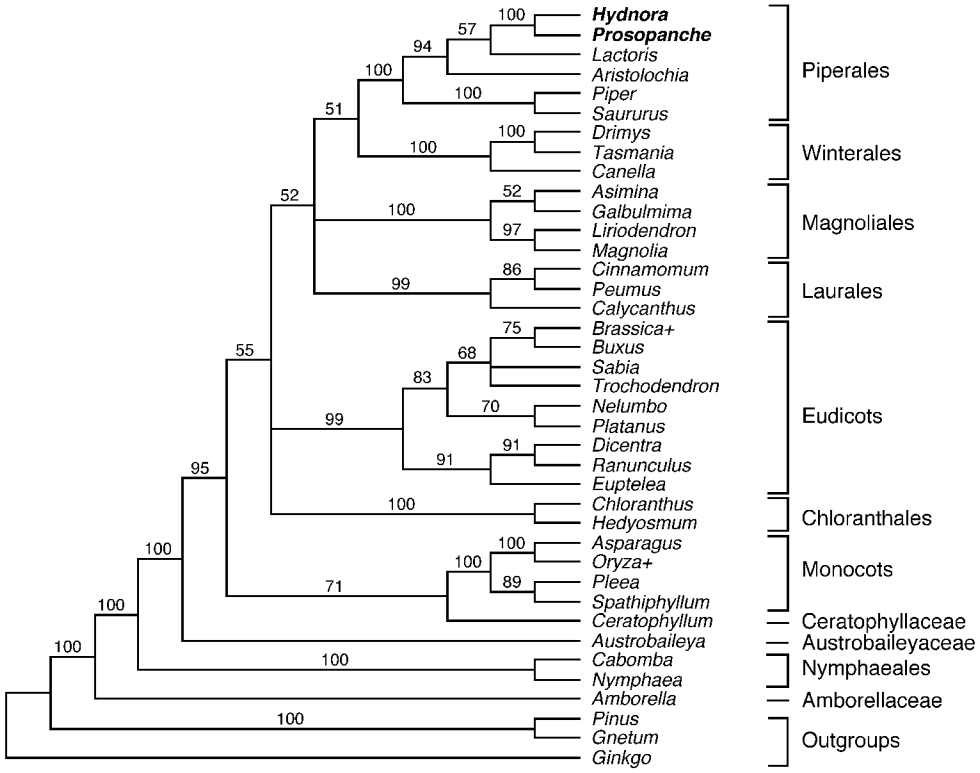

Fig. 3. Bootstrap consensus tree obtained from maximum parsimony analysis of the six-gene, 39-taxon data matrix containing 2389 informative sites. This tree is similar to the strict consensus of four equally parsimonious trees (length = 11 640, consistency index minus uninformative sites = 0.4779) except for the position of Chloranthaceae. Taxa with "+" contain placeholders (see MATERIALS AND METHODS).

within the eudicots and along the ''spine'' of the tree that represents the eumagnoliid clades. It is of interest that these nodes receive low BS support when any of the separate partitions are analyzed separately (trees not shown). Of the total 2389 informative characters, 43% derive from the nuclear rDNA partition, but nearly 5000 base pairs (bp) of sequence was collected to obtain these characters (Table 1). As a percentage of the partition length, the chloroplast genes have more informative characters than the other two partitions (25.5%). As with the 77-taxon data set, analysis of the mitochondrial partition results in more resolved nodes with BS values of 90% or greater (14 of 35). Also agreeing with the 77-taxon analysis, Piperales are strongly monophyletic (100% BS) and are sister to Winterales (but here with lower BS support). Within Piperales, Hydnoraceae still emerge from a paraphyletic Aristolochiaceae s.l. that also includes *Lactoris*. Support for this clade is high (94% BS), an increase apparently attributable to the inclusion of LSU rDNA data.

