

Molecular Phylogeny of Extant Gymnosperms and Seed Plant Evolution: Analysis of Nuclear 18S rRNA Sequences

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To study the evolutionary relationships among the four living gymnosperm orders and the interfamilial relationships in each order, a set of 65 nuclear 18S rRNA sequences from ferns, gymnosperms, and angiosperms was analyzed using the neighbor-joining and maximum-parsimony methods. With *Selaginella* as the outgroup, the analysis strongly indicates that the seed plants form a monophyletic group with the ferns as a sister group. Within the seed plants the angiosperms are clearly a monophyletic group. Although the bootstrap support for the monophyly of the gymnosperm clade is moderate, the monophyly is further supported by its lack of angiosperm-specific indels. Within the gymnosperms there appear to be three monophyletic clades: Cycadales-Ginkgoales, Gnetales, and Coniferales. The cycad-ginkgo clade is the earliest gymnosperm lineage. Given the strong support for the sister group relationship between Gnetales and Coniferales, it is unlikely that Gnetales is a sister group of the angiosperms, contrary to the view of many plant taxonomists. Within Coniferales, Pinaceae is monophyletic and basal to the remaining conifer families, among which there are three monophyletic clades: Phyllocladaceae-Podocarpaceae, Araucariaceae, and Sciadopityaceae-Taxaceae-Cephalotaxaceae-Taxodiaceae-Cupressaceae. Within the latter clade, Sciadopityaceae may be an outgroup to the other four families. Among the angiosperms, no significant cluster at the level of subclass was found, but there was evidence that Nymphaeaceae branched off first. Within the remaining angiosperms, the monocots included in this study are nested and form a monophyletic group. This study attests to the utility of nuclear 18S rRNA sequences in addressing relationships among living gymnosperms. Considerable variation in substitution rates was observed among the ferns and seed plants.

Introduction

The four living gymnosperm orders (Coniferales, Cycadales, Ginkgoales, and Gnetales) are morphologically highly divergent from one another, so that their evolutionary relationships have been uncertain. There are at least three different views. First, from morphological data, Parenti (1980), Crane (1985), and Doyle and Donoghue (1986) proposed that the Coniferales and Ginkgoales are sister groups. Similarly, Page (1990) classified the extant gymnosperms into two subdivisions: Coniferphytina, which includes Coniferales and Ginkgoales, and Cycadophytina, which includes Cycadales and Gnetales. Second, Loconte and Stevenson (1990) drew a different conclusion from the morphological data and regarded the Coniferales and Ginkgoales as two separate groups and the Cycadales as the most archaic seed plants. Third, on the basis of chloroplast and nuclear DNA sequences, Savard et al. (1994) suggested that the conifers and cycads form a clade, representing the earliest lineage among seed plants. In addition to uncertainty about the interordinal relationships, the interfamilial relationships within each gymnosperm order are also controversial (Hart 1987; Page 1990).

Another major issue is whether the gymnosperms are paraphyletic or monophyletic. On the basis of morphological and fossil data, many authors have suggested that the gymnosperms are paraphyletic, arguing that the

ancestor of angiosperms was derived from a certain gymnosperm lineage, which has been assumed to be the Mesozoic Bennettitales (Arber and Parkin 1907, 1908), Glossopteridales (Melville 1969; Retallack and Dilcher 1981), Caytoniales (Cronquist 1988) of the cycadopsids (sensu Cronquist: including the living cycads and several extinct groups), or the Gnetales (Wettstein 1907). The last view was affirmed by cladistic analyses of morphological data (Crane 1985; Doyle and Donoghue 1986; Loconte and Stevenson 1990 and references therein) and parsimony analyses of partial nuclear 18S and 26S rRNAs (Hamby and Zimmer 1992), *rbcL* gene sequences (Chase et al. 1993), and combined morphological and molecular data (Doyle, Donoghue, and Zimmer 1994). However, in contrast to the above viewpoint, phylogenetic analyses of 5S rRNA sequences (Hori, Lim, and Osawa 1985), partial fragments of the small subunits of the nuclear and the plastid rRNAs (Troitsky et al. 1991), *rbcL* gene sequences (Hasebe et al. 1992), and chloroplast intergenic spacer (*cpITS*) sequences (Goremykin et al. 1996) suggested that the extant gymnosperms are monophyletic, implying that none of the extant gymnosperm lineages is a direct ancestor of angiosperms. Troitsky et al. (1991) pointed out that the ancestral form of angiosperms should be searched for among progymnosperms. Generally, all the above molecular data were based on either too few gymnosperm samples, only partial rRNA sequences, or in the case of *rbcL* genes, saturated in synonymous substitutions (Martin et al. 1993; Goremykin et al. 1996).

Finally, whether seed plants (including gymnosperms and angiosperms) are monophyletic or polyphyletic has been another unsettled issue. Beck (1981 and references therein) proposed that cycadopsids and con-

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iferopsids (including the Mesozoic cordaites, the conifers, and ginkgo) are two independent lineages derived from Aneurophytales and Archaeopteridales (both are progymnosperms), respectively. This hypothesis assumes that seed plants are polyphyletic and that the seed has evolved twice. While supporting the polyphyletic hypothesis of seed plants, Meyen (1984) suggested the following two evolutionary lines: the primitively platyspermic Ginkgoopsida (including several progymnosperms, ginkgo, and *Ephedra*), and the secondarily platyspermic Pinopsida (including cordaites and conifers) which were derived from the lyginopterid seed ferns of Cycadopsida (sensu Meyen, including cycads, *Welwitschia*, *Gnetum*, angiosperms, and some other seed ferns). On the other hand, Rothwell (1981, 1982) believed that the seed plants are monophyletic, that the aneurophytes represent an ancestral group from which the pteridosperms evolved; that "coniferopsids" were derived from within the seed fern complex, and that *Archaeopteris* is unrelated to any group of seed plants. Under this hypothesis, the seed originated only once.

To date, no extensive molecular study has been carried out to examine the evolutionary relationships of extant gymnosperm orders or families as a whole, which, undoubtedly, are inextricably related to the origin of angiosperms and early divergence of seed plants. Therefore, we initiated the present study. We obtained 23 new nuclear 18S rRNA sequences from seed plants and ferns to provide a greater representation of the extant gymnosperm families and the major subclasses of angiosperms (table 1). Our objectives were: (1) to reconstruct the phylogenetic relationships of the major lineages of seed plants, of the extant gymnosperm orders and families, and of the major subclasses of angiosperms, respectively; (2) to examine hypotheses concerning the early events in seed plant evolution, and the evolutionary position of Gnetales; and (3) to study the range of rate variation among lineages.

