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Molecular Phylogenetics and Evolution 28 (2003) 47–59
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Using *mat*K sequence data to unravel the phylogeny of Casuarinaceae

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Received 7 May 2002; revised 17 December 2002

Abstract

Casuarinaceae are a Gondwanic family with a unique combination of morphological characters not comparable to any other family. Until recently, the 96 species in the family were classified in a single genus, *Casuarina s.l.* A recent morphological revision of the family resulted in the splitting of *Casuarina s.l.* into four genera—*Allocasuarina*, *Casuarina s.s.*, *Ceuthostoma*, and *Gymnostoma*. This study uses *mat*K sequence data from 76 species of Casuarinaceae and eight outgroup taxa to examine the phylogenetic structure within the Casuarinaceae. The study demonstrates the monophyly of the four genera and examines the relationships within the family; it tests the validity of the infra-generic subdivision of *Allocasuarina*; it discovers geography-based infra-generic subdivisions within *Gymnostoma* and *Casuarina*; and, finally, provides a molecular framework on which to trace the evolution of xeromorphy in the Casuarinaceae.
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1. Introduction

The family Casuarinaceae originally contained a single genus, *Casuarina* L. However, over the last two decades a morphological revision of Casuarinaceae resulted in the splitting of *Casuarina* into four genera (Johnson and Wilson, 1989): *Gymnostoma* L. Johnson (18 species; one in northeastern Queensland, the rest in Malesia, the Solomons, Fiji and New Caledonia), *Ceuthostoma* L. Johnson (two species in Malesia, from Palawan and Borneo to New Guinea), *Casuarina* L. (17 species; six in Australia, the rest extending from the Bay of Bengal to Polynesia) and *Allocasuarina* L. Johnson (endemic to Australia; 58 species, divided among 14 sections). All of these genera grow in tropical climates, but *Casuarina* extends into warm temperate regions of Australia and *Allocasuarina* is concentrated mainly in warm to cool temperate regions (southern Australia). The splitting of *Casuarina* into four genera and the naming of numerous new species of *Allocasuarina* has received some criticism; see for example, exchanges between Hwang (1990, 1991a,b, 1992), Crisp (1991) and Johnson (1991).

Casuarinaceae are a Gondwanic family. Pollen attributed to Casuarinaceae has been found in Paleocene through to Miocene deposits in South Africa (Coetzee and Muller, 1984; Coetzee and Praglowski, 1984), Argentina (Archangelsky, 1973), New Zealand (Mildenhall, 1980) and Australia (Johnson and Wilson, 1989; Macphail et al., 1994). As well as being the second most widely distributed genus of Casuarinaceae today, *Gymnostoma* is the oldest and most broadly distributed genus in the fossil record. Megafosssils of *Gymnostoma* are recorded from Paleocene sediments in New South Wales (Scriven and Hill, 1995), Eocene in South Australia, Victoria and Queensland (Christophel, 1980, 1989), Oligocene in Tasmania (Hill and MacPhail, 1983) as well as the Miocene of New Zealand (Campbell and Holden, 1984) and South America (Frenguelli, 1943). There are only a couple of records of *Casuarina* from the Miocene and Pliocene (Campbell and Holden, 1984; Christophel, 1989) and there is no certain fossil record of *Allocasuarina* until the early Pleistocene (Jordan, 1997), although some fossils currently reported as *Casuarina* may belong to this genus (Dilcher et al., 1990). Casuarinaceae no

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doi:10.1016/S1055-7903(03)00028-9

longer occur in New Zealand, South America or southern Africa.

Phylogenetic relationships among the genera of Casuarinaceae are unclear. Johnson and Wilson (1989) suggested that *Gymnostoma* and *Ceuthostoma* represent the more primitive members of the family, while *Allocasuarina* represents the most derived genus. The extreme morphological reduction seen in this family, as well as the unique combination of morphological traits (e.g., drooping equisetoid twigs, reduced scale-like leaves in whorls forming toothed sheaths at each node, inflorescences with alternating whorls of tooth-like bracts and reduced flowers, wind-pollination, woody 'cone'-like infructescences, winged samaras as fruits), make comparative studies of morphology difficult. Evidence from the fossil record is inconclusive. The oldest megafossils, from Late Paleocene sediments, have been assigned to an extinct species of *Gymnostoma* (see Scriven and Hill, 1995), with non-xeromorphic characters such as stomata in open grooves and few or no trichomes. These plants probably grew in moist environments, ideal for preservation in the fossil record. Fossils of xeromorphic plants are generally rare because the dry environmental conditions in which they exist are not conducive to the preservation of the plants in the fossil record. Xeromorphic Casuarinaceae began to appear in the megafossil record 20–30 million years ago, corresponding with the desiccation of the Australian continent. This change may represent either the adaptation of non-xeromorphic plants to the increasing aridity, or the geographic and taxonomic radiation and increase in population sizes of xeromorphic taxa that were already in existence in small patches of dry habitat, but which expanded their ranges rapidly with the onset of arid conditions.

Morphological character distributions among the genera are complex and preliminary cladistic analyses (Johnson and Wilson, unpub.) have suggested that phylogenetic and biogeographic relationships among genera may not be decipherable from morphology alone. Within Casuarinaceae xeromorphic plants were grouped together (by Poisson, 1874) as 'Cryptostomae' (species of the current genera *Casuarina* and *Allocasuarina*), as distinct from 'Gymnostomae' (*Gymnostoma*). As the name suggests, the stomata of the Cryptostomae (including *Ceuthostoma*) are concealed in deep furrows. Those of Gymnostomae are exposed in shallow furrows and are therefore more prone to water loss. While *Ceuthostoma* shares this xeromorphic feature with *Casuarina* and *Allocasuarina*, its general morphology resembles that of *Gymnostoma* (Johnson and Wilson, 1989). For this reason, the phylogenetic position of *Ceuthostoma* relative to the other three genera remains unresolved.

Not only are the phylogenetic relationships within Casuarinaceae unclear, but the sister group of the family also remains enigmatic, given the isolated position of the family in terms of morphological and molecular data. As stated earlier, the combination of morphological traits (see above) that characterise this family is unique, making comparative studies of morphology difficult. Manos and Steele (1997) in their molecular study of the 'higher' Hamamelids placed Casuarinaceae in a clade with Betulaceae, Myricaceae, and Ticodendraceae. Their combined analysis of *rbc*L and *mat*K sequence data indicated that, of the taxa included in their study, Betulaceae was the most likely sister taxon. These data were verified in an *rbc*L analysis of the Hamamelidae and their allies by Qiu et al. (1998).

Although Casuarinaceae have been thoroughly revised and described, phylogenetic information about the group is limited. Sogo et al. (2001) carried out a study of *rbc*L and *mat*K sequences in the family; their results support the recognition of four genera, but their study was based on a limited number of species. In this study we looked at 76 species of the 96 recognised in the family. We amplified approximately 1500 bp of sequence from the 3′ end of the *mat*K gene (and *trn*K intron; see Fig. 1) from the chloroplast genome (Hilu and Liang, 1997; Neuhaus and Link, 1987; Olmstead and Palmer, 1994), and used the data to reconstruct a more detailed phylogeny of the Casuarinaceae. This phylogenetic framework was used to to examine the evolution of xeromorphy in Casuarinaceae: did it arise just once before the divergence of *Ceuthostoma*, *Allocasuarina*, and *Casuarina*, or did it arise more than once, with *Ceuthostoma* acquiring xeromorphic characters in parallel with *Casuarina* and *Allocasuarina*?

