Relationships of the Wollemi Pine (*Wollemia nobilis*) and a molecular phylogeny of the Araucariaceae

S. Gilmore and K.D. Hill

Abstract

Gilmore, S.[1] *and Hill, K.D. (National Herbarium of New South Wales, Royal Botanic Gardens, Sydney, NSW 2000, Australia;* [1] *Present address: Division of Botany and Zoology, Australian National University, Canberra, ACT 0200, Australia)* 1997. *Relationships of the Wollemi Pine (*Wollemia nobilis*) and a molecular phylogeny of the Araucariaceae*. Telopea *7(3): 275–291*. Sequences were obtained for the *rbc*L gene from chloroplast DNA of the newly discovered Australian conifer *Wollemia nobilis* (Araucariaceae), 5 species of *Araucaria* and 4 species of *Agathis*. Phylogenetic analysis of our new data and other available sequences indicate that 1) Araucariaceae is monophyletic; 2) *Agathis* and *Araucaria* are both monophyletic; 3) *Wollemia* is the sister group to *Agathis*; 4) the Pinaceae are the sister group to all other conifers, although the monophyly of the conifers is not unequivocally demonstrated. The utility of *rbc*L for deriving a robust phylogeny of the Araucariaceae and of the conifers is discussed.

Introduction

A previously unknown conifer recently discovered in the Wollemi National Park (New South Wales, Australia) has been described as a new species, *Wollemia nobilis* W.G. Jones, K.D. Hill & J.M. Allen, placed in a new genus in the family Araucariaceae (Jones et al. 1995). The new monotypic genus brings the total number of accepted extant genera in the family to three (with *Araucaria* Juss. and *Agathis* Salisb.). This study aimed to test the monophyly of the family, of *Araucaria* and *Agathis* , and to resolve the phylogenetic relationships of *Wollemia nobilis*. This would allow assessment of differences in morphological characters in a phylogenetic context.

Our approach has been to sample DNA sequences of a genetic marker and phylogenetically analyse them (Hillis & Moritz 1990). Here we sampled sequences from the plastid-encoded gene for the large subunit of ribulose bis-phosphate carboxylase (*rbc*L). This gene has been the subject of intensive study in recent years (Olmstead & Palmer 1994, Clegg & Zurawski 1992, Hillis & Moritz 1990, Palmer et al. 1988 for reviews) and has proved useful at inter-family and intra-family levels for a number of groups such as the Saxifragaceae s.l. (Morgan and Soltis 1993), Asteraceae (Kim et al. 1992), and an analysis of the primary lines of descent of the angiosperms (Chase et al. 1993). Gadek and Quinn (1993) used *rbc*L sequences to test relationships in the conifer family Cupressaceae sens. str., producing results that are congruent with results from other data (chemical, morphological and anatomical); however, support is low for some groupings of taxa due to the low rate of *rbc*L divergence.

DNA from leaf tissue of *Wollemia nobilis* and several species of both *Araucaria* and *Agathis* was sequenced, and included in the analyses with sequences for other conifers obtained from international databases, to gather evidence to position the Araucariaceae relative to the other conifers. We used this information to select suitable outgroups to test relationships within the family, using different tree building algorithms and different data-weighting regimes.

Extant and fossil Araucariaceae

All members of the Araucariaceae are trees, some species growing to over 60 m tall, though some species are never larger than 10 m. Leaves are spirally arranged with parallel veins and the medium- to large-sized cones are subglobose to ovoid, hard and woody, with each of numerous fused bract-scales bearing one seed (Dallimore et al. 1966). *Wollemia* is placed within the Araucariaceae by the following characters: adult leaves broad, spirally arranged, with several parallel veins and no distinct midvein, microsporophylls peltate with 4–9 pendulous microsporangia, pollen wingless, and female strobili with numerous fused bract-scale complexes, each with a single inverted ovule that develops into a dry winged seed (Jones et al. 1995). Of these characters, the peltate microsporophylls with pendulous microsporangia are unequivocally apomorphic at the family level. The structure of the female cone is unique to the family, but features both apomorphic and plesiomorphic characters (Hart 1987). *Wollemia* is monoecious, as are *Agathis* and *Araucaria* (Jones et al. 1995, Dallimore et al. 1966).

The modern distribution of the Araucariaceae is almost exclusively Southern Hemisphere (Fig. 1). However, the Mesozoic fossil record suggests that the family once had a greater range, extending widely through the Northern Hemisphere. Although many of the leaf, pollen and wood fossils lack clear araucarian apomorphies and may be merely superficially similar, the distinctive female cones are well-known from many localities in the Northern Hemisphere (Stockey 1994). Currently, the greatest number of species of *Araucaria* occur in New Caledonia, but the New Caledonian taxa are a closely related group within one section of the genus (R. Henry, pers. comm.), consistent with this being a local radiation rather than a centre of origin.

It has been suggested that the Araucariaceae appeared in the late Triassic, with a peak of diversity in the Jurassic and a continued decline since the end of the Cretaceous (Miller 1977). Fossils with some *Araucaria*-like features have been recorded from the Triassic (Virginia, USA); however, their affinities to the family are unclear (Stockey 1982). A fossil record showing the female cone apomorphies of *Araucaria* dates to the Jurassic (Miller 1977, Stockey 1981, Stockey 1982, Stockey 1994), and represents one of

Fig. 1. Present-day distribution of the family Araucariaceae.

the oldest extant genera known (Stockey 1981). The fossil record suggests that the genus *Araucaria* had emerged, as had all extant sections, during the Jurassic (Stockey 1981, Stockey 1994), although many of the identifications have been based on sets of unpolarised similarities rather than synapomorphies. Fossils regarded as *Araucaria* have been found in Cretaceous deposits in Japan, as well as Jurassic deposits in England and North America (Stockey 1994), although placement of some of these within extant sections has proved problematic, not least because of the lack of diagnostic apomorphic characters in some of the material. Pollen with *Araucaria*-like characters is also widespread in the Jurassic and Cretaceous of both northern and southern hemispheres, although clear synapomorphies with extant genera are again lacking (Hughes 1969).

The fossil record for *Agathis* is less extensive than that for *Araucaria*, and is restricted to Australia and New Zealand, in Cretaceous to Miocene sediments (Stockey 1982, Cantrill 1992). A fossil described as *Agathis jurassica* from the Jurassic Talbragar Fish Bed Flora in New South Wales (White 1981) was considered the oldest of these but more recent studies indicate that the fossil is not in fact an *Agathis* (Stockey 1990, 1994) It has been suggested that *Agathis* evolved in Gondwana at some time in the late Jurassic or early Cretaceous (Hill 1995). Again, however, no phylogenetic analysis of the characters used to place these fossils in the genus *Agathis* has been made, and they may merely represent shared plesiomorphies.

A form-genus *Araucarioides*, which shares some characters with *Araucaria* and others with *Agathis*, is represented in deposits from the Cretaceous to the Oligocene (Hill 1995, M. Pole pers. comm.). *Araucarioides* would appear to be distinct from *Wollemia* in possibly possessing an abscission layer in the leaf petiole, a feature found in *Agathis* but not *Araucaria* (M. Pole pers. comm.). A pollen form-genus *Dillwynites* that closely resembles the pollen of *Wollemia* is known from mid-Cretaceous to Recent deposits, often in close association with *Araucarioides* leaf fossils (Macphail et al. 1995). Establishment of relationships of both *Araucarioides* and *Dillwynites* has been again on the basis of general phenetic similarities and not phylogenetic assessment of individual characters.