Materials and Methods

In addition to our previously reported 15 nuclear 18S rRNA sequences of gymnosperms (Chaw et al. 1993, 1995), 15 sequences of seed plants from GenBank, and one unpublished rDNA sequence (*Pseudotsuga menziesii*) from Dr. D. E. Soltis, we determined 23 new nuclear 18S rRNA sequences from 2 ferns, 8 gymnosperms, and 13 angiosperms. These 65 samples cover all extant orders and families of gymnosperms, and the majority of subclasses of dicots and monocots (table 1). To simplify our analysis and the presentation of our result, sequences of closely related species and parasitic plants were excluded; inclusion of those sequences did not change our conclusion.

Our experience indicated that PCR amplification of nuclear 18S rRNA genes from genomic DNA often amplifies nonfunctional genes (Chaw et al. 1995), so we used two procedures to obtain the 18S rDNA gene templates prior to polymerase chain reaction (PCR). (1) For the two ferns, all gymnosperms, and four angiosperms (*Ceratophyllum*, *Chloranthus*, *Nuphar japonica*, and

Piper, for which we found nonfunctional genes from PCR amplification of the genomic DNAs of these taxa), total RNAs were first extracted and then transcribed to cDNAs following our previously reported method (Chaw et al. 1995). For each taxon we synthesized three independent cDNAs, which were then used as templates. (2) For the remaining nine angiosperm taxa, total DNAs were extracted with Saghai-Marroof et al.'s (1984) method and used for amplification. PCR amplification and DNA sequencing followed previously established methods (Chaw et al. 1993, 1995), except that PCR products were purified using a Wizard PCR Preps kit (Promega Corp., Madison, Wis.) and subcloned into pBluescript II (KS+ or SK+) (Stratagene, La Jolla, Calif.), or pCR Vector (Invitrogen, San Diego, Calif.) instead. For each species at least one clone from each of three independent PCRs was sequenced.

The alignment of sequences was conducted with the SEQED program in the VOSTORG package (Zharkikh et al. 1990). The distance matrix for the aligned sequences was calculated by using the two-parameter method of Kimura (1980) as implemented in a computer program provided by the second author. This distance matrix (not shown) was then used to reconstruct a phylogenetic tree by the neighbor-joining (NJ) method (Saitou and Nei 1987); the distances were computed using the weight of 40% for transitions and 60% for transversions. Bootstrap estimates of the confidence levels for subsets of taxa (Felsenstein 1985) were conducted (1,000 replicates) by using a program modified from that of T. S. Whittam and a program based on the complete and-partial (CP) bootstrap technique (Zharkikh and L.

Parsimony analyses were also conducted using PAUP 3.1.1 (Swofford 1993). We used the heuristic search with three options—general, branch swapping and stepwise addition—separately to seek for the most parsimonious (MP) trees. For the first two options simple taxon addition was used. We also manually rearranged the taxon order in 10 different data sets to increase the chance of finding the shortest tree. For the last option 10 replicates of random addition were used. All three options were performed with tree-bisection-reconnection (TBR) branch swapping and MULPARS. These searches were run using Macintosh LC630 or Power Macintosh 7500/100 computers. *Selaginella galleanii* was used as an outgroup. To obtain an estimate of the strength of support for the topology of the resultant MP tree, the bootstrap method with heuristic search was also applied to a data set with the taxon order exactly the same as that of the data set used for the NJ method. In this analysis, 100 replicates were conducted and only the groups supported by bootstrap values of 50% or greater were retained in the bootstrap consensus tree. The consistency of these data with NJ data and previously proposed hypotheses of relationships of seed plants was also examined by moving branches of the MP trees to new nodes and then searching above and below the nodes for more parsimonious solutions, using MacClade 3.03 (Maddison and Maddison, 1992). The

Table 1
Sequences Used in This Study and Their Sources

Family ^a	Species	Collection place ^{b/} GenBank Accession No.	Voucher ^{c/} Reference
Fern allies			
Selaginellaceae	<i>Selaginella galleottii</i>	X75517	Kranz et al. (1995)
Ferns			
Marattiaceae	<i>Angiopteris lygodiiifolia</i>	Taipei	CHAW 1398
Aspleniaceae	<i>Asplenium nidus</i>	Taipei	CHAW 1348
Gymnosperms			
Ginkgoales			
Ginkgoaceae	<i>Ginkgo biloba</i>	D16448	Chaw et al. (1993)
Cycadales			
Cycaceae	<i>Cycas taitungensis</i>	Taitung County	Tsou 570
Zamiaceae	<i>Zamia pumila</i>	M20017	Nairn and Ferl (1988)
Gnetales			
Ephedraceae	<i>Ephedra antisiphilitica</i>	L24091	Nickrent and Soltis (1995)
	<i>E. sinica</i>	D38242	Chaw et al. (1995)
	<i>Gnetum leyboldii</i> subsp. <i>wood-sonianum</i>	L24045	Nickrent and Starr (1994)
Welwitschiaceae	<i>Welwitschia mirabilis</i>	Univ. of Tokyo	TI-85-00047
Coniferales			
Araucariaceae	<i>Agathis borneensis</i>	Singapore Bot. Gard.	CHAW 1390
	<i>Araucaria excelsa</i>	D38240	Chaw et al. (1995)
Cephalotaxaceae	<i>Cephalotaxus wilsoniana</i>	D38241	Chaw et al. (1995)
Cupressaceae	<i>Calocedrus formosana</i>	Forest Res. Inst. Taipei	CHAW 1375
	<i>C. decurrens</i>	Vancouver	CHAW 1382
	<i>Juniperus chinensis</i> var. <i>kaizuka</i>	D38243	Chaw et al. (1995)
Phyllocladaceae	<i>Phyllocladus trichomoides</i>	D38244	Chaw et al. (1995)
Pinaceae	<i>Abies lasiocarpa</i>	X79407	Capesius (1994)
	<i>Larix leptolepis</i>	Forest Res. Inst. Tungshih	CHAW 1403
	<i>Pinus elliotii</i>	D38245	Chaw et al. (1995)
	<i>Pinus luchuensis</i>	D38248	Chaw et al. (1995)
	<i>P. wallichiana</i>	X75080	Sensen et al. (unpublished)
	<i>Pseudotsuga menziesii</i>	WSU campus	CAMPBELL s.n.
	<i>Dacrycarpus imbricatus</i>	D38247	Chaw et al. (1995)
	<i>Nageia nagi</i>	D16447	Chaw et al. (1993)
	<i>Podocarpus costalis</i>	D38473	Chaw et al. (1995)
	<i>Sciadopitys verticillata</i>	Taipei Hort. Exp. Center	CHAW 1379
Taxaceae	<i>Amentotaxus formosana</i>	D38248	Chaw et al. (1995)
	<i>Torreya nucifera</i>	D38249	Chaw et al. (1995)
	<i>Taxus mairei</i>	D16445	Chaw et al. (1993)
Taxodiaceae	<i>Cryptomeria japonica</i>	Taipei Hort. Exp. Center	CHAW 1387
	<i>Taiwania cryptomerioides</i>	D38250	Chaw et al. (1995)
Angiosperms			
Magnoliopsida (dicots)			
Magnoliidae			
Aristolochiaceae	<i>Aristolochia tomentosa</i>	L24083	Nickrent and Soltis (1995)
	<i>Asarum hayatanum</i>	Taipei City	CHAW 772
Ceratophyllaceae	<i>Ceratophyllum demersum</i>	Taipei County	CHAW 1397
Chloranthaceae	<i>Chloranthus spicatus</i>	Taipei County	CHAW 1255
Magnoliaceae	<i>Magnolia acuminata</i>	Baton Rouge	CHAW 1507
Nymphaeaceae	<i>Nuphar japonica</i>	Taichung County	CHAW 1171
	<i>N. shimadai</i>	Taoyuan County	CHAW 1177
	<i>Nymphaea tuberosa</i>	L24404	Nickrent and Soltis (1995)
Papaveraceae	<i>Argemone mexicana</i>	Penghu County	PENG 12748
Piperaceae	<i>Peperomia serpens</i>	L24411	Nickrent and Soltis (1995)
	<i>Piper kadsura</i>	Taipei City	CHAW 1142
Ranunculaceae	<i>Ranunculus taiwanensis</i>	Taichung County	CHAW 1368
Saururaceae	<i>Houttuynia cordata</i>	L24147	Nickrent and Soltis (1995)
	<i>Saururus chinensis</i>	Taipei County	CHAW 1169
Hamamelidae			
Betulaceae	<i>Alnus glutinosa</i>	X54984	Savard and Lalonde (1991)
Moraceae	<i>Morus alba</i>	L24398	Nickrent and Soltis (1995)
Caryophyllidae			
Chenopodiaceae	<i>Spinacia oleracea</i>	L24420	Nickrent and Soltis (1995)
Polygonaceae	<i>Polygonum chinense</i>	Taipei City	CHAW 1355
Dilleniidae			
Brassicaceae	<i>Sinapis alba</i>	X17062	Rathgeber and Capiesius (1990)
Malvaceae	<i>Gossypium hirsutum</i>	L24145	Nickrent and Soltis (1995)
Monotropaceae	<i>Monotropa uniflora</i>	L26062	Nickrent and Soltis (1995)