DISCUSSION

The results of analyses of the three-, five-, and six-gene data sets, as well as analyses of separate genomic partitions, all place Hydnoraceae within Aristolochiaceae s.l. (including Lactoridaceae). The monophyly of this expanded family received moderate to low support with the five-gene analysis but gained strong support when additional sequence data from the nuclear partition is included. The overall topology of the 77-taxon tree (Fig. 2) is similar to the 105-species five-gene tree reported by Qiu et al. (2000). In that study, two clades of Aristolochiaceae sensu stricto (s.s.) were resolved at 100% BS: *Aristolochia* + *Thottea* and *Asarum* + *Saruma* clade (*Hydnora* or *Prosopanche* were not sampled). In 1 of the 18 shortest trees, *Lactoris* was shown as sister to the former clade, but with low BS support (58%). In a morphological cladistic study of basal angiosperms (Doyle and Endress, 2000), the following characters were identified as unambiguously changing on the node giving rise to Aristolochioideae (*Aristolochia*), Asaroideae (*Saruma*, *Asarum*), and *Lactoris*: inflorescence a solitary flower (or occasionally with one or two additional flowers) and dry, indehiscent fruits. Aside from the cymose inflorescence, González and Rudall (2001) pointed out the general lack of morphological features linking *Lactoris* to Aristolochiaceae. The features cited by Endress (1994) supporting a close relationship between the families were considered symplesiomorphies for the entire order Piperales by these authors. The molecular data presented here, specifically the six-gene analysis, provide further indication that Aristolochiaceae s.l. are monophyletic and that *Hydnora*, *Prosopanche*, and *Lactoris* are components of this expanded family.

Affinities of Hydnoraceae: historical review and perspectives—First described as a fungus nearly 230 yr ago, *Hydnora* was discovered by Thunberg (1775), a student of Linnaeus. The family name (Hydnorinae) of Agardh (1821) was conserved in the Montreal Code. The relationships between Hydnoraceae and Aristolochiaceae, particularly *Thottea*, were discussed by Meyer (1833). During the same approximate time period (1818), *Rafflesia* was discovered in Sumatra (Brown, 1822), but an explicit relationship between Hydnoraceae and Rafflesiaceae was not suggested until 1844 (Brown, 1844). During the 1860s, *Prosopanche* was first discovered in South America (De Bary, 1868), and later additional species of *Hydnora* were described from Africa (Beccari, 1871; Decaisne, 1873). The association of Hydnoraceae with Rafflesiaceae likely stems mainly from the fact that both are parasites with flesh-colored flowers, although the number of shared morphological features is certainly limited. The association between Hydnoraceae, Rafflesiaceae (or Cytinaceae), and Aristolochiaceae continued through the latter part of the 19th century, and some authors, such as Baillon (1886), actually classified Hydnoraceae as a tribe of Aristolochiaceae. The treatment of Hydnoraceae in *Pflanzenfamilien* by Solms-Laubach (1894, p. 285) considered the evidence favoring the two competing concepts as illustrated by the following translation:

> What was previously said in the case of Rafflesiaceae is also true here. Most authors characterize *Hydnora* as a branch of Rafflesiaceae. Contradicting this is the construction of the androecium and of the fruit. In addition, there is the completely divergent structure of the seed, the presence of a perisperm, as well as the rough composition of the cellulose walls in the 'nutrient tissue' (Nährgewebe), as well as many other significantly divergent aspects. Based on the entire construction of the flower, one could definitely make a case for directly classifying it as Aristolochiaceae, even though this classification does not explain away the differences in the construction of the seed.

By the turn of the century and into the 1920s, additional species of *Hydnora* (Jumelle and Perrier de la Bâthie, 1912) and *Prosopanche* (Chodat, 1915) were described and anatomical studies were published for the ovules and seeds (Tieghem, 1897; Dastur, 1921). In a later version of *Pflanzenfamilien*, Harms (1935, p. 288) wrote the following about familial relationships:

> The family was usually attached to the Tribe Rafflesiaceae (for example of R. Brown) or Cytinaceae. However, the construction of the androecium and gynoecium contradicts this. Additional substantial differentiating features are the different construction of the seed, the presence of a perisperm and the deposit of the reserve materials in the cellulose walls (Solms Laubach). E. Meyer had thoroughly discussed the relationships to Aristolochiaceae (especially *Thottea*); and according to Solms-Laubach as well, the characteristics of the seed construction speak against such a classification, that Baillon, for example (among others), had posited.