Since the general decline of the conifer biota towards the end of the Cretaceous and throughout the Tertiary, the family Araucariaceae was thought to have become extinct in south-eastern Australia until the discovery of *Wollemia* (Jones et al. 1995, Kershaw & McGlone 1995). *Wollemia* thus represents an isolated relic of a once extensive araucarian range, which may provide valuable information on evolution and biogeography of the Araucariaceae and of the Gondwanan biota generally.

A cladistic analysis of conifers at both family and genus levels using a comprehensive data set including morphological, anatomical and chromosomal characters placed the Araucariaceae as the sister group to a clade made up of Cupressaceae and Sciadopitys (Hart 1987). Other studies have differed considerably, placing the Araucariaceae as the sister to Pinaceae (Miller 1988; Nixon et al. 1994), although differing markedly from each other in the relationships of the other conifer families (Fig. 2). Molecular studies using 18S rRNA gene sequences have suggested a sister group relationship to the Podocarpaceae (Chaw et al. 1995). In all cases high degrees of homoplasy obscure resolution of relationships at the generic level.

Materials and methods

Plant material: fresh leaf material of *Wollemia nobilis* was collected from two widely separated trees of one wild population (trees tagged 18 and 19 by New South Wales National Parks and Wildlife Service). Fresh leaf material of *Araucaria angustifolia*

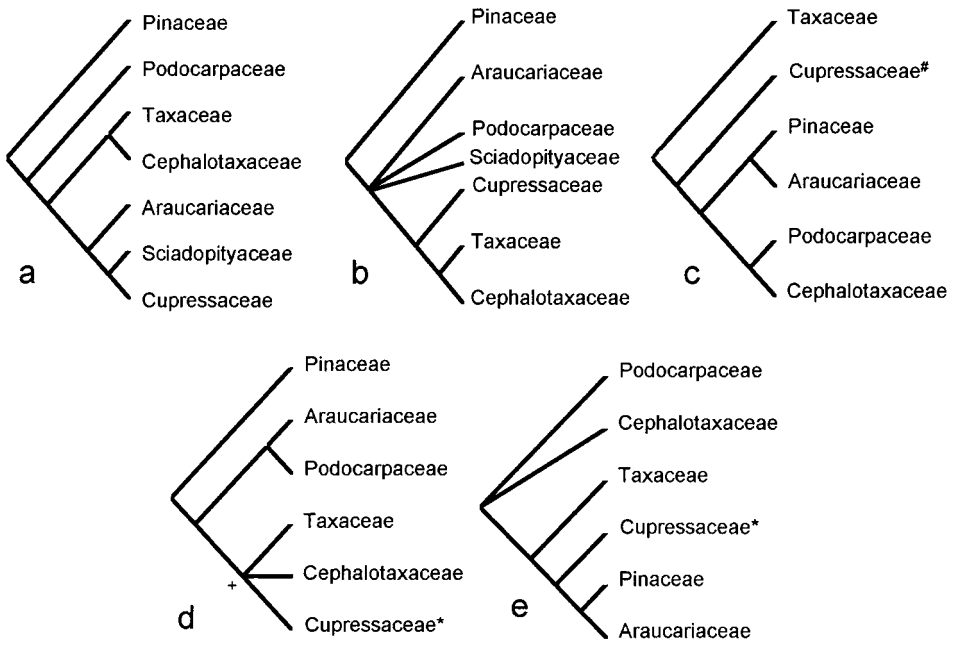

Fig. 2. Trees of relationships within the conifers. **a,** Hart 1987. **b,** Price 1993. **c,** Miller 1988. **d,** Chaw et al. 1995. **e,** Nixon et al. 1994. Analyses d and e did not include *Sciadopitys*.

* sens. lat., including Taxodiaceae

including Taxodiaceae and *Sciadopitys*

+ this analysis shows Taxaceae to be paraphyletic, although no clear resolution is evident at this node

O. Kuntze, *A. bidwillii* Hook., *A. cunninghamii* Aiton ex D. Don, *A. hunsteinii* K. Schumm. et Hollr., *A. muelleri* (Carr.) Brongn. et Gris, *Agathis ovata* (Moore ex Viell.) Warb., *A. lanceolata* Lindl. ex Warb., *A. moorei* (Lindl.) Mast., and *A. macrophylla* (Lindl.) Mast. was gathered from living collections at the Royal Botanic Gardens, Sydney, and voucher specimens deposited at the National Herbarium of New South Wales (NSW). An additional unpublished sequence for *Agathis robusta* was provided by C. Quinn, University of New South Wales. The species analysed are listed in Table 1 along with accession information.

DNA extraction: total genomic DNA was extracted from fresh leaves by an adaption of the method of Doyle and Doyle (1990) following grinding in liquid nitrogen, and was purified by the diatomite method of Gilmore et al. (1992). An additional phenol: chloroform:isoamyl alcohol (25:24:1) extraction was performed following the elution of the DNA from the diatomite matrix, and the DNA reprecipitated. Purified DNA was dissolved in Milli-Q (Millipore) purified water or TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer and stored at -20°C.

Polymerase chain reaction: bases 18 to 1428 of the *rbc*L coding region were amplified as well as the region to 41 base pairs downstream of the gene. The entire region, or a series of overlapping fragments which together covered the equivalent region, were amplified. Primers are listed in Table 2 along with their positions relative to *rbc*L. Amplifications were conducted on either a FTS-1S Capillary Thermal Cycler (Corbett Research) or a Omn-E Thermal Cycler (Hybaid Ltd.). For the FTS-1S, reactions were

Table 1. Species sampled and GenBank accession numbers.

Taxon	RBG (Sydney) Accession	GenBank Accession
Agathis		
A. robusta	Partial sequence supplied by CJ Quinn (UNSW)	
A. ovata	13935 (living collection)	U87754
A. moorei	871593 (living collection)	U87755
A. macrophylla	13930 (living collection)	U87756
Araucaria		
A. angustifolia	911936 (living collection)	U87750
A. bidwillii	15780 (living collection)	U87751
A. hunsteinii	15820 (living collection)	U87749
A. cunninghamii	15819 (living collection)	U87752
A. muelleri	871351 (living collection)	U87753
Wollemia		
W. nobilis (tree 18)	NSW392980 (herbarium)	U87757
W. nobilis (tree 19)	NSW392981 (herbarium)	Not submitted

Table 2. Primers used.

Primer code	Strand	Sequence
rbcL1	→	5′gggatttatgtcaccacaaacaga
861	→	5′tggaccactgtttggaccga
381	→	5′gcagttattgacagacaaaagaaatcatggt
635	← (downstream of rbcL)	5′cttttagtaaaagattgggccgag
496	←	5′ccccaagggtgtcctaaagttcctccaccaaa
497	←	5′accatgattcttctgcctatcaataactgc

carried out on a 18 µL scale (1–10 ng DNA, 0.2 mM each of dNTPs, 4 pmol each primer, 2µL PCR Reaction Buffer with Mg++ (Boehringer Mannheim) and 1.25U Taq DNA polymerase (Boehringer Mannheim)). The best amplifications were achieved using a touchdown PCR protocol (Don et al. 1991) with an initial denaturation step of 94°C for 3 min, followed by annealling for 30 seconds at 64°C and chain extension at 72°C for 1min 50s. In successive cycles the denaturation step was 94°C for 30s and the extension step was retained, but the annealling temperature was successively dropped 2°C after the 1st, 3rd, 5th, 8th, 12th, 16th and 20th cycles. After 20 cycles the annealling temperature was 50°C, and this was retained for the final 15 cycles. Following cycling, an additional extension step (72°C, 5 min) was added.