Table 1
Continued

Family ^a	Species	Collection place ^b / GenBank Accession No.	Voucher ^c / Reference
Rosidae			
Araliaceae	<i>Hedera helix</i>	X16604	Nickrent and Soltis (1995)
Buxaceae	<i>Buxus sempervirens</i>	X16599	Nickrent and Franchina (1990)
Cornaceae	<i>Cornus racemosa</i>	X16602	Nickrent and Franchina (1990)
Fabaceae	<i>Glycine max</i>	X02623	Eckenrode, Arnold, and Meagher (1984)
Linaceae	<i>Linum perenne</i>	L24401	Nickrent and Soltis (1995)
Asteridae			
Polemoniaceae	<i>Gilia capitata</i>	L28138	Nickrent and Soltis (1995)
Solanaceae	<i>Lycopersicon esculent</i>	X51576	Kiss, Szukalek, and Solymosy (1989)
Liliopsida (monocots)			
Arecidae			
Arecaceae	<i>Trachycarpus wagnerianus</i>	Taipei City	CHAW 1370
Commelinidae			
Poaceae	<i>Oryza sativa</i>	X00755	Takaiwa et al. (1984)
	<i>Zea mays</i>	K02202	Messing et al. (1984)
Sparganiaceae	<i>Sparganium eurycarpum</i>	L24419	Nickrent and Soltis (1995)
Zingiberidae			
Cannaceae	<i>Canna coccinea</i>	Taipei City	CHAW 1371

^a The classification system of gymnosperms followed Page (1990), and that of angiosperms followed Cronquist (1988).

^b The new sequences are available from GenBank (accession numbers D85292–D85304, D29773–D29774, D29776–29780, D29782, D29784, and D29787).

^c The collectors' names are in capital letters: all vouchers (except TI-85-0047 and CAMPBELL, s.n., collections of TI and WS, respectively) are deposited in the Herbarium, Academia Sinica, Taipei, Taiwan (HAST).

relative rate test of Wu and Li (1985) was used to compare the rates of nucleotide substitution among lineages.

Results

The Inferred Tree

Because of high sequence similarities, we were able to align the nuclear 18S rRNA sequences of the 65 taxa used in this study by the SEQED program in the VOS-TORG package (Zharkikh et al. 1990) with only some minor manual adjustments; the alignment is available on request from the first or second author. The length of the aligned sequences is 1,857 bp, including gaps. The nucleotide positions at approximately 130–140, 190–280, 660–750, 1380–1400, 1530–1570, and 1730–1760 are highly variable. These regions approximately correspond, respectively, to the area between the helices 8 and 9; the V2 region; the areas including E23-1, E23-2, and E 23-5 helices; the V7 region; the V8 region; and the V9 region of Neefs et al.'s (1993) secondary structural model for eukaryotic small subunit rRNAs.

After elimination of all gaps and unknown sites, 1,423 sites are available for comparison, among which 1,026 sites are invariant and 397 sites are variable. Among the 397 variable sites 249 are phylogenetically informative.

The lycopod *Selaginella galleottii* was selected as the outgroup because lycopods were considered on the basis of both morphological characters (Tippo 1942 and many other taxonomists) and molecular data (Raubeson and Jansen 1992) to have diverged earlier than ferns in the evolution of vascular plants. This was also supported by the tree inferred from nuclear 18S rRNA sequences with the *Chlamydomonas reinhardtii* as the outgroup (not shown).

For all sequence pairs compared the average ratio of transition to transversion is 2.08. For the sequence

divergence between *Selaginella* and each of the other taxa, *Welwitschia* is the highest in both transition (7.2%) and transversion (4.3%) among gymnosperms, and *Trachycarpus* is the highest in transition (8.1%) while maize (*Zea mays*) is the highest in transversion (3.9%) among angiosperms. Among the gymnosperm sequences, the *Welwitschia* sequence deviates the most, having the highest divergence in transitions and transversions from other gymnosperm sequences. Among angiosperms, the maize sequence is the most divergent. Among all sequence pairs the *Nageia* and *Podocarpus* sequences are most similar, differing by only 0.1% in transition and 0% in transversion. Further results on the relative rates among lineages will be given later.

Figure 1 shows the phylogenetic tree inferred by the NJ method with the weights of 40% and 60% for transitional and transversional substitutions, respectively. The two numbers on each node denote the bootstrap proportions obtained by the CP and the traditional bootstrap techniques (only one number is shown when the two values are the same). The upper number is an unbiased estimate and is in general higher than the lower number. Figure 2 depicts the phylogenetic tree constructed by the parsimony method, using heuristic search in PAUP 3.1.1 (Swofford 1993). Our heuristic searches with the options general, branch swapping, and stepwise addition separately all resulted in the four shortest trees, of 1,928 substitutions or steps. We shall call them the most parsimonious trees. They are different from one another in the positions of the *Cephalotaxus* and *Glycine-Sinapis-Hedera-Buxus-Cornus* clades. Nodes leading to these two clades collapse in the strict consensus tree as indicated by arrows in figure 2. As these trees differ only slightly, our discussion will, for simplicity, focus on the tree in figure 2.