It is curious that while paraphrasing Solms-Laubach, Harms focused upon the evidence against a relationship between Hydnoraceae and Aristolochiaceae, even though the original statement (above) appears to have considered this as a viable possibility.

A series of detailed studies of *Prosopanche* was published by Cocucci (1965, 1975, 1976) that treated the taxonomy, floral morphology, and anatomy of the genus. A tendency toward fusion of the stamens and gynoecium as well as embryological features (such as the presence of unitegmic ovules) prompted Coccuci and Cocucci (1996) to propose a relationship between Hydnoraceae and Mitrastemonaceae (Rafflesiales). This scheme showed *Hydnora* and then *Prosopanche* being derived from *Mitrastemon*, which was itself derived from Annonaceae. In parallel, *Aristolochia* (via *Thottea*) and *Rhizanthes* (via *Pilostyles*) were derived from an anonaceous ancestor. Although Aristolochiaceae were peripherally involved, the family was not proposed to be a close relative of Hydnoraceae.

Cronquist (1981) placed Hydnoraceae in Rafflesiales and then allied this order with Santalales (Rosidae). Although he acknowledged that the family had traditionally been associated

TABLE 2. Comparison of Aristolochiaceae and Hydnoraceae.

Character	Aristolochiaceae character states	Hydnoraceae character states
Pollination	Entomophilous (various)	Beetles and blowflies
Flower sex	Bisexual	Bisexual
Flower symmetry	Actinomorphic and zygomorphic	Actinomorphic
Flower merosity	Three	Three to four
Perianth	Mono- and dichlamydous	Monochlamydous
Calyx	Synsepalous, tubular	Synsepalous, tubular
Perianth insertion	Epigynous (Aristolochioideae) and hemi-epigynous (Asaroideae)	Epigynous
Adnation of A and G	Gynostemium in some	Tepalostemon fused to G
Anther dehiscence	Extrorse, longitudinal	Extrorse, longitudinal
Tapetum	Secretory	Secretory
Pollen	Monosulcate, polyporate, polycolpate	Monosulcate (*Hydnora*), 2 to 3-porate or trichotomocolpate (*Prosopanche*)
Carpel number	Four to six	Three to four
Placentation	Parietal and axile	Parietal
Endosperm development	Cellular	Cellular
Embryo	Minute, undifferentiated	Minute, undifferentiated

with Aristolochiaceae (based on perianth features), he considered the groups distinct and stated, ''In my opinion, the Rafflesiales are singularly misplaced in the Aristolochiales.'' The presence of monosulcate pollen in *Hydnora*, however, presented a complication because nearly every other angiosperm with this condition was classified in Magnoliidae. Cronquist escaped this dilemma by proposing that the monosulcate pollen in *Hydnora* represented a reversion to a more primitive type, paralleling other simplification trends seen when plants adopt the parasitic habit. Molecular phylogenetic analyses using nuclear and mitochondrial genes do not suggest a close relationship between Hydnoraceae and Rafflesiaceae; indeed, the most recent data suggest that Rafflesiaceae are a clade in the eudicots (Nickrent, 2002; A. Blarer et al., unpublished data).