The amplification mix for the Omn-E was as for the FTS-1S scaled up to 100 µL. The touchdown protocol was less effective on the Omn-E and different cycling parameters were used. Following an initial denaturation of 94°C for 3 minutes, each cycle consisted of a denaturation step of 94°C for 30s, annealling at 50°C for 30s, and chain extension at 72°C for 1 min 50s. Following cycling an additional extension step (72°C, 5 min) was added. Following PCR, products were visualised by electrophoresing an aliquot in 0.8% agarose stained with ethidium bromide. Most of the desired products were washed of residual nucleotides and primers by the addition of 300 µL sterile water with reconcentration in a Microcon-100 column (Amicon) according to the manufacturer's protocol. From several amplifications, small extraneous products (in concentrations too low to be detected on a stained gel) were preferentially cloned. In these cases, target DNA bands were excised from agarose following electrophoresis of the total product in a 0.8% agarose gel. The band was then purified using the Qiaquick gel extraction kit (Qiagen), according to the manufacturer's instructions.

Cloning and sequencing of PCR products: while some PCR products were directly sequenced, most amplification products were ligated into a vector (pGEM.T) which exploits the tendency of Taq DNA polymerase to adenylate 3' ends of double stranded DNA (Clark 1988). Bacteria were transformed using the pGEM.T Vector System II kit (Promega), with half the manufacturer's specified volumes. The host bacterial strain was JM109. Transformants were screened for insert size (Anonymous 1991) and plasmid DNA was recovered by the alkali lysis method with purification by polyethylene glycol (Sambrook et al. 1989). Alternatively, some plasmids were purified using the Jetstar miniprep system (Genomed). The purified plasmid was used as a template for sequencing with an automatic sequencer (ABI 373A) and Taq Dye Primer and Taq DyeDeoxy Terminator cycle sequencing kits (Perkin Elmer) at Westmead Hospital (Sydney, Australia). Trace and data files were returned for alignment and editing with the aid of the Sequencher vers. 2.1 and 3.0 software packages (Gene Codes).

Phylogeny reconstruction: the *rbcL* gene is highly conserved across the group studied, and sequences were readily aligned by eye. We employed the maximum parsimony method of Fitch (1977) and Maximum Likelihood Estimations (Felsenstein 1981, 1993) with the computer package Phylogenetic Analysis Using Parsimony (PAUP*) version 4 (Swofford unpublished, used with the author's permission). Trees generated by PAUP* were exported into MacClade 3.06 (Maddison & Maddison 1996) to prepare trees for publication. Each individual base position was coded as an unordered character, and missing data were treated as unknown. Sequences for *rbc*L of various conifers, *Gingko biloba* and several angiosperms were recovered from GenBank (see Table 3), to test the monophyly of the family and to select suitable outgroups for a study of generic affinities within the family. Where the data base sequences were longer than our own we trimmed them to correspond to the region we had amplified, and where they were shorter we treated the missing region(s) as unknown.

Parsimony analysis: to test the monophyly of the family Araucariaceae we aligned our 10 sequences with those recovered from GenBank (table 3). When the sequences were aligned, base substitutions at third codon positions were observed approximately three times as often as first or second codon position changes (data not shown). We used this information to apply two different weighting schemes to our data. Firstly we used equal weighting for all characters, and then we weighted first, second and third codon positions in the ratio 3:3:1, with characters from the non-coding region remaining unweighted. The cycads were set as outgroups (Loconte and Stevenson 1990) for heuristic searches conducted using the simple sequence addition option, with tree bisection-reconnection (TBR) branch swapping. Support for the groupings recovered under each weighting scheme was tested by performing one hundred jackknife replicates with deletion of 50% of characters each replicate.

Table 3. *rbcL* sequences recovered from GenBank.

Nomenclature follows GenBank records, but note that most Taxodiaceae, including *Cunninghamia*, are now subsumed within Cupressaceae, and *Sciadopitys* is placed in the monogeneric family Sciadopityaceae.

Taxon	GenBank accession
Cycadaceae	
Cycas circinalis	L12674
Bowenia serrulata	L12671
Ginkgoaceae	
Ginkgo biloba	D1073
Pinaceae	
Pinus pinea	X58133
Pseudotsuga menziesii	X52937
Podocarpaceae	
Podocarpus gracilior	X58135
Taxaceae	
Amentotaxus argotaenia	L12580
Cupressaceae	
Callitris rhomboidea	L12537
***Taxodiaceae**	
**Cunninghamia lanceolata*	L25757
**Sciadopitys verticillata*	L25753
Magnoliaceae	
Magnolia hypoleuca	L12655
Gnetaceae	
Gnetum gnemon	L12680
Gnetum leyboldii	U72820
Papaveraceae	
Papaver orientale	L08764
Poaceae	
Oryza sativa	D00207

Relationships within the Araucariaceae were then more closely examined, using four Agathis terminals (different species), five Araucaria terminals (different species representing four sections), two *Wollemia* terminals (two individuals of the same species), with *Podocarpus* and *Callitris* selected as outgroups on the basis of their position relative to the Araucariaceae in the larger analysis (note that besides *Callitris*, both *Amentotaxus* and/or *Cunninghamia* would have been equally suitable outgroups). By this strategy we sought to avoid artefacts that may have been introduced by using an outgroup too distant from the Araucariaceae. We once again noted a bias in favour of third codon position substitutions, with such changes being observed three times as often as first or second codon position substitutions. The initial analysis utilised a branch-and-bound search using the furthest addition sequence option with all characters weighted equally. The analysis was then repeated after weighting first, second and third codon positions in the ratio 3:3:1 (the non-coding region was unweighted). Support for the groupings under each weighting scheme was tested by performing one hundred jackknife replicates with deletion of 50% of characters each replicate.

Maximum likelihood: relationships within the Araucariaceae were reanalysed using the maximum likelihood criterion for optimality. As before, the same two outgroup

taxa were selected by their position in the larger analysis. Initially, equal nucleotide frequencies were set, with all sites assumed to evolve at the same rate with the molecular clock hypothesis not enforced. Secondly, nucleotide frequencies were set to those observed in the data and the transition/transversion ratio set to 2. All sites were set to evolve at the same rate and the molecular clock was not enforced. Support was tested using jackknife values for the observed groupings.

Results

Ten complete sequences were obtained for the Araucariaceae (Table 1). In all cases the length of coding region sequenced was the same (1411 bp), as was the downstream region (41 bp) . With the data set extended to include sequences from GenBank, we used maximum parsimony to find trees from both unweighted (Fig. 3a) and weighted (3:3:1) characters (Fig. 3b). With both searches, 4 most parsimonious trees were found, differing only in resolution within the Araucariaceae. *Podocarpus* was placed in a sister group position to the Araucariaceae, with a jackknife value of 65 with unweighted characters. The Araucariaceae–Podocarpaceae clade was placed as sister to a clade comprising Cupressaceae (sens. lat.) and Taxaceae. The analysis with weighted characters differed only in the resolution within *Araucaria* to this point, but differed dramatically below this node in placing Pinaceae as the sister to the all other conifers, and the conifers as a monophyletic group. In contrast, the unweighted analysis suggested that the conifers may not be monophyletic.

The small data set (Araucariaceae, *Podocarpus* and *Callitris*) had 1452 characters of which 269 were variable and 94 were potentially parsimony-informative. With the codon positions unweighted, 4 most parsimonious trees of length 324 were recovered (Fig. 4a). According to this result, the Araucariaceae are monophyletic with *Araucaria* the sister group to the remainder of the Araucariaceae, and *Wollemia* the sister group to *Agathis*. There was strong jackknife support for most of these groupings (jackknife values of 100, 91, 55 and 100 repectively for Araucariaceae, *Araucaria*, *Wollemia* + *Agathis*, *Agathis*, Fig. 4a). While the data could not resolve relationships among the terminal *Agathis* taxa, some resolution was observed within *Araucaria*. The genus split into 2 groups, the first containing *A. angustifolia*, *A. bidwillii* and *A. hunsteinii* (jackknife value 68; with further resolution to an *A. bidwillii* and *A. hunsteinii* clade, itself with a jackknife value of 65), and, secondly, *A. cunninghamii* grouped with *A. muelleri* (jackknife value 93).