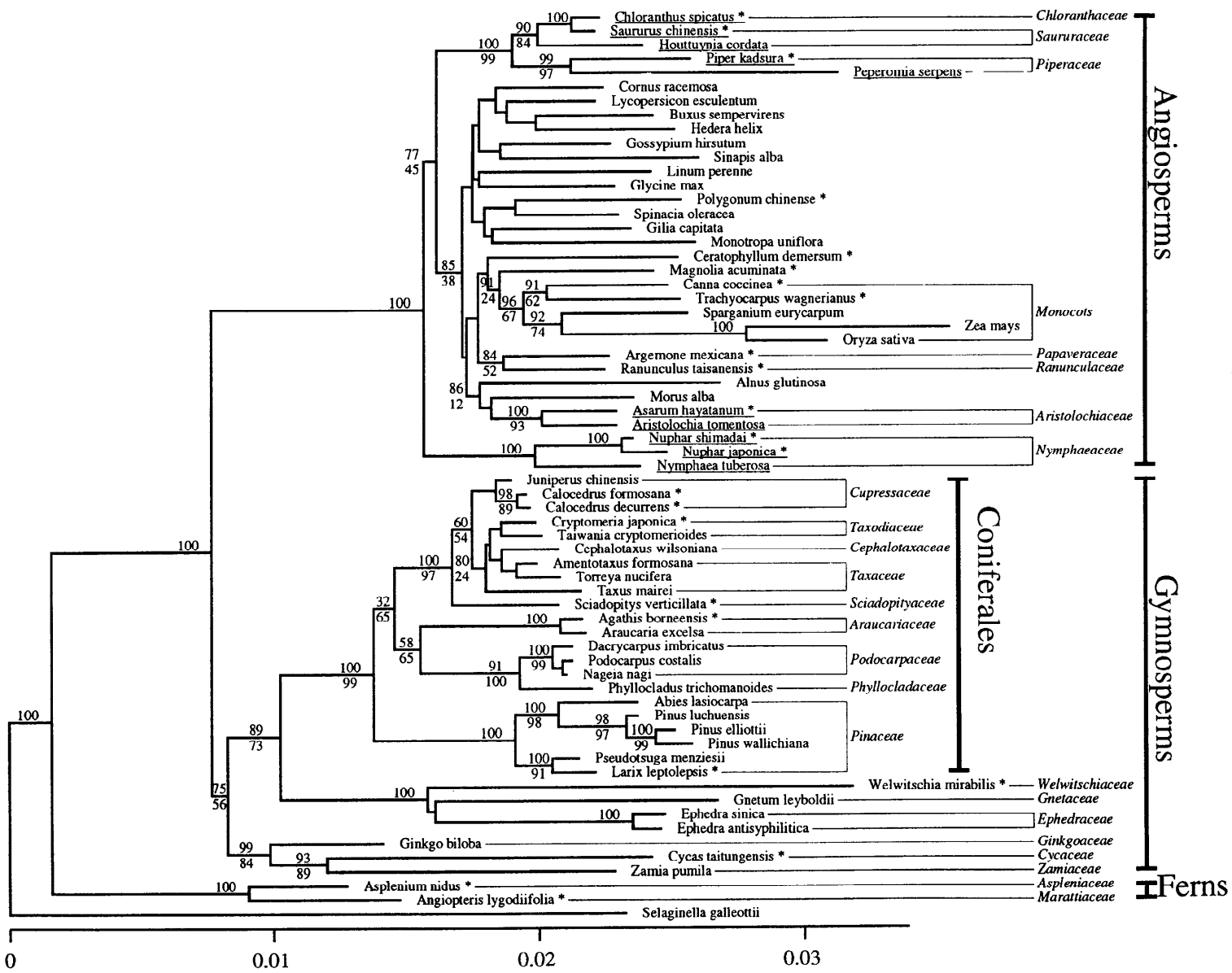


FIG. 1.—The neighbor-joining tree. The branch lengths indicate the number of substitutions per 100 sites. The upper and lower numbers at each node denote the proportions of the CP and the traditional bootstrap replicates that support the monophyly of the taxa in the subset designated by the node. Only bootstrap values higher than 50% are shown. Our new sequences are indicated by asterisks. Paleoherbs, as defined by Taylor and Hickey (1992), are underlined. For dicots only significant clades are labeled with family names.

