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Phylogenetic Position of Hoop Pine (*Araucaria cunninghamii*)

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Abstract

A variety of gymnosperms (13 species of conifer from 5 families and 2 species of non-conifer) were collected and the small ribosomal subunit (18S rRNA) was sequenced by direct sequencing. Fifteen species were examined in the study, which included widely separated provenances of *Araucaria cunninghamii*, to determine the phylogenetic relationship of these gymnosperms. Two of the four conifer families found in Australia that were included in the study (Araucariaceae and Podocarpaceae) separated as expected with further separation of genera and species within these families. Homologies between the gymnosperms varied from 89% to 98%. Four geographically distinct groups within *A. cunninghamii* were separated phylogenetically. This study suggests that *A. cunninghamii* was the most recent *Araucaria* species to evolve, with the Papua New Guinean provenances undergoing the fastest evolutionary changes. This type of analysis has provided molecular evidence to support the taxonomy of the Coniferales. It has shown, contrary to an established understanding in angiosperms, that sequence information of the 18S rRNA genes provides a useful means of studying phylogeny in the gymnosperms at the species and within-species levels.

Introduction

Araucaria cunninghamii (hoop pine) is a forest tree species that occurs in north-eastern Australia (Queensland and New South Wales), Papua New Guinea and Irian Jaya. The species has been the focus of a breeding program to improve both the quality and growth performance characteristics of trees in future plantations (Nikles and Newton 1983; Haines and Nikles 1987; Dieters and Nikles 1991). Hoop pine is grown for its high value timber, having a plantation area in Australia of about 55000 ha, with a harvest cycle of 50 years. Due to the long cultivation times, this species is an obvious target for molecular-assisted breeding protocols.

Use of molecular markers for genetic mapping, molecular taxonomy and evolutionary studies is well established (Tanksley *et al.* 1989; Welsh and McClelland 1990; Williams *et al.* 1990; Helentjaris 1991; Cheliak 1993). Sequence comparisons are only now being used in forestry species to study genetic structures of populations and to establish phylogenies (Chaw *et al.* 1993). Comparisons of morphological characters have most often been used to develop phylogenies. These phylogenetic inferences relate to the evolutionary connection of particular taxa and show the possible natural historic development of groups of families, genera and species. Techniques for phylogenetics using DNA sequences based on highly conserved genes have proliferated in recent years (Hamby and Zimmer 1992). The 18S ribosomal RNA genes (small ribosomal subunit) have been used to develop molecular based phylogenetic inferences of the position of and relationship between related taxa. The

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usefulness of sequence comparison using the 18S ribosomal subunit is attributed to its high degree of conservation, which allows comparisons of ancient evolutionary events. The sequence comparison between 18S ribosomal subunits has been extensively used in a diverse range of organisms, such as insects (Carmean *et al.* 1992), trees (Chaw *et al.* 1993; Savard *et al.* 1993), fungi (Edman *et al.* 1988; White *et al.* 1990), grasses (Hamby *et al.* 1988) and grain legumes (Eckenrode *et al.* 1985). The small ribosomal subunits in flowering plants have been informative and been demonstrated to have a wide applicability (Zimmer *et al.* 1988; Wolf *et al.* 1989).

Until recently, little work has been undertaken to establish phylogenetic relationships of gymnosperms using this gene. Studies of phylogenetic relationships within gymnosperms have been made in a limited number of taxa using sequence comparisons (Chaw *et al.* 1993). In this study, evidence is provided of phylogenetic relationships between *Araucaria cunninghamii* and other gymnosperms. *Araucaria cunninghamii* has the widest distribution of any of the 19 recognised species (Wilde and Eames 1952) in *Araucaria* (Kanowski *et al.* 1985). Four distinct genetic groups have been identified in *A. cunninghamii* (Graham *et al.* 1994*a*). The present study sought to examine the evolutionary relationships between these groups of *A. cunninghamii* and some related conifers to provide a framework to further study the genetic diversity of *A. cunninghamii.*