When codon positions were weighted in the ratio 3:3:1 (512 characters had weight 1 and 940 had weight 3), 4 most parsimonious trees were recovered, of length 524. The strict consensus tree (Fig. 4b) shows that *Agathis* and *Araucaria* each form monophyletic groups, with no resolution within *Agathis*. Within *Araucaria*, the clades consisting of 1) *A. bidwillii* and *A. hunsteinii,* and 2) *A. cunninghamii* and *A. muelleri* were retained, but *A. angustifolia* was placed as sister to the *A. cunninghamii* + *A. muelleri* clade.

Under Maximum Likelihood criteria, we recovered trees with similar topology to those recovered with the parsimony searches with unweighted characters. Assuming equal nucleotide frequencies with no weighting for transitions and transversions and all sites evolving at the same rate, the best tree had a score of 3910 (Fig. 4c). *Agathis* and *Araucaria* both formed separate monophyletic groups, and *Araucaria* once again split into two groups. The first (jackknife value 83) has *A. angustifolia* sister to a clade containing *A. bidwilli* and *A. hunsteinii* (jackknife value 69). The second group had *A. cunninghamii* and *A. muelleri* as a clade with a jackknife value of 95. This tree was identical in topology to one of the parsimony trees when codon positions were

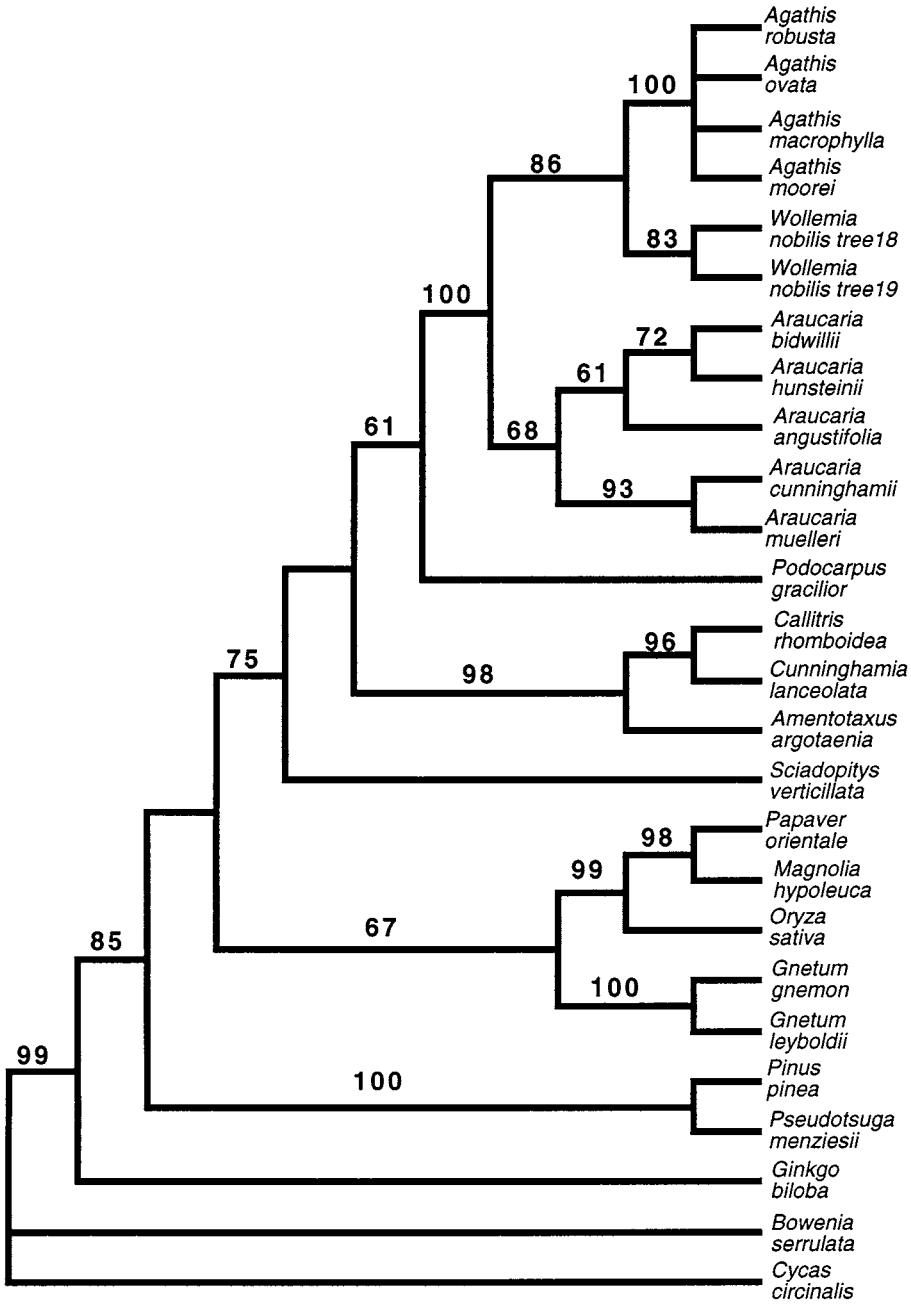

Fig. 3a. Large data set. Strict consensus tree, parsimony, unweighted. Numbers displayed above branches are jackknife (50% character deletion) support values.