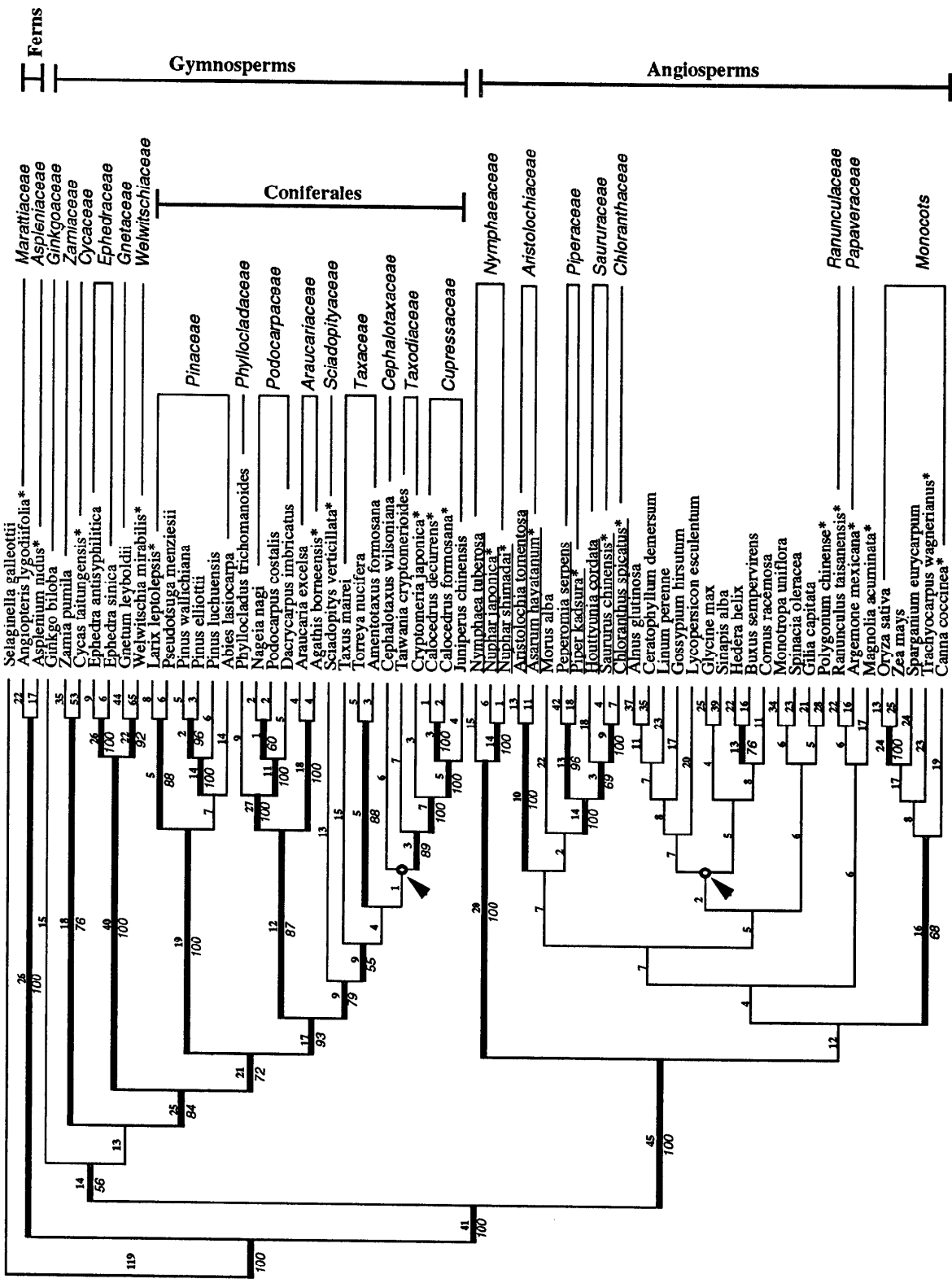


FIG. 2.—One of the four most parsimonious trees. Each of them has a length of 1,928 steps, CI = 0.41 and RI = 0.72. Bold-faced branches lead to the nodes in which the monophyly of the included taxa is supported by more than 50% of the bootstrap replicates. The upper and lower numbers at each node denote the branch lengths and the proportions of the bootstrap replicates, respectively. Arrows indicate nodes not present in the strict consensus of all most parsimonious trees. Our new sequences are indicated by asterisks. Paleotherbs, as defined by Taylor and Hickey (1992), are underlined. For dicots mainly significant clades are labeled with family names.

Relationships Among Major Extant Seed Plant Lineages

Both bootstrap techniques (fig. 1) strongly indicate that the ferns and the seed plants (gymnosperms and angiosperms) are two separate monophyletic groups, supported by 100% of the 1,000 bootstrap replicates. The branching pattern of figure 1 reveals that all modern seed plants share a common ancestor and that the ferns are an outgroup to the seed plants, which are divided into two major sister groups: gymnosperms and angiosperms. The angiosperms form a monophyletic clade strongly supported by the data. The bootstrap support for the monophyly of the gymnosperms is only 75% even after the CP bootstrap correction, so that no definitive conclusion can be drawn. However, since the Gnetales lineage appears to be a sister group of the conifers, with the cycads and ginkgo being an outgroup to them, it is unlikely that the Gnetales are a sister group of the angiosperms. In addition, the monophyly of gymnosperms is supported by the observation that they lack the following angiosperm-specific mutations: three one-nucleotide deletions at positions 136, 681, and 1544, and a one-nucleotide insertion at position 239.

The MP tree in figure 1 also suggests that, to the exclusion of ferns, the seed plants form a monophyletic group, within which gymnosperms and angiosperms are two separate subgroups. This grouping requires only 1,928 nucleotide substitutions, whereas the tree will require 12 additional substitutions if the Gnetales are placed as a sister group of angiosperms (not shown).

Relationships Among the Extant Gymnosperm Orders and Families

Within gymnosperms, the NJ analysis suggests three monophyletic clades: Cycadales-Ginkgoales, Gnetales, and Coniferales, highly supported by both the CP and the traditional bootstrap techniques (99%/84%, 100%/100%, and 100%/99%, respectively) (fig. 1). The sister group relationship between the latter two orders suggests that the Gnetales are not a sister group to the angiosperms. These phylogenetic relationships indicate that the divergence between the cycad-ginkgo and Gnetales-conifer lineages represents the first major split in the evolutionary diversification of extant gymnosperms. The parsimony analysis (fig. 2) also confirms that Gnetales and Coniferales are monophyletic clades, supported by 100% and 72% of the 100 bootstrap replicates, individually; and that the two orders form sister groups, supported by 84% of the bootstrap value. In the MP tree the order Ginkgoales is shown as an outgroup to the rest of the gymnosperm orders, but the bootstrap value is below 50% (not shown), and if Ginkgoales is forced to be the sister group of Cycadales, the tree requires only one step more than the MP tree. Moreover, among the seed plants only cycads and ginkgo share a one-nucleotide deletion with *Selaginella* and ferns at position 1536, giving additional support for the sister-group relationship of the cycads and the ginkgo and for the lineage being basal to other gymnosperms.

In the NJ tree (fig. 1), the two *Ephedra* species form a clade, which together with *Gnetum* form a larger

clade. *Welwitschia* is an outgroup to *Ephedra* and *Gnetum*, but this may be a sampling error because the bootstrap value is only 15% (not shown). Indeed, the MP tree (fig. 2) supports the *Welwitschia*-*Gnetum* clade, with *Ephedra* as an outgroup.

Both the NJ and MP trees (figs. 1 and 2) strongly suggest that all conifer families are monophyletic. Also, all of the genera of Pinaceae examined are monophyletic, supported by 100% of the bootstrap replicates and by a one-nucleotide insertion at position 195. Therefore, Pinaceae is a natural family and an outgroup to the remaining conifer families. Within Pinaceae there are two monophyletic, above-genus clades: the *Pseudotsuga*-*Larix* clade and the *Abies*-*Pinus* clade, supported by more than 90% of the bootstrap replicates (fig. 1). The *Pinus* species also form a well-supported monophyletic genus.

Within conifers, other than Pinaceae three additional highly supported clades are seen in the NJ tree (fig. 1): the Phyllocladaceae-Podocarpaceae and Araucariaceae clades, and a clade composed of Sciadopityaceae, Taxaceae, Cephalotaxaceae, Taxodiaceae, and Cupressaceae. In the MP tree (fig. 2) not only are the above three clades maintained, the first two clades and all of them together are highly supported by bootstrap values. Within the above largest clade, Sciadopityaceae may be basal to the remaining four families, which share a unique one-nucleotide deletion at position 1749. In the NJ tree interfamilial relationships among these four families cannot be determined because the bootstrap support for each node of this clade is low, as is also reflected by the short internal branches among these families. Moreover, the MP tree (fig. 2) has branching patterns that differ from those in the NJ tree (fig. 1). Although the genera of Taxodiaceae and Cupressaceae are grouped in their own clades, the bootstrap values for the familial monophylies are low (fig. 2). Besides, the phylogenetic positions of the taxodiaceous genera *Cryptomeria* and *Taiwania* on the MP tree are different from those on the NJ tree (fig. 1), indicating that the familial relationships between Taxodiaceae and Cupressaceae cannot be resolved by nuclear 18S rRNA data.