2. Materials and methods

Ninety-one samples of Casuarinaceae (representing 53 species of *Allocasuarina*, 11 species of *Casuarina*, 1 species of *Ceuthostoma* and 11 species of *Gymnostoma*) and three samples of two outgroup taxa (*Betula* and

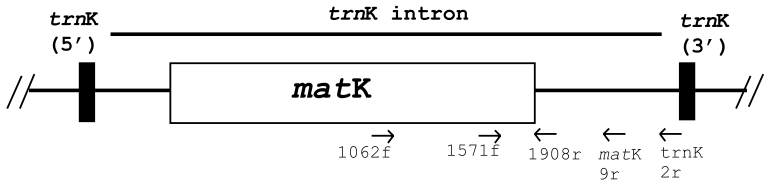

Fig. 1. Location of *mat*K gene within the *trn*K cistron. Solid boxes represent the 5′ and 3′ *trn*K exons; the *mat*K gene, represented by the open box, is part of the *trn*K intron. Approximate locations of the primers used in this study are indicated.

Table 1
Casuarinaceae taxa used in the analysis of *mat*K sequence data

Taxon	Section[a]	Collection No.	GenBank	Source; locality[b]
Allocasuarina				
A. acutivalvis subsp. *acutivalvis*	5. *Ceropitys*	P Jobson 7048	AY191668	Wild; Moorine Rock, WA
A. acutivalvis subsp. *prinsepiana*	5. *Ceropitys*	P Jobson 7123	AY191669	Wild; E of Buntine, WA
A. brachystachya	11. *Cylindropitys*	KLW 3191	AY191647	RBG Sydney; SE of Tingha, NSW
A. campestris	5. *Ceropitys*	KP3	AY191666	Kings Park, WA; source unknown
A. corniculata	11. *Cylindropitys*	NSW 481136	AY191672	RBG Annan; ex Kings Park
A. crassa	11. *Cylindropitys*	DAS 99001	AY191644	RTBG; Cape Pillar, Tas
A. decaisneana	1. *Dolichopitys*	KP1	AY191677	Kings Park, WA; source unknown
A. decussata	6. *Allocasuarina*	CBG 13301	AY191660	CBG; Channybeerup, WA
A. dielsiana	5. *Ceropitys*	KLW 9750	AY191664	RBG Annan; Murchison R, WA
A. diminuta subsp. *annectens*	11. *Cylindropitys*	KLW 9835	AY191637	Wild; SW of Corang River, NSW
A. diminuta subsp. *diminuta*	11. *Cylindropitys*	KLW 9759	AY191643	RBG Annan; Crokers Range, NSW
A. distyla	11. *Cylindropitys*	KLW 9761	AY191638	RBG Annan; Glowworm Tunnel road, NSW
A. duncanii	11. *Cylindropitys*	RTBG 970397	AY191625	RTBG; Snug Tiers, Tas
A. emuina	11. *Cylindropitys*	KLW 9767	AY191634	RBG Sydney; Mt Emu, Qld
A. eriochlamys subsp. *eriochlamys*	5. *Ceropitys*	KP5	AY191663	Kings Park, WA; source unknown
A. fibrosa	2. *Oxypitys*	KP6	AY191675	Kings Park, WA; source unknown
A. fraseriana	7. *Amorphopitys*	NSW 481139	AY191658	RBG Annan; Kings Park, WA
A. glareicola	11. *Cylindropitys*	KLW 9764	AY191641	RBG Annan; Castlereagh area, NSW
A. globosa	5. *Ceropitys*	KP4	AY191661	Kings Park, WA; source unknown
A. grampiana	11. *Cylindropitys*	PG Abell 451	AY191626	RBG Annan; Mt William, Vic.
A. grevilleoides	2. *Oxypitys*	P Jobson 7237	AY191676	Wild; N of Mogumber, WA
A. gymnanthera	11. *Cylindropitys*	KLW 9785	AY191635	Wild; NW of Denman, NSW
A. helmsii	5. *Ceropitys*	P Jobson 6948	AY191667	Wild; Kimba, SA
A. huegeliana	8. *Oopitys*	KLW 9763	AY191655	RBG Annan ex Gordon Inlet Road, WA
A. humilis	13. *Trachypitys*	KP2	AY191619	Kings Park, WA; source unknown
A. inophloia	10. *Inopitys*	D Blaxell 88/199	AY191653	RBG Sydney; Stannary Hills, Qld
A. inophloia	10. *Inopitys*	DAS 99034	AY191652	Wild; Mt Garnett, N. Qld
A. lehmanniana subsp. *ecarinata*	11. *Cylindropitys*	KLW 9757	AY191640	RBG Annan; NE of Hopetoun, WA
A. littoralis	11. *Cylindropitys*	DAS 99002	AY191627	RTBG; source unknown
A. littoralis	11. *Cylindropitys*	KP7	AY191651	Kings Park, WA; source unknown
A. luehmannii	3. *Platypitys*	KLW 9782	AY191673	Wild; NE of Singleton, NSW
A. mackliniana subsp. *hirtilinea*	11. *Cylindropitys*	KLW 9883	AY191633	Wild; Wonwondah-Dadswells Bridge Road, Vic.
A. mackliniana subsp. *xerophila*	11. *Cylindropitys*	KLW 9884	AY191629	Wild; N of Gymbowen, Vic.
A. media	11. *Cylindropitys*	KLW 9745	AY191623	RBG Annan; Wilsons Promontory, Vic.
A. microstachya 7216	13. *Trachypitys*	P Jobson 7216	AY191621	Wild; Green Head to Coorow Road, WA
A. microstachya 7238	13. *Trachypitys*	P. Jobson 7238	AY191620	Wild; N of Mogumber, WA
A. misera	11. *Cylindropitys*	KLW 9882	AY191630	Wild; Stawell, Vic.
A. monilifera	11. *Cylindropitys*	DAS 99005	AY191624	RTBG; Safety Cove, Tas
A. muelleriana subsp. *muelleriana*	11. *Cylindropitys*	KLW 9754	AY191648	RBG Annan; Lobethal, SA
A. nana	12. *Nanopitys*	KLW 9762	AY191622	RBG Annan; Newnes State Forest, NSW
A. ophiolitica	11. *Cylindropitys*	KLW 9774	AY191639	Wild; NW of Curricabark, NSW
A. paludosa	11. *Cylindropitys*	DAS 99007	AY191649	RTBG; Gladstone, Tas
A. paradoxa	11. *Cylindropitys*	KLW 9752	AY191632	RBG Annan; Cranbourne, Vic.
A. pinaster	2. *Oxypitys*	KP8	AY191674	Kings Park, WA; source unknown
A. portuensis	11. *Cylindropitys*	KLW 9744	AY191645	RBG Sydney; Neilsen Park, NSW
A. pusilla	11. *Cylindropitys*	KLW 9760	AY191631	RBG Annan; Murrayville, Vic.
A. rigida subsp. *rigida*	11. *Cylindropitys*	KLW 9758	AY191650	RBG Annan; Barren Mtn, NSW
A. rupicola	11. *Cylindropitys*	KLW 9766	AY191646	RBG Annan; Mt Norman, Qld
A. scleroclada	5. *Ceropitys*	MD Crisp 4842A	AY191665	CBG; Mt Ragged Range, WA
A. simulans	11. *Cylindropitys*	KLW 9770	AY191636	Wild; near Nabiac, NSW
A. spinosissima	4. *Echinopitys*	MD Crisp 5566	AY191671	CBG; E of Southern Cross, WA
A. tessellata	5. *Ceropitys*	KLW 9769	AY191670	RBG Annan; Mt Singleton, WA
A. thalassoscopica	11. *Cylindropitys*	P. Sharpe C2	AY191642	Wild; Mt Coolum Qld
A. thuyoides	14. *Acanthopitys*	KLW 9787	AY191618	RBG Annan; Stirling Range, WA
A. tortiramula	5. *Ceropitys*	KP9	AY191662	Kings Park, WA; source unknown