The tendency to consider Hydnoraceae closely related to Rafflesiaceae also likely influenced the circumscription and scoring of character states in previous morphological cladistic analyses. Although both Aristolochiaceae and Hydnoraceae were included in the study by Dahlgren and Bremer (1985), the two did not form a clade. Instead, the latter was sister to Rafflesiaceae on a clade supported by seven characters. For one character (plants without chlorophyll), the states scored for the two holoparasites were not homologous and in others the range of character states present in the families was not adequately captured by the scoring. For example, pollen type for the two families was given as ''monosulcate, inaperturate or ulcerate'' despite the presence of colporate or porate pollen in *Cytinus* (Takhtajan et al., 1985) and two- to three-porate pollen in *Prosopanche* (see Table 2). The presence of monosulcate pollen in *Hydnora* compelled Takhtajan (1997) to follow a more traditional classification and place Hydnoraceae among the magnoliids. With reference to Hydnorales, he states, ''Together with the next order, Rafflesiales are related to the Aristolochiaceae, especially to the Asaroideae, and have probably originated directly from their immediate ancestors.'' This placement is in agreement with molecular data with respect to Hydnoraceae, but again it has not escaped the pitfall of associating this family with Rafflesiaceae.

Classification of Piperales and Aristolochiaceae s.l.—Two strongly supported clades emerged from these analyses within the order Piperales: (1) Piperaceae plus Saururaceae and (2) Aristolochiaceae s.l. Recovery of the latter clade agrees with previous results from nuclear SSU rDNA (Nickrent and Duff, 1996; Nickrent et al., 1998) but bootstrap support is higher, particularly with the six-gene analysis. Here, Aristolochiaceae s.l. includes the shrub *Lactoris* (Lactoridaceae), Aristolochiaceae s.s., and Hydnoraceae. Given these results, the two clades will here be discussed Piperaceae + Saururaceae and Aristolochiaceae s.l.

Despite the use of over 11 kb of sequence data derived from genes representing all three subcellular genomes, relationships within Aristolochiaceae s.l. are still unresolved. Thus, additional (molecular) data will be required to provide greater resolution of these relationships. Three possible tree topologies could emerge that would allow the three component families to remain monophyletic: (Hydnoraceae (Aristolochiaceae, Lactoridaceae)), (Aristolochiaceae (Hydnoraceae, Lactoridaceae)), or (Lactoridaceae (Hydnoraceae, Aristolochiaceae)). Given that it is unlikely that the monophyly of Hydnoraceae will be disrupted by any additional data, and given that *Lactoris* is monospecific, the other possible topologies that could result from further analysis would all involve paraphyly of Aristolochiaceae. Without additional data, it is premature to propose a reclassification of Aristolochiaceae s.l. based on phylogenetic principles.

The inclusion of Hydnoraceae in Aristolochiaceae s.l. adds yet another dimension to an already morphologically diverse order. Parasitism in Hydnoraceae represents one out of approximately ten independent evolutionary events that lead to this nutrition mode in flowering plants. The possible tree topologies discussed above gain additional interest when framed around the question of the origin of parasitism in Aristolochiales.

It is likely that overall flower morphology (flesh-colored tubular flowers) suggested to early workers an association between Hydnoraceae and Aristolochiaceae. There are a number of morphological features that are potential synapomorphies between these two families (Table 2). Characters that are compatible with both families (but not representing the entire range) are: entomophily; bisexual, epigynous, three-merous, monochlamydous, synsepalous, flowers; anther dehiscence extrorse and longitudinal with a secretory tapetum; pollen mon-

osulcate; placentation parietal; endosperm development cellular; and embryo minute and undifferentiated. Ovary position varies in Aristolochiaceae, and it has been suggested that the epigynous condition in *Asarum* can be reversed (Kelly, 1997). For pollen features, both monosulcate and inaperturate types occur in Aristlochiaceae and *Lactoris*, and pollen morphology is equally diverse in Hydnoraceae (Table 2). Moreover, during the course of evolution of holoparasitic angiosperms, morphological structures are often lost, reduced, or convergent. For these reasons, it is prudent to not engage in excessive speculation as to the homology of the morphological features that remain (e.g., epigynous perianth insertion, monochlamydous perianths, fusion of androecium and gynoecium, etc.) until more detailed analyses of the genetic basis for such characters are conducted.