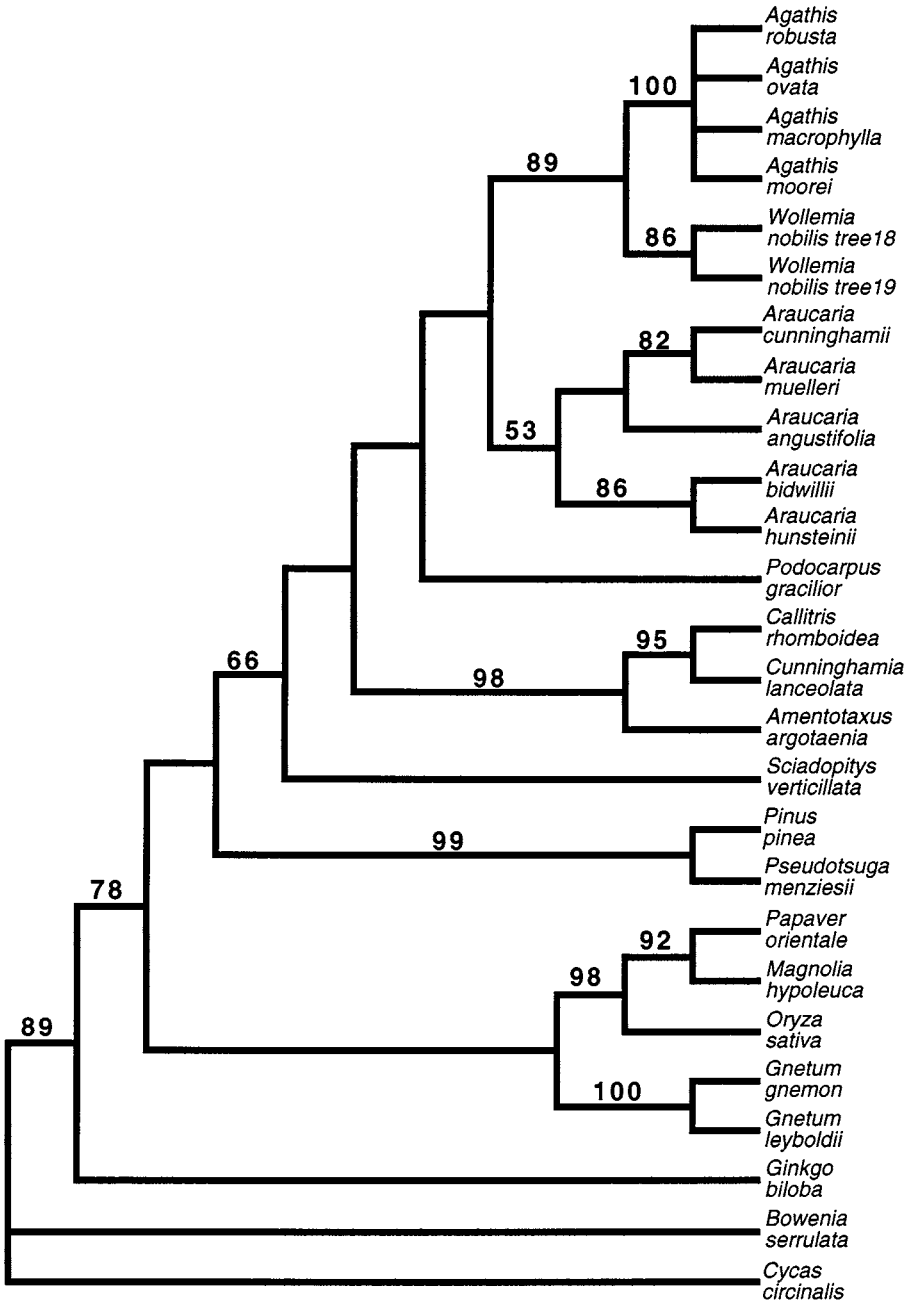

Fig. 3b. Large data set. Strict consensus tree, parsimony, weighted.

unweighted. In general, equal or stronger jackknife support was found for groupings than was found under parsimony.

The same topology was produced when nucleotide frequencies were estimated from the data and the transition to transversion ratio set to 2, although this tree differed in having a likelihood score of 3815, and slightly different jackknife support values (Fig. 4d).

Discussion

Our analysis strongly supported the monophyly of the family Araucariaceae and of *Agathis* and *Araucaria*. The phylogenetic position of *Wollemia* is thus concordant with its description (Jones et al. 1995) as a new genus in that family. An alternative classification, consistent with our phylogenetic results, would be to treat *Wollemia nobilis* as a species of *Agathis*. However, the level of jackknife support for the *Wollemia* + *Agathis* clade (55 to 89 in our analyses) is much lower than that for *Agathis* (100 in all analyses). Therefore, the recognition of *Wollemia* as a distinct genus is preferable on grounds of likely taxonomic stability.

Both *Wollemia* and *Agathis* are known only from Australasia and the south-west Pacific as either extant plants or fossils. This suggests that a radiation occurred within the Araucariaceae in the later Mesozoic and possibly early Tertiary in the Australasian precinct of Gondwana.

The resolution within *Araucaria* is slight, but some support is shown for the placement of the scale-leaved species as a monophyletic group (Section *Eutacta*, represented by *A. cunninghamii* and *A. muelleri*) as the sister to the broad-leaved species groups, which themselves then constitute a monophyletic group. This latter is the only group within the Araucariaceae to possesses hypogeal germination (Burrows et al. 1993), previously suggested to be an apomorphic character (Stockey 1994), and now corroborated by the molecular data.

Araucariaceae is placed as sister to the Podocarpaceae, and this clade in turn is placed as sister to a clade made up of Cupressaceae (sens. lat.) and Taxaceae. This is in overall agreement with Chaw et al. (1995) and Price et al. (1993), but differs from Hart (1987), Nixon et al. (1994) and Miller (1988). However, the analysis published by Miller was based on a limited number of characters from one organ only (the seed cone) and that of Hart showed a high level of homoplasy. Nixon et al. used only 5 characters that were informative within the conifer clade.

By including other conifer taxa in our data set we were able to show that, while *rbc*L did not give good resolution at the intrageneric level within the Araucariaceae, we could build a phylogeny within the conifers that agrees with data from some other sources. While some relationships are not fully resolved and sampling is minimal within groups, it appears that:

1. the Podocarpaceae is the sister to the Araucariaceae,

2. Cupressaceae (sens. lat.) and Taxaceae are part of a robust clade that is the sister to the Podocarpaceae–Araucariaceae clade, and

3. Pinaceae is separate from all other extant conifer families, either as the basal branch or a separate clade.

The analysis of the primary lines of descent of the angiosperms using *rbc*L sequences by Chase et al. (1993) suggested that the conifers were paraphyletic. However, the analyses of Price et al. (1993) and Savard et al. (1994) using the same gene and a larger number of conifer taxa support the monophyly of the conifers. Our results do not