Table 1 (*continued*)

Taxon	Section[a]	Collection No.	GenBank	Source; locality[b]
A. torulosa	6. *Allocasuarina*	DAS 99036	AY191659	Wild; Lake Tinaroo, Qld
A. trichodon	9. *Trachypitys*	MD Crisp 5109	AY191654	CBG; NW of Cape Riche, WA
A. verticillata	8. *Oopitys*	KLW 9753	AY191657	RBG Annan; Orford, Tas
A. verticillata	8. *Oopitys*	KLW 9873	AY191656	Wild; Wombeyan Caves road, NSW
A. zephyrea	11. *Cylindropitys*	RTBG 97.0398	AY191628	RTBG; W. Coast Tas
Casuarina				
C. collina		KLW 7722	AY191697	Cult. Balmain; Riviere des Pirogues, New Caledonia
C. cristata		DAS 99004	AY191698	RTBG; source unknown
C. cristata		KLW 9748	AY191699	RBG Annan; Mongarilby, Qld
C. cunninghamiana subsp. *unninghamiana*		KLW 9826	AY191714	Wild; Jackadgery, NSW
C. cunninghamiana		DAS 99006	AY191707	RTBG; source unknown
C. equisetifolia subsp. *equisetifolia*		DAS 99010	AY191702	Wild; Sarawak, Borneo
C. equisetifolia subsp. *equisetifolia*		Phil. Sp. 5	AY191703	Wild, Philippines
C. equisetifolia subsp. *equisetifolia*		DAS 99037	AY191701	Wild; Port Douglas Beach, Qld
C. equisetifolia subsp. *incana*		KLW 9765	AY191700	RBG Annan; Peregian Beach, QLD
C. glauca		KLW 9739	AY191705	Wild; RBG Sydney
C. glauca		KLW 9755	AY191704	Wild; RBG Annan
C. obesa		KLW 9743	AY191709	RBG Sydney; source unknown
C. obesa		KLW 9751	AY191708	RBG Annan; Leeman, WA
C. oligodon subsp. *oligodon*		KLW 9799	AY191706	RBG Sydney; source unknown
C. 'parapotamia' ms		Phil. Sp. 6	AY191712	Wild; Mt. Victoria, Palawan, Philippines
C. pauper		NA Leist 82	AY191711	Wild; Nymagee—Cobar road, NSW
C. 'riparia' ms		Phil. Sp. 14	AY191713	Wild; Luzon, Philippines
C. 'timorensis' ms		KLW 9808	AY191710	Crossmaglen, NSW; Timor
Ceuthostoma				
C. palawanense		Phil. Sp. 7	AY191696	Wild; Mt Bloomfield, Palawan, Philippines
C. terminale			AY 033838	Sogo et al. (2001)
Gymnostoma				
G. australianum		DAS 99024	AY191678	Wild; Cape York, Qld
G. australianum		KLW 9742	AY191679	RBG Sydney; Roaring Meg Creek, Qld
G. chamaecyparis		KLW 9961	AY191692	Wild; Paagoumene, New Caledonia
G. deplancheanum		KLW 7704	AY191681	RBG Sydney; Riviere des Lacs, New Caledonia
G. deplancheanum		KLW 9741	AY191682	RBG Sydney; Riviere Bleue, New Caledonia
G. glaucescens		DAS 99025	AY191684	Wild; Mt. Des Sources, New Caledonia
G. leucodon		KLW 9936	AY191683	Wild; Riviere des Pirogues, New Caledonia
G. 'mesostrobilum' ms		Phil. Sp 1	AY191685	Wild; Mt Victoria, Palawan, Philippines
G. 'mesostrobilum' ms		T Livshultz 0064	AY191686	Wild; Tenom, Sabah
G. nobile		DAS 99008	AY191687	Wild; Sarawak
G. nobile		FRI 43903	AY191688	Cult; Peninsular Malaysia
G. nodiflorum		KLW 9917	AY191691	Wild; Kone-Tiwaka road, New Caledonia
G. papuanum		KLW 9740	AY191695	Moluccas, New Guinea
G. poissonianum		DAS 00013	AY191689	Wild; Mt Dzumac, New Caledonia
G. poissonianum		DAS 00014	AY191690	UTAS; New Caledonia
G. sumatranum		FRI 43901	AY191693	Cult; Peninsular Malaysia
G. sumatranum		K Hill NSW 442215	AY191694	Cult; Bogor BG. Java
G. webbianum		KLW 7724	AY191680	RBG Sydney; Riviere des Pirogues, New Caledonia
Betula papyrifera		DAS 00015	AY191716	RTBG; source unknown
Betula papyrifera			U92853	Manos and Steele (1997)
Betula utilis		RTBG 92.0414	AY191717	RTBG; source unknown
Myrica cerifera			U92857	Manos and Steele (1997)

Table 1 (*continued*)

Taxon	Section[a]	Collection No.	GenBank	Source; locality[b]
Myrica gale		KLW 9788	AY191715	RBG Tomah; source unknown
Nothofagus cunninghamii			U92859	Manos and Steele (1997)
Ticodendron			U92855	Manos and Steele (1997)
Trigonobalanus			U92866	Manos and Steele (1997)

[a] Section refers to *Allocasuarina* only.

[b] Bogor BG—Bogor Botanic Gardens, Java; CBG—Australian National Botanic Gardens, Canberra; DAS—D.A. Steane; FRI—Forestry Research Institute Malaysia (FRIM), Kuala Lumpur, Malaysia; KP—Kings Park and Botanic Garden, Perth; E—east; N—north; NE—northeast; NSW—New South Wales; Qld—Queensland; R—River; RBG Annan—Royal Botanic Gardens Sydney (Mt Annan site); RBG Sydney—Royal Botanic Gardens Sydney (Sydney site); RBG Tomah—Royal Botanic Gardens Sydney (Mt Tomah site); RTBG—Royal Tasmanian Botanical Gardens, Hobart; SA—South Australia; SE—southeast; SW—southwest; Tas—Tasmania; UTAS—School of Plant Sciences, University of Tasmania; WA—Western Australia.