Materials and Methods

Plant Material

Foliage samples were collected from the conifer collection maintained by the University of Queensland, Australia, and from the Queensland Forest Research Institute collection, Indooroopilly, Queensland. Provenances of *Araucaria cunninghamii* came from a trial established by the Queensland Forest Research Institute in Imbil, Queensland, Australia. The samples included individuals from the families Araucariaceae, Podocarpaceae, Pinaceae and Cupressaceae (Table 1). The Australian species examined were *Agathis robusta* (C.Moore ex F.Muell.) (Araucariaceae), *Araucaria cunninghamii* (Aiton ex D.Don) (Araucariaceae) (four provenances, two from Queensland and two from Papua New Guinea), *Araucaria heterophylla* (Salisb.) (Araucariaceae) and *Podocarpus elatus* (R.Br. ex Endl.) (Podocarpaceae). Species examined that were not native to Australia were *Araucaria columnaris* (Forster) Hooker (Araucariaceae), *Araucaria luxurians* (Brongn. and Gris) (Araucariaceae), *Cupressus macrocarpa* (Cupressaceae) (Hartw.) and *Pinus elliottii* (D.Don), (Pinaceae).

Table 1. Species used for phylogenetic analysis, including those sequenced in this work and those taken from Genebank

Family	Species	Provenance	Genebank no.
Araucariaceae	*Araucaria cunninghamii*	Eastern Australia, Papua New Guinea	–
Araucariaceae	*Araucaria heterophylla*	Norfolk Island	–
Araucariaceae	*Araucaria columnaris*	New Caledonia	–
Araucariaceae	*Araucaria luxurians*	New Caledonia	–
Araucariaceae	*Agathis robusta*	Queensland	–
Podocarpaceae	*Podocarpus nakai*	Japan	D16447
Podocarpaceae	*Podocarpus elatus*	Eastern Australia	–
Cupressaceae	*Cupressus macrocarpa*	Western USA	–
Pinaceae	*Pinus elliottii*	South-eastern USA	–
Pinaceae	*Pinus wallichiana*	North America	X75080
Pinaceae	*Pinus luchuensis*	Ryukyu Islands	D16446
Pinaceae	*Picea mariana*	Canada, Northern USA	L01782
Taxaceae	*Taxus marei*	China	D16445
Ginkgoaceae	*Ginkgo biloba*	China	D16448
Zamiaceae	*Zamia pumila*	Cuba	M20017

DNA Extraction

DNA was extracted using a modified method from Graham *et al.* (1994*b*). Two grams of foliage from a single individual were cut into pieces of about 10 mm in length and placed in 10 mL homogenising buffer (1) (50 mM ethylenediamine tetraacetic acid (EDTA), 50 mM Tris-HCl (pH 5.5), 1% (w/v) PVP 40, 0.6 M sucrose). The tissue was homogenised using a Polytron at 10000 rpm for 5 min on ice. The unbroken cells and cell walls were removed from the smooth homogenate by passing through a 125 μm sieve and collected into a 50 mL tube. The resulting homogenate was centrifuged at 1000 *g* for 15 min at 4°C. The pellet containing cell organelles such as nuclei and chloroplasts was resuspended in homogenising buffer and centrifuged at 1000 *g* for 15 min at 4°C to further clean the organelle preparation. All of the buffers in the DNA extraction steps were adjusted to pH 5.5. A 2 mL aliquot of CTAB buffer (2% (w/v) CTAB, 100 mM MES-HCl, 50 mM EDTA, 1.4 M NaCl, 1% (w/v) PVP 40) was added to the pellet, mixed and incubated at 55°C for 20 min. After incubation, the mixture was centrifuged at 10000 *g* for 10 min at 4°C and the supernatant transferred to a clean centrifuge tube. An equal volume of chloroform and iso-amyl alcohol (24:1 (v:v)) was added and mixed to an emulsion by gentle inversion before centrifugation at 10000 *g* for 5 min to separate the phases. The upper aqueous phase was taken and, if cloudy, the chloroform step was repeated. After the chloroform treatment, 0.1 volume of 7.5 M ammonium acetate and 1 volume of ice-cold absolute ethanol were added to precipitate the DNA. After gentle inversion for about 15 s, the DNA was visible as a cotton-wool-like material in the tube. The DNA was removed with a wide-bore pipette tip, taking care not to compact the DNA, into a fresh microcentrifuge tube and washed twice with 70% (v/v) ethanol. Following centrifugation at 12000 *g* the DNA pellet was dried and resuspended in TE buffer (100 mM Tris HCl, 20 mM EDTA) (pH 8.0) containing RNAase to a final concentration of 1 ng mL^{-1}. The DNA was used in the subsequent PCR (polymerase chain reaction) amplification of the 18S genes.