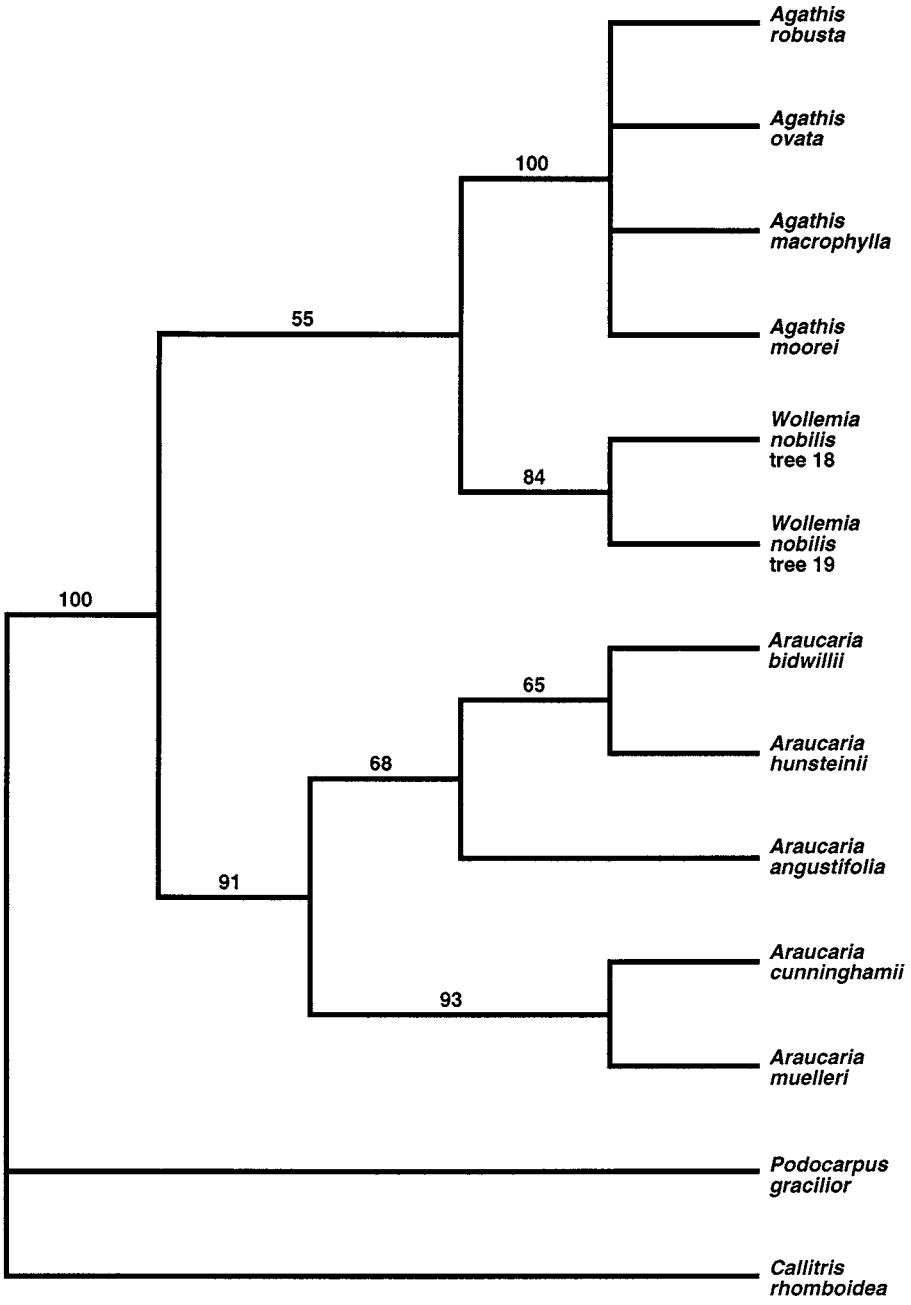

Fig. 4a. Small data set. Strict consensus tree, parsimony, unweighted. Numbers displayed above branches are jackknife (50% character deletion) support values.

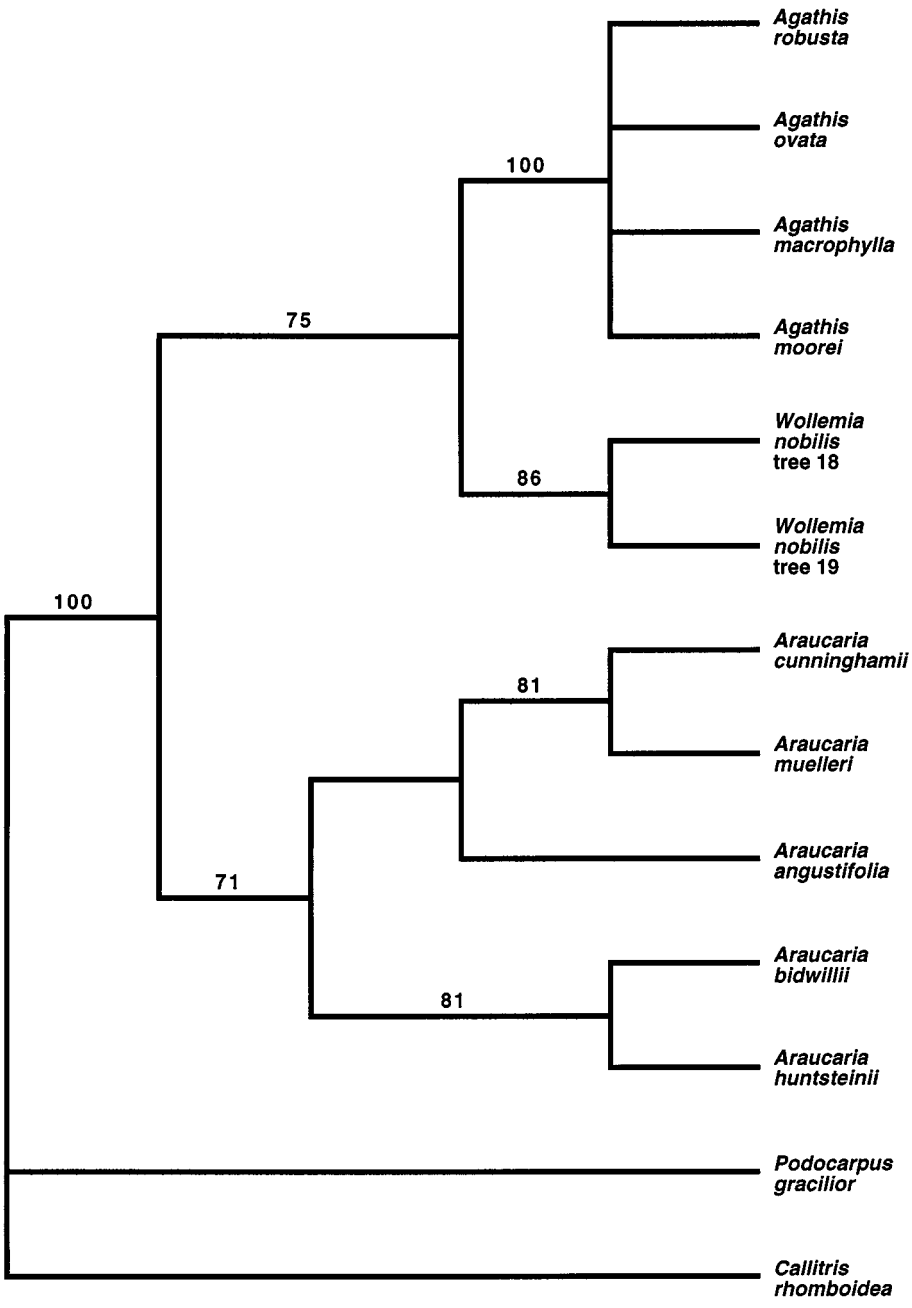

Fig. 4b. Small data set. Strict consensus tree, parsimony, weighted.