Myrica) were collected from the wild or from cultivated specimens in botanic gardens (Table 1). Tissue was frozen in liquid nitrogen and stored at −70 °C, dried using silica gel (Chase and Hills, 1991) or preserved in a CTAB/salt solution (Thomson, 2002).

DNA was extracted using a modified CTAB protocol (Doyle and Doyle, 1990). Approximately 0.1 g of green tissue ('needles') was ground under liquid nitrogen and was transferred to a 1.5 ml eppendorf tube. Five hundred μl of hot (65 °C) CTAB buffer (0.02 M EDTA, 1.4 M NaCl, 0.1 M Tris pH 8.0, 2% CTAB, 0.7% v/v DTT, 2% soluble PVP) was added. The slurry was incubated at 65 °C for 30 min with occasional shaking, followed by extraction with an equal volume of chloroform:isoamyl alcohol (24:1). Phases were separated by centrifugation for 10 min at 20,000*g*. The aqueous phase was removed and re-extracted with chloroform:isoamyl alcohol. Two volumes of cold 95% ethanol were added to the aqueous phase, mixed gently, and incubated on ice for 10 min. The DNA was pelleted at 20,000*g* for 5 min. The pellet was washed briefly in 76% ethanol/0.01 M sodium acetate and was re-centrifuged for 5 min. The supernatant was removed, the pellet was air-dried and resuspended in 100 μl TE (10 mM Tris, pH 8.0, 1 mM EDTA). When necessary, DNA was cleaned using a Prep-A-Gene DNA purification kit (Bio-Rad, USA) according to manufacturer's instructions.

A 1500 bp fragment from the 3′ end of the *mat*K gene was amplified using primers 1062f and *trn*K 2r (Fig. 1, Table 2) in the PCRs. Each PCR had a final volume of 50 μl and contained 10–20 ng genomic DNA, 160 μM each dATP, dCTP, dTTP, and dGTP, 4 mM $MgCl_2$, 0.5 μM forward (1062f) and reverse (*trn*K 2r) primers, 1.25 U Taq DNA polymerase (Qiagen, Germany) and 1× Qiagen Taq DNA polymerase buffer. Cycling conditions were: initial melting at 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 45 °C for 1 min, 72 °C for 2 min; final extension at 72 °C for 15 min. More recalcitrant samples (e.g. those prepared from silica-dried tissues) were amplified using Advantage 2 DNA polymerase (Clontech, USA). The 25 μl reactions were prepared following the recommendations of the manufacturer: 0.4 μM of each primer (1062f and *trn*K 2r), 400 μM each dNTP, 1× Advantage 2 Polymerase mix and 1× Advantage 2 polymerase buffer. Cycling conditions were as follows: 95 °C for 1 min; 35 cycles of 95 °C for 30 s, 54 °C for 30 s, 68 °C for 3 min; final extension at 68 °C for 3 min. PCR products were cleaned using a QIAquick DNA Cleanup System (Qiagen, Germany).

Table 2
Primer sequence and location

Primer	Sequence	Start[c]
1062f	5′ GTGGAAATTCCGTTTTCTCTACG 3′	1062
1571f	5′ GGATCCTTTCATTCATT 3′	1571
1908r	5′ ACTAAYGGGATGGCCTRATGC 3′	1908
*mat*K 9r[a]	5′ CAATCATTCGTGATTGGCCAG 3′	2282
*trn*K 2r[b]	5′ AACTAGTCGGATGGAGTAG 3′	2573

[a] Primer designed by Manos and Steele (1997).

[b] Primer designed by Steele and Vilgalys (1994).

[c] The base position at which the primer begins is relative to the *Nicotiana* sequence (Sugita et al., 1985).

PCR products were sequenced in both directions using a suite of 3–5 primers (Fig. 1, Table 2), including three that were custom-designed for this study (1062f, 1571f, 1908r) and two more conserved primers (*mat*K9r and *trn*K2r; Manos and Steele, 1997; Steele and Vilgalys, 1994). PCR products were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, USA) following the recommendations of the manufacturer. Sequencing products were fractionated on a Perkin–Elmer 373 DNA sequencer. The *mat*K partial sequences for each sample were aligned and checked using Sequence Navigator version 1.0.1 (Applied Biosystems, USA). All sequences are lodged in GenBank (Accession Nos. AY191618–AY191717). The data set is lodged in TreeBASE (study Accession No. S838; matrix Accession No. M1354).

Complete sequences from all samples were aligned by eye. Some of the sequences were identical or differed only by autapomorphies. To simplify the data set and accelerate analyses, taxa with identical sequences (ignoring autapomorphies) were pooled into single terminal units (*Gymnostoma* 1, *Allocasuarina* Group 1, *Allocasuarina* Group 2, *Allocasuarina* Group 3; see legend to Fig. 2). Additional *mat*K sequences for *Ceuthostoma terminale* (Sogo et al., 2001) and outgroup taxa