18S PCR Amplification and Sequencing

Polymerase Chain Reaction

PCR with 18S primers was conducted in a reaction volume of 25 μL containing approximately 100 ng of DNA template from each species, *Taq* DNA polymerase (1 I.U.), dATP, dTTP, dGTP and dCTP (200 μM of each, Promega Corporation), primers with sequences 18sn1 5′-GTAGTCATATGCTTGTCTC-3′ and 18sc1 5′-GAAACCTTGTTACGACTT-3′ (1 μM of each; synthesised by the Centre for Molecular and Cell Biology, University of Queensland) and PCR buffer to a final concentration of 0.01 M Tris-HCl, 1.5 mM $MgCl_2$, 0.05 M KCl, 0.1 mg mL^{-1} gelatine, pH 8.3. The reaction mixture was cycled in a Perkin Elmer 9600 GeneAmp PCR system with the following amplification conditions: an initial denaturation step of 2 min at 94°C, followed by 35 cycles of annealing for 30 s at 55°C, extension for 1 min at 72°C and denaturation for 30 s at 94°C, and a final cycle of 25°C for 2 min. The PCR products were visualised on a 1.5% (w/v) agarose gel in 1 X TBE buffer at 16 V cm^{-1}. After staining with ethidium bromide for 40 min and destaining for 10 min in 1 X TBE buffer, photographs were taken.

Sequencing

Sequencing primers were designed from published material (Hamby *et al.* 1988; Bult *et al.* 1992; Chaw *et al.* 1993) and trialed. Redesign of several primers was necessary to avoid secondary structure problems at the published primer annealing sites. Alternative primer annealing sites were chosen near but not in the secondary structure locations. All of the amplification and sequencing primers, including both coding and non-coding strand oligonucleotides, are found in Table 2. Sequencing was done using a *Taq* DNA polymerase cycle DyeDeoxy terminator sequencing protocol and run on the Applied Biosystems Model 373A DNA sequencing system.

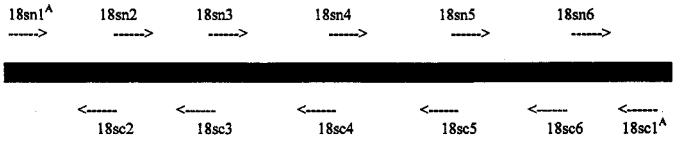
DNA of the single amplicons produced in the PCRs were purified using a Microcon 100 centrifuge column purification system (Amicon), before quantification of the DNA by ultraviolet spectrophotometry. Each of the DNA samples was diluted to a concentration of 100 ng μL^{-1}, of which 1 μL was added to the sequencing reaction mix. Sequencing reaction mixtures were prepared by adding 9.5 μL of Applied Biosystems terminator mix, 1 μL of the purified PCR amplified DNA (100 ng), 3.2 μL of a single primer (1 μM) and made up to 20 μL with sterile Milli-Q water in a 500 μL reaction tube. This was done for each of the sequencing primers (Table 2) in combination with each of the DNA samples. All of the reactions were subjected to 25 cycles of the following conditions: an initial step of denaturation at 94°C for 10 s, followed by annealing at 50°C for 5 s and extension at 60°C for 4 min. A final step of 15°C was included to hold the PCR reactions before purification. Purification was achieved by phenol chloroform extraction using non-fluorescing phenol and ethanol precipitation for 15 min at room temperature. The samples were dried before preparation for electrophoresis on the Applied Biosystems Model 373A DNA sequencing system.