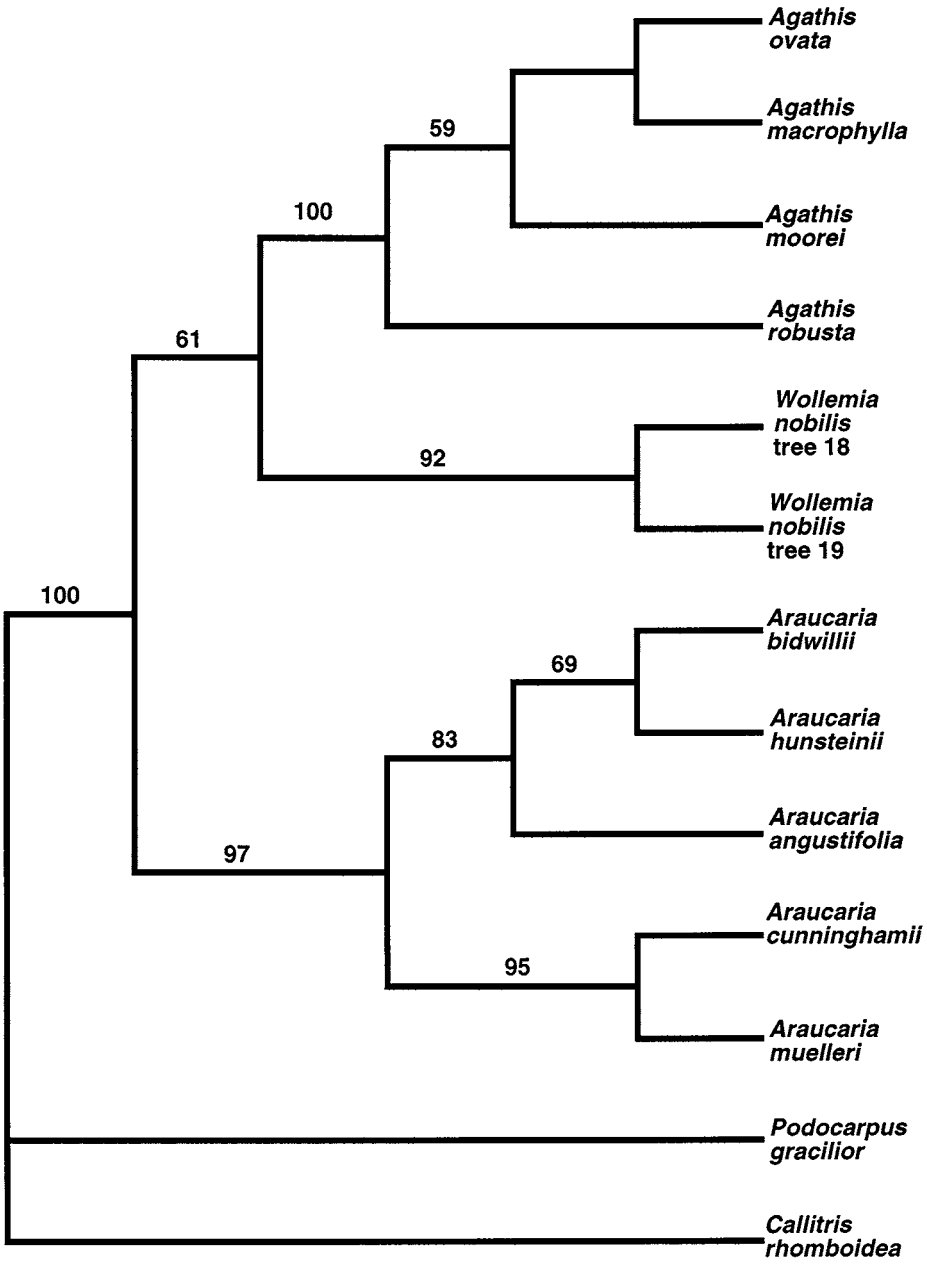

Fig. 4c. Small data set. Maximum likelihood tree, unweighted. Numbers displayed above branches are jackknife (50% character deletion) support values.

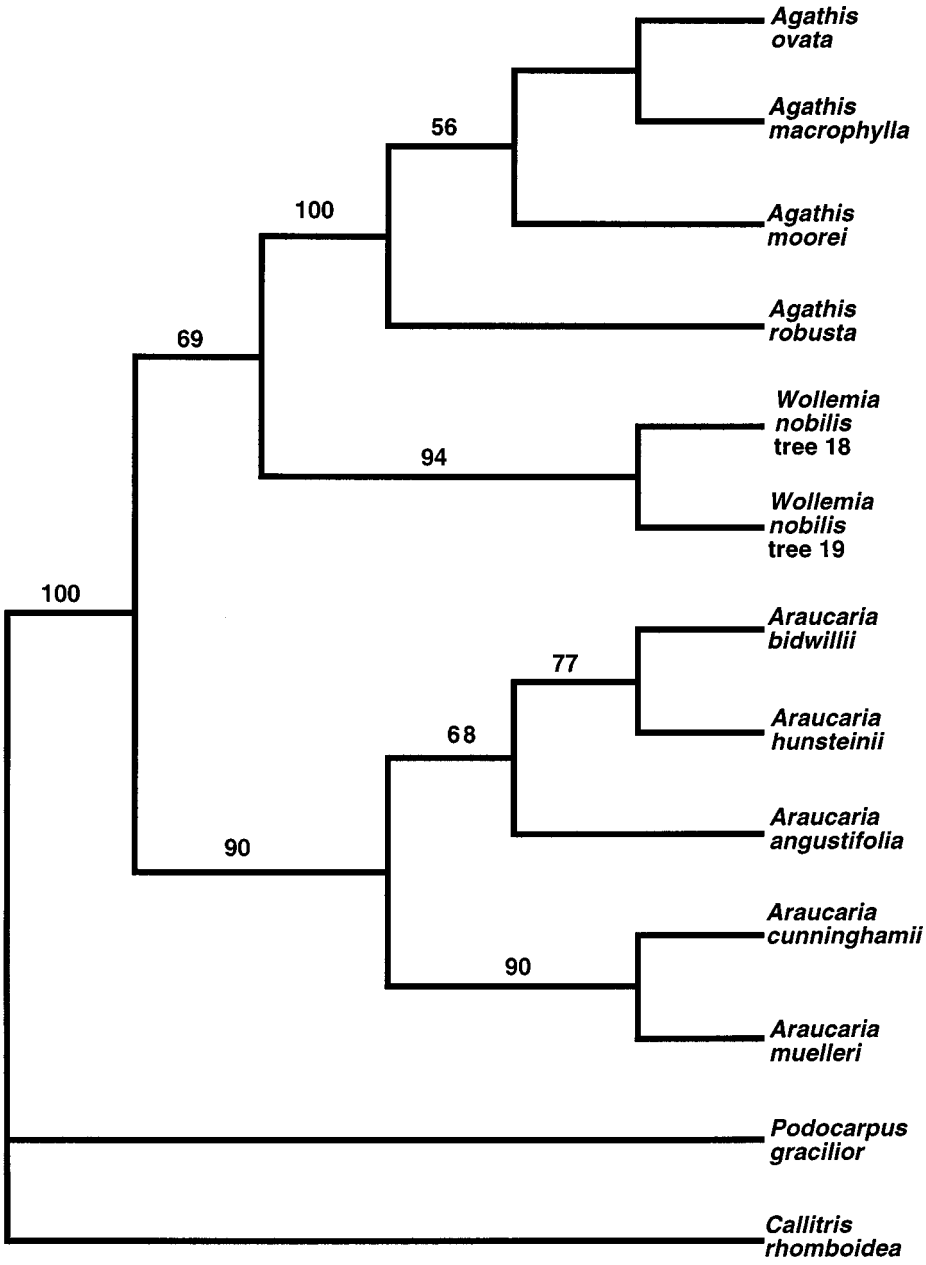

Fig. 4d. Small data set. Maximum likelihood tree, weighted.

clearly resolve the conifers from the angiosperms, and show some agreement with Chase et al. in the unweighted analysis. However, the weighted analysis indicates rather that the conifers are monophyletic. This suggests that the degree of divergence in the rbcL gene at this point is insufficient for a reliable separation.

Acknowledgments

The Royal Botanic Gardens and Domain Trust and the Friends of the Royal Botanic Gardens provided financial support for this study. Dr Peter Weston assisted with advice and critical comment on the manuscript. Kathi Downs is gratefully acknowledged for excellent technical support, and Prof. John Thomson is thanked for helpful discussion and advice. Dr Mike Pole is thanked for helpful discussion of the fossil record. Dr David Swofford is thanked for access to unpublished versions of PAUP 4.0 for the phylogenetic analyses. Dr David Morrison made helpful comments on an earlier draft of the manuscript.

References

Anonymous (1991) *Promega Protocols and Applications Guide*. (Promega Corporation: Madison, WI, USA).

Burrows, G.E., Boag, T.S. and Stockey, R.A. (1992) A morphological investigation of the unusual cryptogeal germination strategy of the Bunya Pine (*Araucaria bidwillii*) – an Australian rainforest conifer. *Int. J. Plant Sci.* 153: 503–512.

Cantrill, D.J. (1992) Araucarian foliage from the Lower Cretaceous of southern Victoria, Australia. *Internat. J. Pl. Sci.* 153: 622–645.

Chase, M.W., Soltis, D.E., Olmstead, R.G., Morgan, D., Les, D.H., Mishler, B.D., Duvall, M.R., Price, R.A., Hills, H.G., Qiu, Y.-L., Kron, K.A.,Rettig, J.H., Conti, E., Palmer, J.D., Manhart, J.R., Sytsma, K.J., Michaels, H.J., Kress, W.J., Karol, K.G., Clark, W.D., Hendren, M., Gaut, B.S., Jansen, K.R., Kim, K.-J., Wimpee, C.F., Smith, J.F., Furnier, G.R., Strauss, S.H., Xiang, Q.-Y., Plunkett, G.M., Soltis, P.S., Swensen, S.M., Williams, S.E., Gadek, P.A., Quinn, C.J., Eguiarte, L.E., Golenburg, E., Learn, G.H., Graham, S.W., Barrett, S.C.H., Dayanandan, S. and Albert, V.A. (1993) Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbc*L. *Ann. Missouri Bot. Gard.* 80: 528–580.