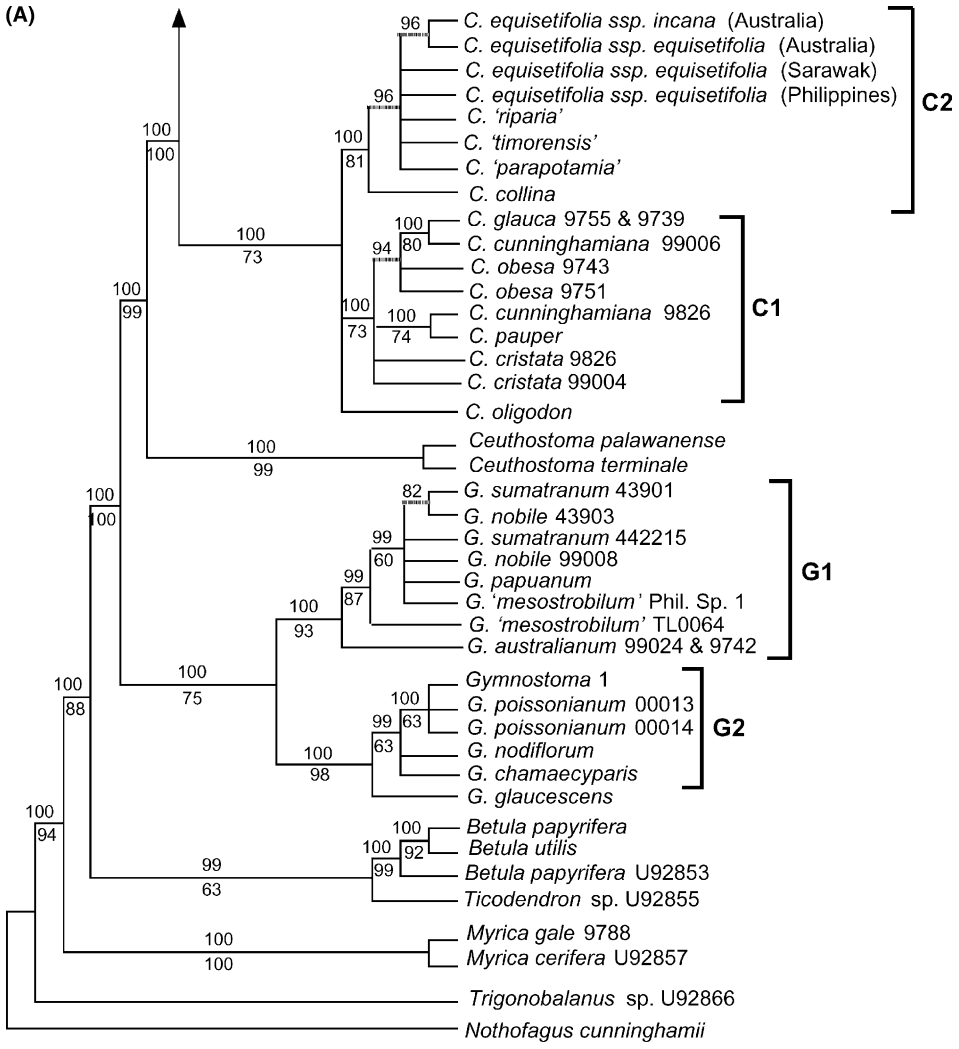

Fig. 2. Bayesian consensus of 8701 trees, and strict consensus cladogram of 93137 most parsimonious trees (length excluding autapomorphies = 464; length including autapomorphies = 700; CI, excluding autapomorphies = 0.679) derived from analyses of *mat*K sequence data from 98 samples of Casuarinaceae, and eight outgroup representatives. Bayesian posterior probability values greater than 50% are shown above branches; bootstrap values greater than 50% for the cladistic analysis are shown below branches. Dotted lines indicate branches that were supported by the Bayesian analysis but collapsed in the cladistic strict consensus. An asterisk indicates a clade that was found in the strict consensus of the cladistic analysis, but was not found by Bayesian analysis. (A) Lower portion of the consensus tree, showing outgroup taxa, *Gymnostoma* and *Casuarina*. '*Gymnostoma* 1' includes four samples of *Gymnostoma* that have identical sequences: *G. deplancheanum* KLW 7704, *G. deplancheanum* KLW 9741, *G. leucodon* KLW 9936 and *G. webbianum* KLW 7724. See text for discussion of clades Gl, G2, C1, and C2. (B) Upper portion of the consensus tree, showing *Allocasuarina*. The number in front of each species name indicates the section of *Allocasuarina* to which the species belongs (see Table 1). '*Allocasuarina* Group 1,' '*Allocasuarina* Group 2,' and '*Allocasuarina* Group 3' comprise six, four, and four samples, respectively, of section *Cylindropitys* (Section 11) that have identical sequences. '*Allocasuarina* Group 1': *A. duncanii*, *A. grampiana*, *A. littoralis* DAS 99002, *A. media* (contains an autapomorphy), *A. monilifera* and *A. zephyrea*. '*Allocasuarina* Group 2': *A. simulans*, *A. diminuta* subsp. *annectens*, *A. distyla*, *A. ophiolitica*. '*Allocasuarina* Group 3': *A. misera*, *A. mackliniana* 9883 (contains an autapomorphy), *A. mackliniana* 9884, *A. pusilla* and *A. paradoxa*. See text for discussion of clades A1, A2, and A3.

[*Betula*, *Myrica*, *Nothofagus*, *Ticodendron*, and *Trigonobalanus*; (Manos and Steele, 1997)] were obtained from GenBank (Table 1) and added to the data set. Fifteen indels (insertion/deletion events), of which seven were autapomorphic, were coded as binary characters. The sequence characters for these indels were excluded from the analysis, such that each indel received equal weighting regardless of the number of nucleotides involved. Phylogenetic analyses were carried out using PAUP* 4.0 b3 (Swofford, 1999).

Percentage pairwise base differences were calculated using the PAIRWISE BASE FREQUENCIES option in the DATA menu of PAUP* 4.0 b3. These values are corrected for gaps and ambiguities.

Maximum parsimony analyses involved heuristic search strategies as described by Catalán et al. (1997);

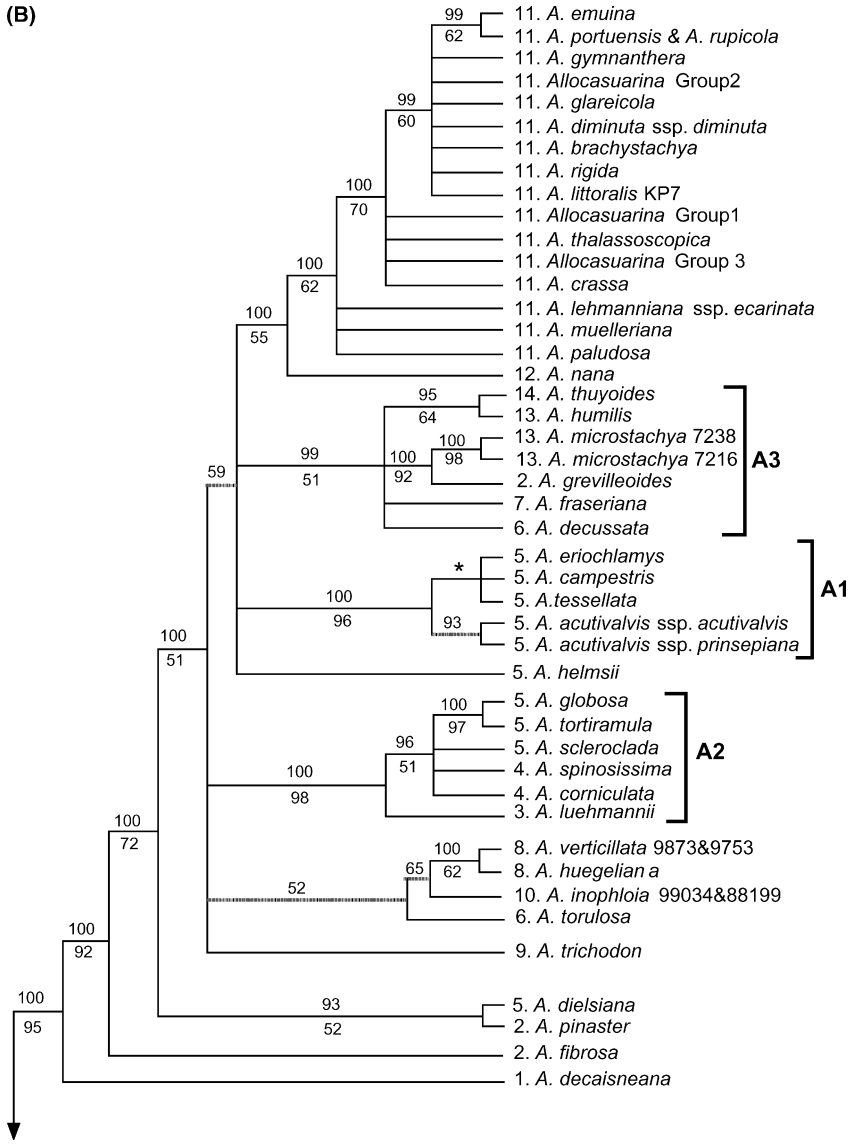

Fig. 2. *(continued)*

(see also Steane et al., 2002). The data set was bootstrapped using 10,000 replicates of the 'fast, stepwise' option of PAUP* 4.0b3 (see Mort et al., 2000).