Table 2. Amplification and sequencing primers and their locations for the small subunit of nuclear rDNA

The design of primers was based on previous work by Hamby *et al.* (1988), Bult *et al.* (1992) and Chaw *et al.* (1993). Tm = temperature

Name	Sequence	Size (bases)	Tm (°C)	Location
18sn1[A]	5′–GTAGTCATATGCTTGTCTC–3′	19	51	1–19
18sn2	5′–ACGGGTGACGGAGAATTAG–3′	19	55	340–359
18sn3	5′–AGGGCAAGTCTGGTGCCA–3′	18	55	535–553
18sn4	5′–GCTCTGAATACATTAGCATGG–3′	21	57	752–773
18sn5	5′–GCTGAAACTTAAAGGAATTGACGGAAGGGC–3′	30	85	1116–1136
18sn6	5′–CAATAACAGGTCTGTGATGCCCTTAGA–3′	27	75	1412–1439
18sc1[A]	5′–GAAACCTTGTTACGACTT–3′	18	47	1752–1770
18sc2	5′–CTAATTCTCCGTCACCCGT–3′	19	55	359–340
18sc3	5′–TGGCACCAGACTTGCCCT–3′	18	55	553–535
18sc4	5′–CGAGACTTATGTAATCGTACC–3′	21	57	793–772
18sc5	5′–GCCCTTCCGTCAATTCCTTTAAGTTTCAGC–3′	30	85	1146–1116
18sc6	5′–TCTAAGGGCATCACAGACCTGTTATTG–3′	27	75	143–1411

18S gene: schematic diagram of sequencing primers



[A]Original PCR (polymerase chain reaction) amplification primers.

Data Analysis

The 18S rRNA gene sequences were aligned by eye using regions of complete conservation for initial comparison. While a number of computer programs are available for sequence alignment, the reliability of their algorithms has yet to be proven for complete computer processing. The matrices of pairwise comparisons, calculated by the two-parameter method of Kimura (1980), were then submitted to the nearest neighbor-joining method of phylogenetic tree construction (Saitou and Nei 1987). Standard Wagner parsimony as well as weighted parsimony were conducted using the Branch and Bound algorithm of PAUP (Swofford 1990). Maximum likelihood was also used (Felsenstein 1981). Bootstrap confidence intervals (Swofford 1990) were estimated from 100 replications for neighbour-joining, standard and weighted parsimony and maximum likelihood. In each of the analyses, two outgroups, *Zamia pumila* and *Ginkgo biloba*, were used when generating trees. A number of systems exist for use in determining phylogenetic relatedness, and generation of phylograms have been compared and reviewed in the literature (Hillis and Dixon 1991).

Results

Sequence Alignment

Figure 1 shows the sequence alignments of the parsimonious sites of 18S rRNA genes from all of the gymnosperms included. The fully aligned sequence length was 1747 bp. From Chaw *et al.* (1993), the forward primary primer annealing site occurs at position 20–39 while the reverse primary primer annealing site occurs at position 1771–1790 of the published sequence from *Zamia pumila*, Genebank accession number M20017.