Chaw, S.-M., Sung, H.-M., Long, H., Zharkhikh, A. and Li, W.-H. (1995) The phylogenetic positions of the conifer genera *Amentotaxus*, *Phyllocladus* and *Nageia* inferred from 18S rRNA sequences. *J. Mol. Evol.* 41: 224–230.

Clark, J.M. (1988) Novel non-templated nucleotide addition reactions catalysed by procaryotic and eucaryotic DNA polymerases. *Nucleic Acid Res.* 16: 9677.

Clegg , M.T. and Zurawski, G. (1992) Chloroplast DNA and the study of plant phylogeny: Present status and future prospects. Pp. 1-13 in Soltis, P.S., Soltis, J.E., Doyle, J.J. (eds), *Molecular Systematics of Plants*. (Chapman & Hall: New York).

Dallimore, W., Jackson, A.B. and Harrison, S.G. (1966) *A Handbook of Coniferae and Ginkgoaceae*. (Edward Arnold: London).

Don R.H., Cox P.T., Wainwright B.J., Baker K., and Mattick J.S. (1991). Touchdown PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* 19: 4008.

Doyle, J.J. and Doyle, J.L. (1990) Isolation of plant DNA from fresh tissue. *Focus* 12: 13–15.

Felsenstein, J. (1981) Evolutionary trees from DNA sequences: A maximum likelihood approach. *J. Mol. Evol.* 17: 368–376.

Felsenstein, J. (1993) *PHYLIP (Phylogenetic Inference Package)*, version 3.5c. (Department of Genetics, University of Washington: Seattle).

Fitch, W.M. (1977) On the problem of discovering the most parsimonious tree. *Am. Nat.* 111: 223–257.

Gadek, P.A. and Quinn, C.J. (1993) An analysis of relationships within the Cupressaceae sensu stricto based on *rbc*L sequences. *Ann. Missouri Bot. Gard.* 80: 581–586

Gilmore, S., Weston P.H. and Thomson, J.A. (1992) A simple, rapid, inexpensive — and widely applicable technique for purifying plant DNA. *Austral. Syst. Bot.* 6: 139–148.

Hart, J.A. (1987) A cladistic analysis of conifers: preliminary results. *J. Arnold Arboretum* 68: 269–307.
Hill, R.S. (1990) *Araucaria* (Araucariaceae) species from Australian Tertiary sediments – a micromorphological study. *Austral. Syst. Bot.* 3: 203–220.
Hill, R.S. (1995) Conifer origin, evolution and diversification in the southern hemisphere. Pp. 10-29 in Enright, N.J. and Hill R. S. (eds), *Ecology of the Southern Conifers*. (Melbourne University Press: Carlton).
Hillis, D. and Moritz, C. (eds) (1990) *Molecular Systematics*. (Sinauer Associates: Sunderland).
Hughes, N.F. (1969) Jurassic and early Cretaceous pollen and spores. Pp. 311-329 in Tschudy, R.H. & Scott, R.A. (eds), *Aspects of Palynology*. (Wiley: New York).
Jones, W.G. Hill, K.D. and Allen, J.M. (1995) *Wollemia nobilis*, a new living Australian genus and species in the Araucariaceae. *Telopea* 3: 173–176.
Kershaw, A. P. and McGlone, M. S. (1995) The Quaternary History of the Southern Conifers. Pp. 30-63 in Enright, N.J. and Hill R. S. (eds), *Ecology of the Southern Conifers*. (Melbourne University Press: Carlton).
Kim, K.-J., Jansen, R.K., Wallace, R.S., Michaels, H.J. and Palmer, J.D. (1992) Phylogenetic implications of *rbc*L sequence variation in the Asteraceae. *Ann. Missouri Bot. Gard*. 79: 428–445.
Kimura, M. (1983) The neutral theory of molecular evolution. Pp. 208-233 in Nei, M. and Koehn, R.K. (eds), *Evolution of Genes and Proteins*. (Sinauer Associates: Sunderland).
Loconte, H. and Stevenson, D.W. (1990) Cladistics of the spermatophyta. *Brittonia* 42: 197–211.
Macphail, M., Hill, K., Partridge, A., Truswell, E. and Foster, C. (1995) 'Wollemi Pine' – old pollen records for a newly discovered genus of gymnosperm. *Geology Today* 11: 48–49.
Maddison, W. P. and Maddison, D. R. (1996) MacClade: analysis of phylogeny and character evolution, version 3.06. (Sinauer Associates: Sunderland).
Miller, C.N. (1977) Mesozoic Conifers. *Botanical Review* 43: 217–80.
Miller, C.N. (1988) The origin of modern conifer families. Pp. 448-86 in Beck, C.B. (ed.), *Origin and Evolution of Gymnosperms*. (Columbia University Press: New York).
Morgan, D.R. and Soltis, D.E. (1993) Phylogenetic relationships among members of Saxifragaceae s.l. based on *rbc*L sequence data. *Ann. Missouri Bot. Gard.* 80: 631–660.
Nixon, K. C., Crepet, W. L., Stevenson, D. and Friis, E. M. (1994) A reevaluation of seed plant phylogeny. *Ann. Missouri Bot. Gard.* 81: 484–533
Olmstead, R. G. and Palmer, J. D. (1994) Chloroplast DNA systematics: a review of methods and data analysis. *Amer. J. Bot.* 81: 1205–1224.
Palmer, J.D., Jansen, R.K., Michaels, H.J., Chase, M.W., and Manhart, J.R. (1988) Chloroplast DNA variation and plant phylogeny. *Ann. Missouri Bot. Gard.* 75: 1180–206.
Price, R.A., Thomas, J., Strauss, S., Gadek, P.A., Quinn, C.J. and Palmer, J.D. (1993) Familial relationships of the conifers from *rbc*L sequence data. *Amer. J. Bot.* 80: 172.
Sambrook, E., Fritsch, F. and Maniatis, T. (1989) *Molecular Cloning*. (Cold Spring Harbor Press: New York).
Savard, L., Li, P., Strauss, S., Chase, M.W., Michaud, M. and Bousquet, J. (1994) Chloroplast and nuclear gene sequences indicate late Pennsylvanian time for the last common ancestor of extant seed plants. *Proc. Natl Acad. Sci. USA* 91: 5163–5167.
Stockey, R. A. (1981) Some comments on the origin and evolution of conifers. *Canadian J. Bot.* 59: 1932–1940.
Stockey, R. A. (1982) The Araucariaceae: An evolutionary perspective. *Rev. Palaeobot. Palynol.* 37: 133–154.
Stockey, R.A. (1990) Antarctic and Gondwanan conifers. Pp. 179-191 in Taylor, T.N., Taylor, E.L., (eds) *Antarctic Palaeobiology*. (Springer: New York).
Stockey, R.A. (1994) Mesozoic Araucariaceae: morphology and systematic relationships. *J. Plant Res.* 107: 493–502.
Swofford, D.L., (unpublished). PAUP: phylogenetic analysis using parsimony, version 4 (demonstration).
White, M.E. (1981) Revision of the Talbragar Fish Bed flora (Jurassic) of New South Wales. *Rec. Austral. Mus.* 33: 965–721.

Manuscript received 19 September 1997
Manuscript accepted 10 November 1997