Bayesian phylogenetic analyses were conducted with MrBayes 2.01 (Huelsenbeck and Ronquist, 2001). The equal rates model (Kimura, 1981) with unequal base frequencies (BAEFREQ = EMPIRICAL) was selected as the best fit model of nucleotide substitution (Modeltest v. 3.06; Posada and Crandall, 1998). Bayesian analysis was started from a random tree and run for 10^6 generations. We used four incrementally heated Markov chains, employing the default heating values. The Markov chains were sampled at intervals of 100 generations, resulting in a final set of 10,001 sample points. Stationarity was reached after 130,000 generations; 1300 sample points were discarded as burn-in. The remaining sample points were used to generate a 50% majority rule consensus. The percentage of sample points recovering any particular clade represents that clade's posterior probability (Huelsenbeck and Ronquist, 2001).

3. Results

A total of 106 sequences (98 Casuarinaceae and eight outgroup sequences) were included in the data set. Pooling of identical sequences resulted in 86 operational taxonomic units (OTUs) and 1502 aligned bases. Actual sequence lengths were generally much shorter than 1502 bp because of the large number of gaps introduced by highly divergent taxa. Most sequences were 1400 bp. There were 244 potentially phylogenetically informative characters included in the analysis.

Table 3
Corrected percentage pairwise differences within and between genera

	1	2	3	4	5	6	7	8
1. Allocasuarina	0–1[a]							
	1–2[b]							
2. *Casuarina*	2–3	0–1						
3. *Ceuthostoma*	4	3	0					
4. *Gymnostoma*	4	3–4	3	0–1				
5. *Betula*	7	7	5–6	5–6	0			
6. *Trigonobalanus*	7–8	7	6	6	4	—		
7. *Myrica*	9	8	7–8	7	5	7	1	
8. *Ticodendron*	13	13	12	11	10	10	11	—
9. *Nothofagus*	15	15	14	14	12	13	12	16

[a] Within sections.
[b] Between sections.

Pairwise sequence differences, corrected for gaps and ambiguities, are shown in Table 3. Within Casuarinaceae these values ranged from 0% between species within a genus to 4% between genera. Among the outgroup genera, *Betula* was the most similar to Casuarinaceae (5–7% base differences), as was also found by Manos and Steele (1997) and other workers; *Nothofagus* was the least similar, with 14–15% pairwise differences. Despite the low percentage difference among Casuarinaceae taxa, there were sufficient phylogenetically informative characters to produce well-resolved consensus cladograms with good statistical support for many clades (Fig. 2).

Maximum parsimony (MP) and Bayesian inference yielded consensus cladograms with highly congruent topologies, the latter being slightly more resolved. The strict consensus from the MP analysis and the majority rule consensus from the Bayesian analysis are shown in Fig. 2. Bayesian analysis resulted in a mean ln L value of −6509.36, variance of 88.94 and a 95% credibility interval of −6528.90 to −6491.99. Two other Bayesian analyses, one using equal rates and equal base frequencies, the other using a gamma distribution of rate variation, yielded respectively identical and almost identical topologies to that shown in Fig. 2, but lower ln L values (results not shown). The heuristic MP analysis yielded 93137 most parsimonious trees of length 464 (excluding autapomorphies; 700 including autapomorphies), consistency index excluding autapomorphies, CI = 0.679 and retention index, RI = 0.919.

Within the ingroup, the four genera are supported as monophyletic, with moderate (73% in *Casuarina*, 75% in *Gymnostoma*) to strong (95% in *Allocasuarina*, 99% in *Ceuthostoma*) bootstrap support and strong (100%) posterior probability values (Fig. 2). *Allocasuarina* and *Casuarina* form a clade (100% bootstrap support, 100% posterior probability, Fig. 2; branch support = 12 steps, Fig. 3A). *Ceuthostoma* is sister to *Allocasuarina* + *Casuarina* (this clade has 99% bootstrap support, 100% posterior probability, Fig. 2A; branch support = 42 steps, Fig. 3A), and *Gymnostoma* is sister to *Ceuthostoma* + *Casuarina* + *Allocasuarina* (this clade has 100% bootstrap support, 100% posterior probability, Fig. 2A; branch support = 21 steps, Fig. 3A). Within each of the large clades (i.e., *Gymnostoma*, *Casuarina*, and *Allocasuarina*) there is distinct phylogenetic structure. *Gymnostoma* comprises two major clades, G1 and G2, both of which have good bootstrap (Fig. 2A) and branch (Fig. 3A) support and 100% posterior probability (Fig. 2A). Clade G1 comprises Malesian species (*G. nobile*, *G. sumatranum*, and *G.* '*mesostrobilum*') as well as *G. australianum* from Northern Australia. The other clade, G2, is purely New Caledonian (*G. chamaecyparis*, *G. deplancheanum*, *G. glaucescens*, *G. leucodon*, *G. nodiflorum*, *G. poissonianum*, and *G. webbianum*).

Similarly, geographic partitioning of taxa also occurs in *Casuarina*. Clade C1 comprises only Australian species of *Casuarina*. Clade C2 is more cosmopolitan, with species from Timor, the Philippines and New Caledonia, as well as the widespread *Casuarina equisetifolia* (four representatives from Australia, Philippines and Sarawak (Borneo)). The relationships within these two main clades remain unresolved, as does the phylogenetic position of the New Guinean species, *Casuarina oligodon* (in a hard polytomy, i.e. there are insufficient data to resolve the node; there is no conflict between characters).

Within *Allocasuarina* several small clades have high bootstrap values but most clades have bootstrap proportions less than 70% (Fig. 2B) and there are frequent hard polytomies. Posterior probability values for many of these clades, however, is high (>95%). Clades that were found by Bayesian analysis that were not found by cladistic analysis usually had a relatively low posterior probability (Fig. 2B, branches with dotted lines). Similarly, the clade that was found by cladistic analysis that was not found by Bayesian analysis had a bootstrap value <50% (Fig. 2B, branch marked with asterisk). The taxonomic sections delimited by Wilson and Johnson (1989) exhibit some phylogenetic integrity. The largest section, *Cylindropitys* (section 11; 27 out of 30 species represented) appears to be monophyletic; the monotypic