Araucariaceae, represented by *Araucaria* and *Agathis*, is supported by 100% of the bootstrap replicates, so the family can be regarded as a natural family. The monogeneric family Phyllocladaceae is a sister group of the Podocarpaceae (represented by *Dacrycarpus*, *Podocarpus*, and *Nageia*). The strong phylogenetic congruence of these two families on the NJ and MP trees reinforce these phylogenetic inferences.

Relationships Among the Extant Angiosperm Orders and Families

Figure 1 suggests that among the angiosperms the Nymphaeaceae diverged first, and then the Piperales (including the Chloranthaceae, the Saururaceae, and the Piperaceae) diverged from the remaining angiosperms. The monophylies of the first two clades are highly supported by both bootstrap procedures; however, bootstrap support for the clade containing Piperales and the rest of angiosperms is only 77% even after CP bootstrap correction. The large angiosperm clade (excluding Nym-

Table 2
Relative Rate Tests

Lineage 1	Lineage 2	Lineage 3	K_{12}	$K_{13} - K_{23}$
<i>Zamia</i>	<i>Angiopteris</i>	<i>Selaginella</i>	0.077	0.0149 ± 0.0074*
<i>Zamia</i>	<i>Ginkgo</i>	<i>Angiopteris</i>	0.042	0.0166 ± 0.0053**
<i>Welwitschia</i>	<i>Gnetum</i>	<i>Ginkgo</i>	0.066	0.0135 ± 0.0066*
<i>Pinus wallichiana</i>	<i>Pseudotsuga</i>	<i>Nageia</i>	0.020	0.0064 ± 0.0036
<i>Zea mays</i>	<i>Oryza</i>	<i>Nymphaea</i>	0.022	0.0108 ± 0.0038**
<i>Peperomia</i>	<i>Piper</i>	<i>Nymphaea</i>	0.031	0.0225 ± 0.0044**
<i>Oryza</i>	<i>Saururus</i>	<i>Nymphaea</i>	0.048	0.0127 ± 0.0054*

NOTE.— K_{ij} = the number of nucleotide substitutions per site computed by Kimura's (1980) two-parameter method.
* Significant at the 5% level.
** Significant at the 1% level.

phaeaceae and Piperales) is supported by 85% of the CP bootstrap replicates and might be a monophyletic group, within which all the monocots surveyed are nested and form a monophyletic clade, supported by 96% of the CP bootstrap replicates (fig. 1). The MP tree (fig. 2) is concordant with the NJ tree in support of the monophylies of Nymphaeaceae, Piperales, and monocots and the basal position of Nymphaeaceae, but disagrees with the NJ tree in the evolutionary position of Piperales and the monophyly of the above large angiosperm clade. On the MP tree, instead of Piperales, the monocots are suggested to be the second clade diverged from other angiosperms. This status, however, is not supported by bootstrap values.

Both the NJ and MP trees (figs. 1 and 2) place *Ceratophyllum* and *Magnolia*, genera of the subclass Magnoliidae, in a relatively derived position. On the NJ tree they are grouped with the monocots; however, the bootstrap values (CP bootstrap, 42%; standard bootstrap 13%) for this clade are low and the phylogenetic positions of these two genera on the MP tree (fig. 2) are disparate from those on the NJ trees. Therefore, neither of these two genera can be regarded as a sister group of the monocots.

The NJ tree (fig. 1) shows that within monocots *Canna* (of the subclass Zingerberidae) and *Trachycarpus* (of the subclass Arceidae) form a monophyletic clade and are an outgroup to the monophyletic subclass Commelinidae (here represented by Poaceae of Cyperales, and *Sparganium* of Typhales), which is supported by 92% of the CP bootstrap replicates. However, in the MP tree (fig. 2) *Canna* and *Trachycarpus* are paraphyletic. Both the NJ and MP trees suggest (figs. 1 and 2) that within Commelinidae the family Poaceae (represented by *Oryza sativa* and *Zea mays*) constitutes a monophyletic family with *Sparganium* as an outgroup. The monophyly of Poaceae is supported by 100% of the CP bootstrap replicates and further confirmed by their unusual one-nucleotide insertion at position 668.

Within dicots there is no significant grouping above or at subclass level (sensu Cronquist 1988) (figs. 1 and 2). Thus, nuclear 18S rRNA sequences cannot unambiguously resolve the relationships among and within the major subclasses of angiosperms. However, within the subclass Magnoliidae sampled, Nymphaeaceae, Aristolochiaceae, and the order Piperales (sensu Cronquist 1988, including Piperaceae, Saururaceae, and Chloran-

thaceae) are each revealed as monophyletic clades, supported by 100% of the CP bootstrap replicates in the NJ tree and 100% of traditional bootstrap replicates in the MP tree. Within the Piperales the family Piperaceae (represented by *Peperomia* and *Piper*) is suggested to be an outgroup to the monophyletic clade consisting of Saururaceae (represented by *Saururus* and *Houttuynia*) and Chloranthaceae (represented by *Chloranthus*). Furthermore, 100% of the bootstrap replicates support a closer relationship of *Chloranthus* to *Saururus* than to *Houttuynia*, indicating that Chloranthaceae is derived from within the Saururaceae. Therefore, Saururaceae is likely a paraphyletic family.

Both the NJ and MP trees also show that the paleoherbs studied (underlined taxa in figs. 1 and 2, as defined by Taylor and Hickey 1992) are paraphyletic. At least the aquatic Nymphaeaceae are rooted at the base of the angiosperms, which seems to suggest that the ancestral angiosperms had a herbaceous, rhizomatous habit rather than a shrubby habit.

Comparison of Substitution Rates Among Lineages

The branch lengths shown in figure 1 clearly reveal considerable variation in substitution rates among lineages. The rates in the two fern lineages are obviously lower than those of most of the seed plant lineages. For example, with *Selaginella* as a reference, one can show that the rate in the *Angiopteris* lineage is significantly lower than that in the *Zamia* lineage (table 2). Among the gymnosperms, the ginkgo shows a very slow rate, being significantly lower than that in the *Zamia* lineage (table 2). In contrast, the *Welwitschia* lineage shows the fastest rate, being significantly higher than that in the *Gnetum* lineage.

Among the conifers, the Pinaceae lineages tend to have higher rates than do the other conifer lineages. The highest rate among the Pinaceae lineages is seen in *Pinus wallichiana*; although the test using a single reference (*Nageia nagi*) does not show a significantly higher rate in this lineage than in the *Pseudotsuga* lineage (table 2), a test using multiple references should show a significant difference. The rate variation in the other conifers is less conspicuous, except that the Cupressaceae lineages have shown lower rates (fig. 1).