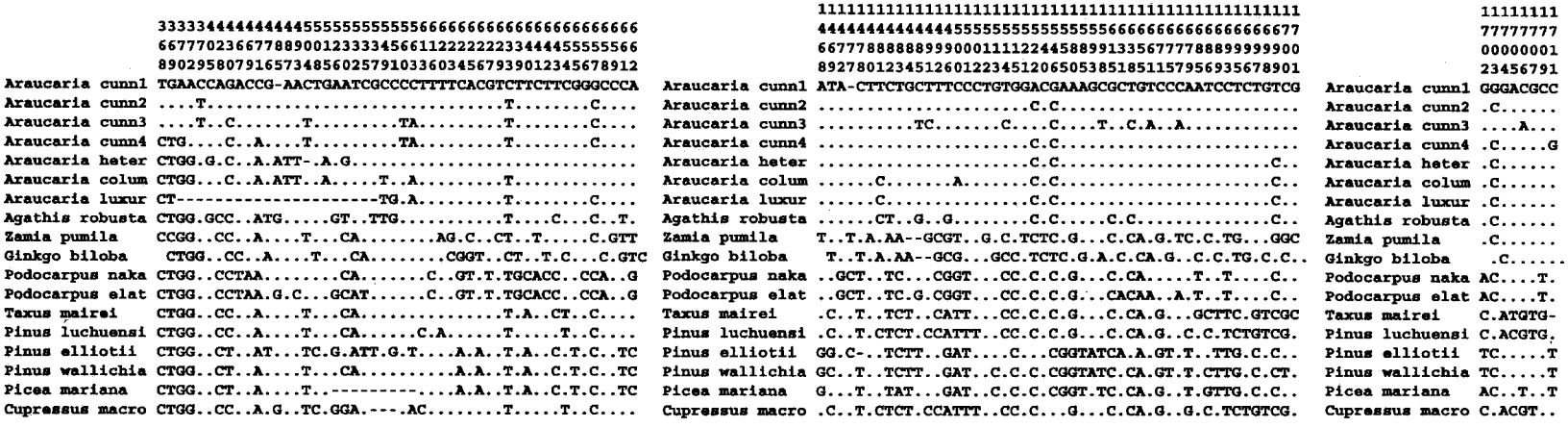


Fig. 1. Sequence alignments of the parsimonious sites across the entire 18S rRNA gene for the 18 accessions. ., denotes equivalent base; –, denotes a gap. Araucaria cunn1 = *A. cunninghamii* (Coonoon), Araucaria cunn2 = *A. cunninghamii* (Cape Flattery), Araucaria cunn3 = *A. cunninghamii* (Bumbu) and Araucaria cunn4 = *A. cunninghamii* (Pimaga), Podocarpus naka = *P. nakai*, Podocarpus elat = *P. elatus*, Cupressus macro = *C. macrocarpa*.

Table 3. Pairwise estimates of dissimilarities between DNA sequences, including the accessions (upper diagonal) and total base pair difference comparisons (lower diagonal)

Species and provenance	Species no.																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 *Araucaria cunninghamii*, Coonoon, Queensland	–	0.006	0.024	0.015	0.024	0.036	0.025	0.057	0.051	0.073	0.052	0.053	0.052	0.066	0.059	0.060	0.086	0.061
2 *Araucaria cunninghamii*, Cape Flattery, Queensland	11	–	0.021	0.011	0.022	0.033	0.023	0.053	0.051	0.071	0.052	0.051	0.053	0.064	0.059	0.059	0.085	0.060
3 *Araucaria cunninghamii*, Bumbu, Papua New Guinea	41	36	–	0.018	0.036	0.046	0.039	0.069	0.062	0.079	0.060	0.062	0.062	0.075	0.066	0.070	0.094	0.069
4 *Araucaria cunninghamii*, Pimaga, Papua New Guinea	26	19	31	–	0.021	0.032	0.024	0.055	0.051	0.071	0.051	0.048	0.051	0.062	0.057	0.058	0.085	0.059
5 *Araucaria columnaris*	41	39	63	36	–	0.029	0.022	0.053	0.056	0.073	0.058	0.055	0.059	0.068	0.063	0.065	0.091	0.066
6 *Araucaria luxuraens*	62	57	80	55	51	–	0.035	0.055	0.063	0.074	0.064	0.062	0.067	0.071	0.066	0.072	0.094	0.073
7 *Araucaria heterophylla*	43	40	67	42	39	61	–	0.059	0.058	0.078	0.059	0.057	0.060	0.069	0.066	0.066	0.091	0.066
8 *Agathis robusta*	99	92	119	95	93	96	102	–	0.076	0.094	0.079	0.073	0.079	0.085	0.082	0.084	0.112	0.085
9 *Podocarpus nakaii*	89	88	108	88	98	110	101	133	–	0.028	0.043	0.043	0.039	0.060	0.056	0.048	0.075	0.049
10 *Podocarpus elatus*	127	123	138	123	127	129	135	163	49	–	0.068	0.060	0.064	0.075	0.072	0.073	0.100	0.073
11 *Pinus luchuensis*	90	91	104	89	101	112	102	138	75	118	–	0.039	0.018	0.058	0.018	0.046	0.071	0.048
12 *Cupressus macrocarpa*	102	103	114	99	109	115	114	142	98	126	31	–	0.038	0.020	0.059	0.048	0.070	0.044
13 *Taxus mairei*	91	92	107	89	103	117	103	138	68	112	32	57	–	0.054	0.033	0.041	0.070	0.048
14 *Pinus elliottii*	114	111	130	108	118	124	119	148	104	130	101	109	94	–	0.063	0.023	0.089	0.062
15 *Pinus wallichiana*	104	103	122	101	113	126	114	146	84	128	80	103	72	41	–	0.015	0.086	0.060
16 *Picea mariana*	92	88	106	83	94	107	98	125	75	104	68	83	65	34	26	–	0.076	0.050
17 *Zamia pumila*	149	148	163	148	158	164	157	195	131	173	124	149	122	154	132	121	–	0.049
18 *Gingko biloba*	106	105	119	103	114	128	115	148	85	127	83	105	84	109	87	76	92	–