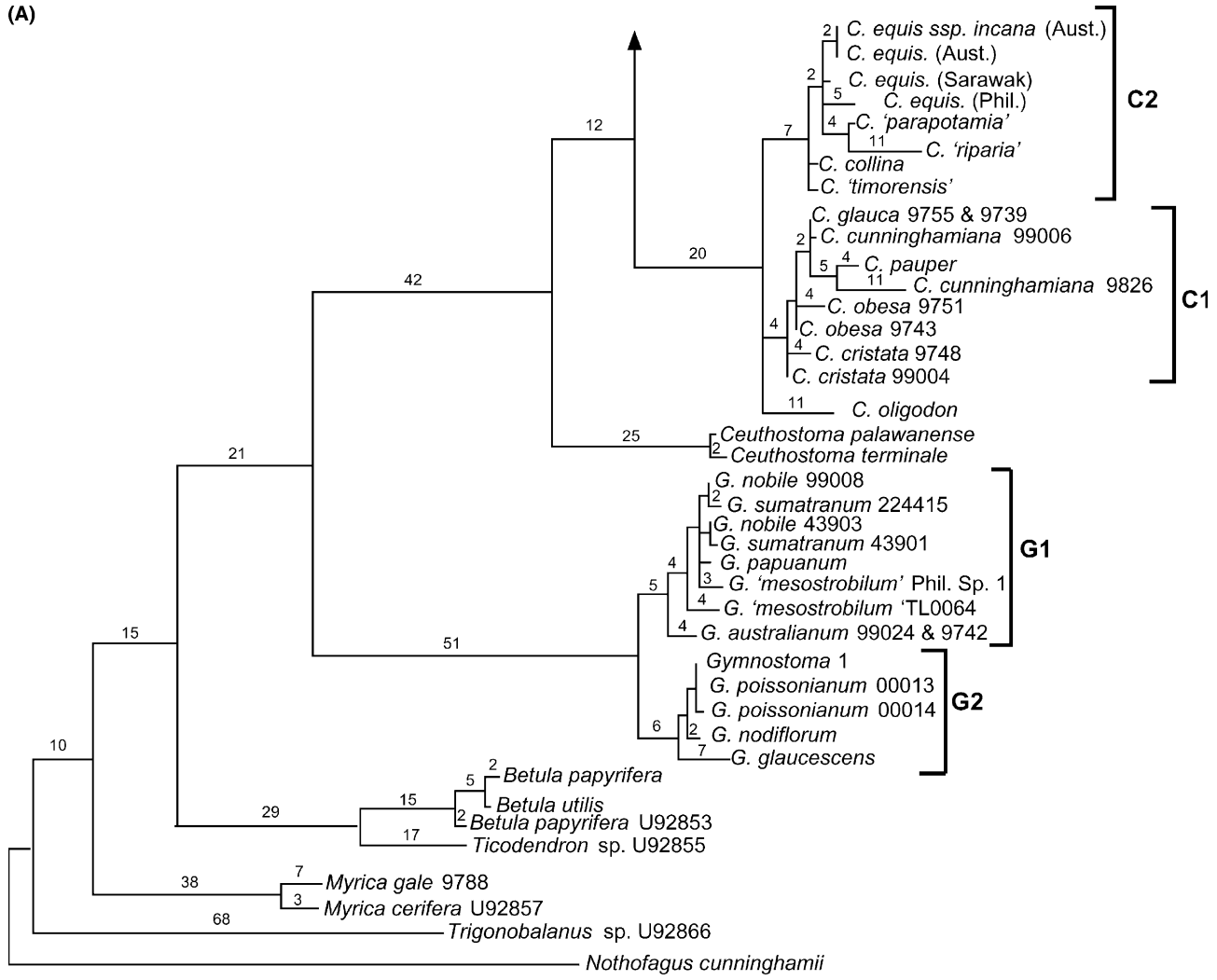

Fig. 3. Phylogram (including autapomorphies) of one of the 57120 trees (see legend to Fig. 2) obtained by cladistic analysis of *mat*K data from 97 samples of Casuarinaceae and eight outgroup representatives. Branch lengths are shown above branches. Branches without digits above them are 1 step long. (A) Lower portion of phylogram, showing outgroup taxa, *Gymnostoma* and *Casuarina*. '*C. equis*.' = *C. equisetifolia* subsp. *equisetifolia*. See legend to Fig. 2 for further details of annotations. (B) Upper portion of the phylogram showing *Allocasuarina*. See legend to Fig. 2 for explanation of annotations.

section *Nanopitys* (section 12) appears to be sister to *Cylindropitys* (11). The small section *Trachypitys* (section 13; two of three species represented) appears to be polyphyletic: *Allocasuarina microstachya* and *A. humilis* appear in a clade (A3; Fig. 2B) with the monotypic sections *Acanthopitys* (section 14) and *Amorphopitys* (section 7), as well as representatives of sections *Oxypitys* (section 2) and *Allocasuarina* (section 6). Of the remaining sections, *Ceropitys* (section 5) is the largest with nine species, and appears to be polyphyletic (Figs. 2 and 3). Species from section *Ceropitys* arise from four nodes on the cladogram. Within *Ceropitys* there is a well-supported monophyletic group (A1; bootstrap support = 96%, posterior probability = 100%, Fig. 2B; branch support = 5, Fig. 3B) comprising *A. eriochlamys*, *A. campestris*, *A. tessellata*, and two subspecies of *A. acutivalvis*. The other species of section *Ceropitys* associate with species of sections *Oxypitys* (section 2), *Platypitys* (sect. 3) and *Echinopitys* (sect. 4). Three other members of section 5, *Ceropitys* (*A. globosa*, *A. tortiramula*, and *A. scleroclada*) appear in a well-supported clade (A2; bootstrap percentage = 98%, posterior probability = 100%, Fig. 2B; branch support = 4, Fig. 3B) with *Allocasuarina luehmannii* (the sole member of section 3, *Platypitys*, from eastern Australia) plus both species from section 4, *Echinopitys* (*A. corniculata* and *A. spinosissima*). The position of *A. helmsii* (section *Ceropitys*) is unresolved, while *A. dielsiana* grouped with *A. pinaster* (section 2, *Oxypitys*; but bootstrap support is only 50%). Another apparently polyphyletic section is *Oxypitys* (section 2), with one of its species, *Allocasuarina fibrosa*, apparently sister to all *Allocasuarina* except

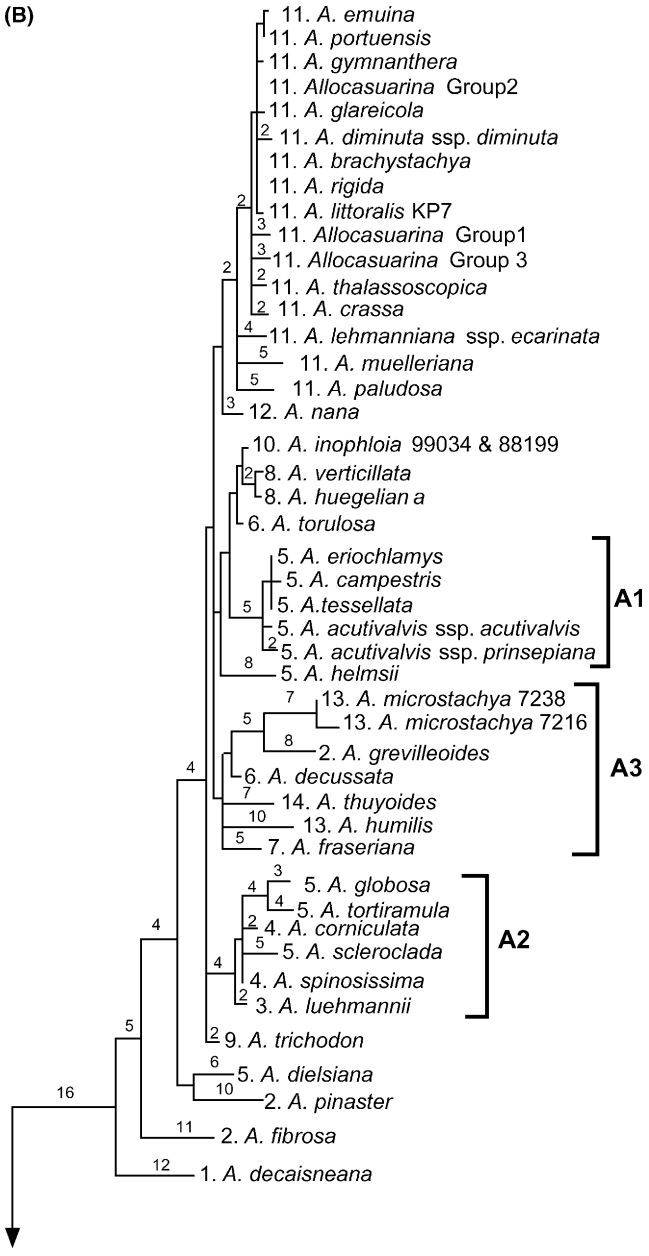

Fig. 3. (*continued*)