Conclusions—*Hydnora* and *Prosopanche* comprise a clade that is resolved as a component of Aristolochiaceae s.l. (including Lactoridaceae). This clade is sister to another composed of Saururaceae and Piperaceae, together comprising Piperales. Separate analyses of nuclear and mitochondrial gene partitions result in generally congruent topologies for the resulting shortest parsimony trees. Combinations of gene sequences gave higher bootstrap support for the Aristolochiaceae s.l. plus Hydnoraceae. Although several morphological similarities can be found between Aristolochiaceae and Hydnoraceae, determining whether such shared character states are homologous is difficult owing to high levels of morphological variation within the families. This study highlights the use of molecular data in placing morphologically derived and therefore phylogenetically problematic plant families as has been done with other groups such as Podostemaceae (Soltis et al., 1999), Hydrostachyaceae (Albach et al., 2001), Aphloiaceae, and Ixerbaceae (Soltis et al., 2000).

LITERATURE CITED

AGARDH, C. A. 1821. (Hydnorinae), nom. cons. *In* Aphorismi botanici, pp. 87–102. Literis Berlingianis, Lund, Germany.

ALBACH, D., D. SOLTIS, M. CHASE, AND P. SOLTIS. 2001. Phylogenetic placement of the enigmatic angiosperm Hydrostachys. *Taxon* 50: 781–805.

APG. 1998. An ordinal classification for the families of flowering plants. *Annals of the Missouri Botanical Garden* 85: 531–553.

BAILLON, H. E. 1886. Hydnoraceae trib. Aristolochiacearum. Librairie Hachette, Paris, France.

BECCARI, O. 1871. Descizione die due nuove specie d'*Hydnora* l'Abissinia. *Nuovo Giornale Botanico Italiano* 3: 6–7.

BROWN, R. 1822. An account of a new genus of plants, named *Rafflesia*. *Transactions of the Linnaean Society London* 13: 201–234.

BROWN, R. 1844. Description of the female flower and fruit of *Rafflesia arnoldi*, with remarks on its affinities; and an illustration of the structure of *Hydnora africana*. *Transactions of the Linnaean Society of London* 19: 221–238.

CHASE, M. W., ET AL. 1993. Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbc*L. *Annals of the Missouri Botanical Garden* 80: 528–580.

CHODAT, R. 1915. Les espèces du genre *Prosopanche*. *Bulletin de la Société Botanique de Genève* 7: 65–66.

COCUCCI, A. E. 1965. Estudios en el género *Prosopanche* (Hydnoraceae) I. Revisión taxonómica. *Kurtziana* 2: 53–74.

COCUCCI, A. E. 1975. Estudios en el género *Prosopanche* (Hydnoraceae) II. Organización de la flor. *Kurtziana* 8: 7–15.

COCUCCI, A. E. 1976. Estudios en el género *Prosopanche* (Hydnoraceae) III. Embriología. *Kurtziana* 9: 19–39.

COCUCCI, A. E., AND A. A. COCUCCI. 1996. *Prosopanche* (Hydnoraceae): somatic and reproductive structures, biology, systematics, phylogeny and potentialities as a parasitic weed. *In* M. T. Moreno, J. I. Cubero, D. Berner, D. Joel, L. J. Musselman, and C. Parker [eds.], Advances in parasitic plant research, 179–193. Junta de Andalucia, Dirección General de Investigación Agraria, Cordoba, Spain.

CRONQUIST, A. 1981. An integrated system of classification of flowering plants. Columbia University Press, New York, New York, USA.

CRONQUIST, A. 1988. The evolution and classification of flowering plants. New York Botanical Gardens, Bronx, New York, USA.

DAHLGREN, R., AND K. BREMER. 1985. Major clades of angiosperms. *Cladistics* 1: 349–368.

DASTUR, R. H. 1921. Notes on the development of the ovule, embryosac, and embryo of *Hydnora africana* Thunb. *Transactions of the Royal Society of South Africa* 10: 27–31.