Phylogenetic Analysis

From Table 3, the largest absolute distance (base pair substitutions) was found between *Zamia pumila* and *Agathis robusta* with homologies of 89%, while the closest absolute distance was found between *Araucaria cunninghamii* (Coonoon, southern Queensland) and *Araucaria cunninghamii* (Cape Flattery, northern Queensland) with homologies of greater than 99%. The phylogenetic analysis by neighbour-joining indicates clear separation of the two Australian families represented, the Araucariaceae and Podocarpaceae. Within the Araucariaceae, further separation of the genus *Agathis* was found from *Araucaria*. Within *Araucaria*, two further separations could be found. These were the Pacific Ocean *Araucaria* species, including *A. heterophylla* (Norfolk Island) and *A. columnaris* (New Caledonia), and *A. cunninghamii*. Within *A. cunninghamii*, two further separations were indicated (Fig. 2), the Australian *A. cunninghamii* (southern Queensland and northern Queensland) and the Papua New Guinean *Araucaria cunninghamii* (northern occurrence and southern occurrence).

The neighbor-joining and parsimony methods indicate a strong monophyletic tree. The tree is supported by bootstrap confidence of 100 replicates indicating that the relative branch points occur 100% of the time at the genus level and fall as far as 64% for branches in the Pacific region species.

Maximum Parsimony

The maximum parsimony method resulted in a very similar tree to the neighbour-joining tree. It placed each of the gymnosperm families included in this study into similar phylogenetic arrangements. To determine the relative robustness of the tree, bootstrapping analysis was undertaken using 100 replicates, with the least significant branch being that of the four *A. cunninghamii* occurrences. The *A. cunninghamii* occurrences showed phylogenetic branching with bootstapping confidences of at least 64%, as shown in Fig. 2. Most of the higher order branches have bootstrap confidences of 85–100%. Among the 1747 sites, there was a total of 101 phylogenetically significant sites across 18 taxa with the most parsimonious tree having 622 steps.