Among the angiosperms, the maize (*Zea mays*) lineage shows the highest rate, being even significantly higher than that in the rice (*Oryza sativa*) lineage; the

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rate difference becomes less conspicuous if the GenBank maize sequence is replaced by a sequence recently obtained by D. A. Soltis (personal communication). The rate for the rice sequence is in turn significantly higher than that in the *Saururus chinensis* lineage (table 2). The *Peperomia serpens* lineage also shows a high rate, being significantly higher than that in the *Piper kadsura*. In summary, rate constancy does not hold among the ferns and seed plants.

Discussion

Evolution Among Major Extant Seed Plant Lineages

Our phylogenetic analyses of the nuclear 18S rRNA sequence data strongly indicate that the living seed plants are a monophyletic clade with the ferns as an outgroup. Therefore, modern seed plants share a common ancestor and the seed evolved only once. This result confirms Rothwell's (1981, 1982) hypothesis, but contradicts Beck's (1981) and Meyen's (1984) view that seed plants are polyphyletic.

Within modern seed plants, the angiosperms are clearly a monophyletic lineage. Although the bootstrap support for the monophyly of the gymnosperm clade is moderate, the monophyly is further supported by its lack of angiosperm-specific indels (see above). Within the extant gymnosperms there appear to be three monophyletic clades: Cycadales-Ginkgoales, Gnetales, and Coniferales. Our results contradict the view that the Gnetales and the angiosperms are sister groups (Crane 1985; Donoghue and Doyle 1989; Loconte and Stevenson 1990; Doyle and Donoghue 1992; Hamby and Zimmer 1992; Chase et al. 1993; Albert et al. 1994; Doyle, Donoghue, and Zimmer 1994; Nixon et al. 1994; Crane, Friis, and Pedersen 1995). In other words, our data suggest that none of the extant gymnosperms is an ancestor of the angiosperms and that the diversification of modern gymnosperm orders probably did not occur until a split between the gymnosperms and the angiosperms had taken place. This conclusion is consistent with the phylogenetic analyses of Hori, Lim, and Osawa (1985), Troitsky et al. (1991), Hasebe et al. (1992), and Goremykin et al. (1996), although our study differs from the others in the detailed relationships among gymnosperms.

Phylogeny Within the Extant Gymnosperm Orders and Families

Strong reproductive resemblances between ginkgo and cycads have been well documented (Foster and Gifford 1974; Stewart and Rothwell 1993 and references therein) but often neglected. These include: (1) the strict dioecy, (2) the monocolpate pollen, (3) the development of haustorial pollen tubes and large multiflagellated sperm cells, (4) the occurrence of a coenocytic phase and the large four-neck-celled archegonia in megasporophyte development, and (5) the extensive period of free nuclear divisions during early embryogeny. Our analyses give additional support for ginkgo being closer to cycads than to either Coniferales or Gnetales, a relationship also supported (81% of the bootstrap propor-

tion) by the *cpITS* data (Goremykin et al. 1996, fig. 8). In addition, our data indicate the cycad-ginkgo clade to be the earliest gymnosperm lineage. Congruent to this is a one-nucleotide deletion (position 1536) that is shared by the ginkgo-cycad clade, ferns, and the outgroup, *Selaginella*. Coincidentally, these taxa (with *Lycopodium* substituting for *Selaginella*) also share a five-nucleotide insertion (positions 499–504) in their *cpITS* sequences (Goremykin et al. 1996, fig. 4). The phylogenetic distribution of these two indels adds substantial support to the inference discussed above. Therefore, nuclear 18S rRNA and *cpITS* data refute Page's (1990) proposal that ginkgo and conifers constitute the subdivision Coniferophytina, being separated from the Cycadophytina (including the cycads and the Gnetales); and reject Savard et al.'s (1994, p. 5166) proposal that among the seed plants conifers and cycads form a clade that represents the earliest lineage.

In a study of 19 gymnosperm sequences with *Chlamydomonas* as the outgroup, we (Chaw et al. 1995) concluded that *Ephedra* and *Gnetum* form a monophyletic group. The present study supports this conclusion and shows that these two genera and *Welwitschia* form a natural, monophyletic order, the Gnetales, although they are morphologically distinct and geologically separated. Recently, on the basis of *cpITS* and *rbcL* sequences, Goremykin et al. (1996) also found strong support for the monophyly of Gnetales. Thus, there is no support for either Meyen's (1984) classification, based on fruitification and fossil evidence, that *Gnetum* and *Welwitschia* belong to the class Cycadopsida and *Ephedra* belongs to the class Ginkgoopsida or Nixon et al.'s (1994) proposal, on the basis of a cladistic analysis of morphological characters, that Gnetales are paraphyletic, with *Gnetum* and *Welwitschia* being sister to angiosperms.

Gnetales has traditionally been regarded by taxonomists as a sister group to the angiosperms since the time of Haeckel (1894) (e.g., Martens 1971; Friedman 1990, 1994) because of its angiosperm-like features, such as the dicotyledonous seeds, vessels in the secondary wood, reduced female gametophytes, double-integumented ovules, and net-veined leaves. However, the nuclear 18S rRNA sequence data presented here strongly indicate that Gnetales is a sister group to the conifers rather than to the angiosperms. This is in agreement with Goremykin et al.'s (1996) finding from their *cpITS* sequence data that *Pinus* and Gnetales possess three unique nucleotide deletions at positions 555, 556, and 557. A phylogenetic analysis of the *cpITS* sequences and the first and second codon positions of *rbcL* genes (Goremykin et al. 1996) also showed no evidence for a sister group relationship between angiosperms and Gnetales. Therefore, morphological resemblances between Gnetales and angiosperms had better be considered as homoplasy; and the inclusion of Gnetales with cycads in the subclass Cycadopsida (Meyen 1984) or the subdivision Cycadophytina (Page 1990) seems inappropriate.

With more sequences incorporated in the present study, the monophyly of the conifer families, which was supported by previous studies (Hart 1987; Raubeson and

Jansen 1992; Chaw et al. 1993, 1995), is strengthened. Our data further suggest that Pinaceae is the earliest and the Araucariaceae-Podocarpaceae-Phyllocladaceae clade is the next to split off from the rest of the conifers, and that Sciadopityaceae is an outgroup to the clade containing Taxaceae, Cephalotaxaceae, Taxodiaceae, and Cupressaceae. These familial relationships are congruent with the analysis of Price et al. (1993) using *rbcL* sequences. Page (1990) and Brunsfeld et al. (1994) have enumerated a number of the distinctive features from embryology (Lawson 1910), wood anatomy (Peirce 1936), pollen morphology (Ueno 1951), chromosome number (Schlarbaum and Tsuchiya 1984), phloem protein (Alosi and Park 1983), and immunology (Price and Lowenstein 1989) to maintain *Sciadopitys* as a separate family, Sciadopityaceae, apart from the Taxodiaceae. The nuclear 18S rRNA sequence data provide additional evidence for this proposal.