DE BARY, A. 1868. *Prosopanche burmeisteri*, eine neue Hydnoree aus Süd-Amerika. *Abhandlungen der Naturforschende Gesellschaft zu Halle.* 10: 243–272.

DECAISNE, M. J. 1873. Note sur trois espèces d'*Hydnora*. *Bulletin de Societie Botanique Francois* 20: 75–77.

DOYLE, J. A., AND P. K. ENDRESS. 2000. Morphological phylogenetic analysis of basal angiosperms: comparison and combination with molecular data. *International Journal of Plant Sciences* 161(Supplement 6): S121–S153.

ENDRESS, P. K. 1994. Floral structure and evolution of primitive angiosperms: recent advances. *Plant Systematics and Evolution* 192: 79–97.

FELSENSTEIN, J. 1978. Cases in which parsimony or compatibility will be positively misleading. *Systematic Zoology* 27: 401–410.

GONZÁLEZ, F., AND P. RUDALL. 2001. The questionable affinities of *Lactoris*: evidence from branching pattern, inflorescence morphology, and stipule development. *American Journal of Botany* 88: 2143–2150.

HARMS, H. 1935. Rafflesiaceae, Hydnoraceae, and Balanophoraceae. *In* A. Engler and H. Harms [eds.], Die Natürlichen Planzenfamilien, 243–281, 282–295, 296–339. Duncker & Humblot, Leipzig, Germany.

HOOT, S. B., S. MAGALLÓN, AND P. R. CRANE. 1999. Phylogeny of basal eudicots based on three molecular data sets: *atpB*, *rbcL*, and 18S nuclear ribosomal DNA sequences. *Annals of the Missouri Botanical Garden* 86: 1–32.

JUMELLE, H., AND H. PERRIER DE LA BÂTHIE. 1912. Quelques phanérogames parasites de Madagascar. *Revue Générale de Botanique* 24: 321–328.

KELLY, L. M. 1997. A cladistic analysis of *Asarum* (Aristolochiaceae) and implications for the evolution of herkogamy. *American Journal of Botany* 84: 1752–1765.

KUZOFF, R. K., J. A. SWEERE, D. E. SOLTIS, P. S. SOLTIS, AND E. A. ZIMMER. 1998. The phylogenetic potential of entire 26S rDNA sequences in plants. *Molecular Biology and Evolution* 15: 251–263.

MEYER, E. H. F. 1833. De Hydnora. *Nova Acta Physico-medica Academiae Caesareae Leopoldino Carolinae Naturae Curiosorum* 16: 765.

MUSSELMAN, L. J., AND J. H. VISSER. 1986. The strangest plant in the world! *Veld and Flora* 71: 109–111.

MUSSELMAN, L. J., AND J. H. VISSER. 1989. Taxonomy and natural history of *Hydnora* (Hydnoraceae). *Aliso* 12: 317–326.

NICKRENT, D. L. 1994. From field to film: rapid sequencing methods for field collected plant species. *Biotechniques* 16: 470–475.

NICKRENT, D. L. 2002. Orígenes filogenéticos de las plantas parásitas. *In* J. A. López-Sáez, P. Catalán, and L. Sáez [eds.], Plantas Parásitas de la Península Ibérica e Islas Baleares, 29–56. Mundi-Prensa Libros, S. A., Madrid, Spain.

NICKRENT, D. L., AND R. J. DUFF. 1996. Molecular studies of parasitic plants using ribosomal RNA. *In* M. T. Moreno, J. I. Cubero, D. Berner, D. Joel, L. J. Musselman, and C. Parker [eds.], Advances in parasitic plant research, 28–52. Junta de Andalucia, Dirección General de Investigación Agraria, Cordoba, Spain.

NICKRENT, D. L., R. J. DUFF, A. E. COLWELL, A. D. WOLFE, N. D. YOUNG, K. E. STEINER, AND C. W. DEPAMPHILIS. 1998. Molecular phylogenetic and evolutionary studies of parasitic plants. *In* D. E. Soltis, P. S. Soltis, and J. J. Doyle [eds.], Molecular systematics of plants II. DNA sequencing, 211–241. Kluwer Academic, Boston, Massachusetts, USA.