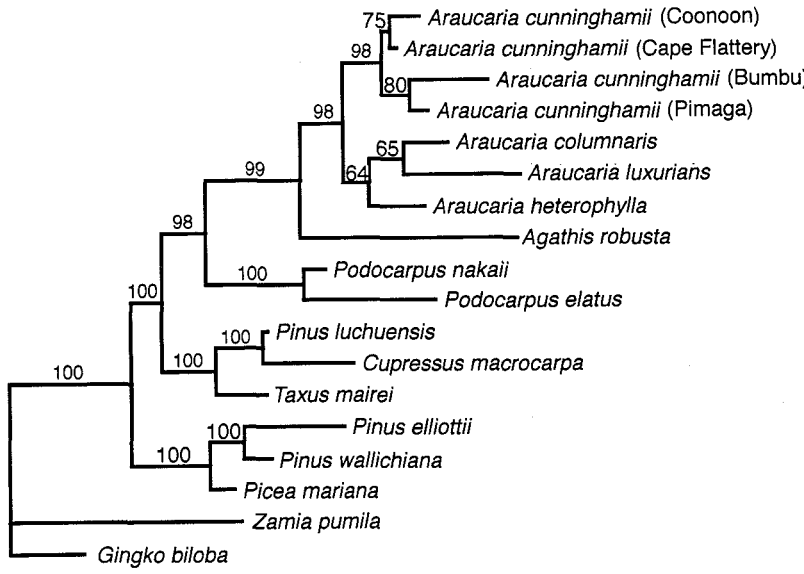


Fig. 2. Most parsimonious phylogenetic tree comprising 18 gymnosperm accessions, including four provenances of *Araucaria cunninghamii*. The numbers at the nodes refer to the percentage of sampled bootstraps in which this branch was observed.

Discussion

The usefulness of phylogenetic analysis based on 18S rRNA gene sequences in angiosperms and gymnosperms has been clearly demonstrated. A number of studies examining the phylogenetic relationships between divergent taxa have demonstrated the applicability of sequence-based phylogenetics (Hori *et al.* 1985; Savard *et al.* 1993; Smith and Klein 1994). Most literature concentrates on the angiosperms, with only a few accounts reported within the gymnosperms (Chaw *et al.* 1993). Many of these studies have shown the general applicability to phylogenetic separation at the level of families and genera, but interspecific phylogenetic analysis with the 18S rRNA gene sequencing has been not been considered. Savard *et al.* (1993) argue that it is not possible to establish relationships between closely related taxa due to a high degree of conservation. However, two species within the same genus can be distinguished with sequence information from the 18S gene. It has also been shown here that the use of 18S rRNA gene sequences in gymnosperms can discriminate between populations within a species.

Evolutionary Divergence

There are two general hypotheses on the evolutionary divergence within the Spermatophyta. The first (Margulis and Swartz 1982) suggests that after divergence from ferns the evolutionary ancestor of Spematophytes split into two groups, one being the Ginkgopsida (maidenhair tree), the Coniferopsida (coniferous trees) and the Gnetopsida, and the other group containing the Cycadopsida (cycads) and angiosperms (flowering plants). The second hypothesis (White 1994) suggests that Ginkgophytes and cordate conifers split away from common ancestors (*Cooksonia*) while the Glossopterids (Southern Hemispheric conifers, cycadopsids and angiosperms) evolved and diverged within this group at approximately the same time into the three lineages. The major differences between these two hypotheses is the position of the Cycadopsida and the Southern Hemispheric conifers. Hori *et al.* (1985) argue from sequencing based on the 5S rRNA genes that the Cycadopsida, chordate conifers and Ginkgophytes are more closely related to each other than to angiosperms, which suggests that the second of the two hypotheses is the more likely. The second hypothesis is also more likely to explain the phylogenetic relationships found in this work (Fig. 2). The Ginkgophytes and Cycadopsida diverged from a common ancestor, followed by divergence of the Northern Hemispheric conifers and then of the podocarps and, lastly, of the Southern Hemispheric conifers. Figure 2 shows that podocarps shared a common ancestor with Southern Hemispheric gymnosperms, suggesting that the podocarps evolved prior to the *Agathis* and *Araucaria* genera. The *Agathis* and *Araucaria* genera share a common ancestor, as would be expected, and all of the *Araucaria* species share a common ancestor. Separation of the Pacific *Araucaria* species from the Australian *Araucaria* species also appears to have occurred. While the ancestors of these species shared a common geographical connection, plate tectonic theory has it that the Pacific region separated from the Australian mainland approximately 90 million years ago, forming a physical barrier (the ocean) and creating genetic isolation (Burrett *et al.* 1991; Veevers 1991).

Phylogenetics may hold valuable information for research work into reconstruction of the fossil record of *Araucaria.* These phylogenetic relationships could be used as a measure of comparison for characters shared between extinct species relative to those same characters shared between extant species. If several species sharing particular morphological characters show relationships to other species, similar conclusions could be made about phylogenetic relationships of extinct species. Additional species from the *Araucaria* genus, particularly those from South America and the Pacific, may help build a better picture of evolutionary origins and relationships of this genus and provide an additional indicator for speculations of tectonic plate theory.

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