So far 12 genera—*Abies*, *Cathaya*, *Cedrus*, *Hesperopeuce*, *Keteleeria*, *Larix*, *Nothotsuga*, *Picea*, *Pinus*, *Pseudolarix*, *Pseudotsuga*, and *Tsuga*—have been assigned to the family Pinaceae (Page 1988, 1990). Grouping of these genera was uncertain and previous researchers have emphasized different characters such as the possession or absence of short shoot, the foliage morphology and the position of strobili (Pilger 1926; Melchior and Werdermann 1954), the leaf phenolics (Niemann and Van Genderen 1980), the cladistic analysis of morphological data (Hart 1987), and the structure of cone and seed (Frankis 1988). The monophyly of *Larix* and *Pseudotsuga* inferred in the present analysis is congruent with the conclusion of Niemann and Van Genderen (1980), Hart (1987), and Frankis (1988) derived from morphological data, the phylogenetic analysis of *rbcL* sequences by Chase et al. (1993), and the karyotype study of Li (1993, 1995), but does not agree with the classification of Pilger (1926), Melchior and Werdermann (1954) and many others who placed *Pseudotsuga* with *Abies* and some other four genera in the subfamily Abietoideae. The nuclear 18S rRNA sequence data strongly indicate that *Abies* is closer to *Pinus* than to the *Larix-Pseudotsuga* clade, which is concordant with the study of Niemann and Van Genderen (1980), but contradicts the view (Hart 1987; Frankis 1988) that *Abies* is closer to subfamily Laricoideae (including *Larix*, *Cathaya*, and *Pseudotsuga*) than to *Pinus*. Because of the few sequences analyzed, the subfamilial relationships within the Pinaceae could not be inferred conclusively.

According to Khoshoo's (1961, 1962) karyological studies, the ancestral state of the chromosome number in conifers is highly likely to be $x = 12$, which is also preserved in Pinaceae, Cephalotaxaceae, *Taxus*, and ginkgo, and the chromosome counts of $x = 11$ in *Torreya*, Cupressaceae, and Taxodiaceae; of $x = 10$ in *Sciadopitys*; and of $x = 7$ in *Amentotaxus* (Chuang and Hu 1963) may represent derived conditions. This trend of reduction in chromosomal number seems to correlate well with the divergence pattern depicted in the nuclear 18S rRNA trees (figs. 1 and 2), which shows Pinaceae to be an outgroup to the other conifers, connoting the ancient status of the family and the chromosomal re-

duction experienced by the terminal clade Sciadopityaceae-Taxaceae-Cephalotaxaceae-Taxodiaceae-Cupressaceae.

Page (1990, p. 305) opposed a total merger of Cupressaceae and Taxodiaceae, arguing that the two families "differ in many fundamental aspects of vegetative and reproductive morphology, such as the decussate scale-leaf habit, . . . which is not developed in the Taxodiaceae." In contrast, on the basis of *rbcL* sequences, Brunsfeld et al. (1994) claimed that the two families form a monophyletic group, and Cupressaceae was derived from the Taxodiaceae. They also quoted evidence from Eckenwalder's (1976) phenetic analysis, Hart's (1987) cladistic analysis, and Price and Lowenstein's (1989) immunological studies to strengthen their view. Our NJ tree (fig. 1) indicates that the cupressaceous and taxodiaceous genera are separate lineages, whereas the MP tree suggests that the two families are paraphyletic. Therefore, their relationships cannot be resolved by the nuclear 18S rRNA sequences.

Phylogeny Within the Extant Angiosperms

The monophyly of angiosperms is not only strongly supported by the present nuclear 18S rRNA sequence data, but also by the cladistic analysis of morphological data (Crane, Friis, and Pedersen 1995 and references therein) and the parsimony analyses of molecular data (Hamby and Zimmer 1992; Chase et al. 1993; Goremykin et al. 1996) and combined morphological and molecular data (Doyle, Donoghue, and Zimmer 1994; Albert et al. 1994). Within the angiosperms, no significant cluster at the level of subclass was recovered by using the nuclear 18S rRNA sequences (fig. 2). However, the basal position of Nymphaeaceae (i.e., an outgroup to the other angiosperms) and the derived status of *Magnolia* and *Ceratophyllum* deserve attention. This was also supported by Doyle, Donoghue, and Zimmer's (1994) analysis of combined morphological and rRNA sequence data and Goremykin et al.'s (1996) analysis of *cpITS* sequence data, but is contradictory to the view that *Ceratophyllum* represents the basal clade of extant angiosperms (Les, Garvin, and Wimpee 1991; Chase et al. 1993; Qiu et al. 1993; Albert et al. 1994; Endress 1994). Although the phylogenetic positions of *Magnolia* and *Ceratophyllum* are ambiguous (figs. 1 and 2), these two genera are clearly nested within the angiosperms rather than being a sister group to the remaining angiosperms. In their analysis of *rbcL* genes, Chase et al. (1993) suggested that the major division of the angiosperms is not monocots versus dicots, but the uniaperturate pollen type versus the triaperturate type, the latter being termed eudicots. Doyle, Donoghue, and Zimmer (1994) and Albert et al. (1994) also found eudicots to be monophyletic. However, these findings are not supported by the present study.

In both the NJ and MP trees the Piperales, including Piperaceae, Saururaceae, and Chloranthaceae, are revealed as a highly monophyletic order. The monophyly of the former three families and a close affinity between the second and third families are strongly supported by the present data, which add additional evidence to the

view of Burger (1977), who retained Chloranthaceae in the Piperales rather than in the Laurales on the basis of morphological and floral features. Cronquist (1988) considered that Chloranthaceae is taxonomically isolated from Piperaceae and Saururaceae, but still defined the Piperales as comprising the above three families. In contrast, Okada (1995) provided karyological data and quoted much other evidence from morphology (e.g., Endress 1986, 1987), cladistic analysis (Donoghue and Doyle 1989), and *rbcL* gene data (Chase et al. 1993, Qiu et al. 1993) to argue for a distant affinity between Chloranthaceae and the other two Piperales families. These conflicts cannot be resolved at present.

The monocots included in the present study are nested, as a monophyletic group, within the remaining larger clade of angiosperms, suggesting that these monocots were derived from dicots. On the basis of *cpITS* sequences and *rbcL* genes, Goremykin et al. (1996) also placed the monocots within the dicots, in agreement with our results. Our data also reject the view (Burger 1977; Taylor and Hickey 1992) that Piperaceae and Chloranthaceae are basal among the most primitive angiosperms and that the monocots were derived from them. Therefore, the rRNA sequence data lend support to Cronquist's (1988, p. 451) view that "the single cotyledon, parallel-veined leaves, absence of a cambium, the dissected stele, and the adventitious root system of monocots are all regarded as apomorphies within the angiosperms, and any plant more primitive (plesiomorphic) than the monocots in these several respects would certainly be a dicot."

In conclusion, these analyses provide further evidence for the utility of nuclear small-subunit rRNA sequences in addressing relationships among living gymnosperms, especially at the taxonomic level of families and above. In contrast, this molecule should be used with caution for phylogenetic studies of angiosperms because the internal branch lengths leading to each clade within the angiosperms are comparatively short.

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