NICKRENT, D. L., AND E. M. STARR. 1994. High rates of nucleotide substitution in nuclear small-subunit (18S) rDNA from holoparasitic flowering plants. *Journal of Molecular Evolution* 39: 62–70.

PARKINSON, C. L., K. L. ADAMS, AND J. D. PALMER. 1999. Multigene analyses identify the three earliest lineages of extant flowering plants. *Current Biology* 9: 1485–1488.

QIU, Y.-L., M. W. CHASE, D. H. LES, AND C. R. PARKS. 1993. Molecular phylogenetics of the Magnoliidae: cladistic analysis of nucleotide se-

quences of the plastid gene *rbcL*. *Annals Missouri Botanical Garden* 80: 587–606.

QIU, Y., Y. LEE, F. BERNASCONI-QUADRONI, D. E. SOLTIS, P. S. SOLTIS, M. ZANIS, E. A. ZIMMER, Z. CHEN, V. SAVOLAINEN, AND M. W. CHASE. 1999. The earliest angiosperms: evidence from mitochondrial, plastid and nuclear genomes. *Nature (London)* 402: 404–409.

QIU, Y.-L., J. LEE, F. BERNASCONI-QUADRONI, D. E. SOLTIS, P. S. SOLTIS, M. ZANIS, E. A. ZIMMER, Z. CHEN, V. SAVOLAINEN, AND M. W. CHASE. 2000. Phylogeny of basal angiosperms: analyses of five genes from three genomes. *International Journal of Plant Sciences* 161: S3–S27.

SAVOLAINEN, V., M. W. CHASE, AND Y.-L. QIU. 2000. Phylogenetics of flowering plants based on combined analysis of plastid *atpB* and *rbcL* gene sequences. *Systematic Biology* 49: 306–362.

SOLMS-LAUBACH, H. 1894. Hydnoraceae. *In* A. Engler and K. Prantl [eds.], Die Natürlichen Planzenfamilien, Part III, 282–285. Wilhelm Engelmann, Leipzig, Germany.

SOLTIS, D. E., M. E. MORT, P. S. SOLTIS, C. HIBSCH-JETTER, E. A. ZIMMER, AND D. MORGAN. 1999. Phylogenetic relationships of the enigmatic angiosperm family Podostemaceae inferred from 18S rDNA and rbcL sequence data. *Molecular Phylogenetics and Evolution* 11: 261–272.

SOLTIS, D. E., ET AL. 2000. Angiosperm phylogeny inferred from 18S rDNA, *rbcL*, and *atpB* sequences. *Botanical Journal of the Linnaean Society* 133: 381–461.

SWOFFORD, D. L. 2001. PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4.0b9. Sinauer, Sunderland, Massachusetts, USA.

TAKHTAJAN, A. 1997. Diversity and classification of flowering plants. Columbia University Press, New York, New York, USA.

TAKHTAJAN, A. L., N. MEYER, R. KOSENKO, AND V. N. KOSENKO. 1985. Pollen morphology and classification in Rafflesiaceae s. l. *Botanichnyi Zhurnal* 70: 153–162.

THORNE, R. F. 1992. An updated phylogenetic classification of the flowering plants. *Aliso* 13: 365–389.

THUNBERG, C. P. 1775. Beskrifning paa en ganska besynnerlig och obekant svamp, *Hydnora africana*. *Konglia Vetenskaps Akademiens Handlingar* 36: 69–75.

TIEGHEM, P. V. 1897. Sur la structure de l'ovule et de la graine chez les Hydnoracees. *Journal de Botanique* 11: 233–238.

ZANIS, M., D. SOLTIS, P. SOLTIS, S. MATHEWS, AND M. DONOGHUE. 2002. The root of angiosperms revisited. *Proceedings of the National Academy of Sciences, USA* 99: 6848–6853.