A. decaisneana (section 1, *Dolichopitys*). One species of section *Oxypitys*, *Allocasuarina grevilleoides*, is sister to *A. microstachya* (section 13, *Trachypitys*) (bootstrap support = 92%, posterior probability = 100%, Fig. 2B; branch support = 5 steps, Fig. 3B). This clade falls within a partly unresolved clade (A3), comprising species from sections *Allocasuarina* (section 6—one of two species), *Amorphopitys* (section 7—monospecific), *Trachypitys* (section 13—two out of three species were sampled), and *Acanthopitys* (section 14—monospecific). Clade A3 has poor bootstrap support (51%) but high posterior probability (99%; Fig. 2B). The scattered placing of species of section *Oxypitys* suggests the need for re-evalution of this section, notably whether the morphological similarities such as unusual branchlet arrangement are convergent.

4. Discussion

Bayesian inference is a relatively recent addition to the analytical toolbox for phylogenetics. Like maximum likelihood analysis, Bayesian estimation is based on the likelihood function. However, whereas a maximum likelihood value represents the probability of the data given a hypothesis (i.e., a tree), Bayesian inference provides the probability of a hypothesis (i.e., a tree) given the data (Lewis, 2001). One very attractive advantage of Bayesian analysis over maximum likelihood is that it requires fewer computational resources, so that large data sets can be analysed more readily. Also, because the estimation of branch support accompanies tree estimation, additional bootstrap analyses are not required. Likelihood-based phylogenetic analyses provide alternatives to parsimony analysis that tend to be less sensitive to artifacts like long branch attraction. In this study a maximum likelihood analysis was not possible because of the large size of the data set. Bayesian inference provided a practical alternative, with the resulting phylogeny providing additional support for the major clades identified by maximum parsimony analysis.

The phylogeny of the Casuarinaceae presented here offers strong support for the four genera defined by Johnson and Wilson (1989). *Gymnostoma* is sister to the other three genera, and this supports the hypothesis that the encryption of stomata in the other three genera has a single origin. This transition can probably be dated to the Late Oligocene at least, based on the recent discovery of Casuarinaceae branchlets with encrypted stomata in sediments of this age from Riversleigh in northeastern Australia (Guerin, 2001). The fossil record of *Gymnostoma* significantly precedes this date (Late Paleocene; Scriven and Hill, 1995), but it still cannot be determined from the fossil record whether encryption of stomata is the ancestral or derived condition in the family. Within the clade containing *Ceuthostoma* + *Allocasuarina* + *Casuarina*, *Ceuthostoma* is sister to the other two genera, suggesting that four leaves (represented by the longitudinal phyllichina; Johnson and Wilson, 1989) per whorl is the ancestral condition, since this also occurs in *Gymnostoma*. This suggests that more than four leaves per whorl is the derived condition. Increasing the number of leaves per whorl allows for more developed encryption of stomata. In plants with four leaves per whorl, the stems tend to be square and although shallow furrows may develop (e.g., in some *Gymnostoma* species), the stomata do not tend to be inside the furrows. Increasing the number of articles

allows for a rounder, more sclerenchymatous stem (see Johnson and Wilson, 1989) and reduces the amount of space between the leaves. Furrows in such a stem take up a greater proportion of the room between phyllichnia, increasing the likelihood that stomata will occur inside the furrows. This encryption of stomata, and increased amounts of sclerenchyma, would have had a selective advantage in a dry climate, eventually leading to very closed furrows with highly protected stomata.

Within *Gymnostoma*, two clades can be identified, one in New Caledonia and one in Australia/Malesia. Recently, Swenson et al. (2001) hypothesised that *Nothofagus* had reached New Caledonia via long distance dispersal from New Zealand, and that the closely related group of extant species there have probably evolved from a single colonist species. A similar scenario may well be true for *Gymnostoma*—as well as New Caledonian species of *Araucaria*, *Agathis* (Setoguchi et al., 1998) and *Metrosideros* (Wright et al., 2000, 2001)—and would explain the well defined clade of extant species in New Caledonia. This hypothesis requires further testing to distinguish it from the possiblity that the New Caledonian species are descendents from a single Gondwanan ancestor. It is significant that while the Casuarinaceae have a fossil record in New Zealand that dates back to the Paleocene (ca 55–65 mya; Macphail et al., 1994), it does not extend back to the time when New Zealand is believed to have separated from Gondwana, ca. 85–90 mya. This suggests a requirement for dispersal (from Australia to New Zealand; see Winkworth et al., 2002) in the family at an early stage (or a poorly known fossil record, e.g., see Crisp (1991)). The wide distribution of *C. equisetifolia* today is a modern example of the ability of species within the family to achieve dispersal. The two subspecies of *C. equisetifolia*, subsp. *equisetifolia* and subsp. *incana*, in this study, collected from Queensland, Australia, group with other *Casuarina* species from the Indomalesian region. *Casuarina equisetifolia* is dispersed by wind and sea (and possibly also by humans) and is found on tropical and subtropical coastlines of northern and northeastern Australia, Burma to Vietnam, Malesia, Melanesia and Polynesia; records from India, the Mascarenes and other tropical areas are regarded as the result of relatively recent introductions, either deliberate or accidental (Johnson and Wilson, 1989). The grouping of *C. equisetifolia* with Indomalesian species (Clade C2; Fig. 2A) rather than the endemic Australian species (Clade C1; Fig. 2A) suggests that *C. equisetifolia* is either a relatively new species that came to Australia from Indomalesia, or evolved in Australia (from an ancestor that was also common to the other Indomalesian taxa) and then dispersed to other regions.

The *mat*K results of Sogo et al. (2001) do not support the division of *Casuarina* into clades C1 and C2. This appears to be because their data set (1014 bp) did not include a highly informative region of ca. 300 bp at the 3′ end of the *mat*K gene. Inclusion of the Sogo et al. (2001) *Casuarina* sequences in our data set resulted in conspecific samples grouping together in clades C1 and C2 (data not shown).

On morphological grounds, *C. cunninghamiana* and *C. oligodon* might be expected to group with clade C2 and *C. collina* with clade C1. Our molecular data, however, suggest that phylogenetic groupings coincide more closely with the species' biogeography than with morphological traits, suggesting morphological convergence between species. Similar phenomena have been reported for other taxa [e.g., *Banksia* (Mast and Givnish, 2002); *Clerodendrum* (Steane et al., 1999); Costaceae (Specht et al., 2001)]. Our results call for a reexamination of morphological characters in *Casuarina* and study of additional genes to verify the results reported here.

Casuarina and *Allocasuarina* are sister taxa, quite similar both in morphology and in *mat*K sequence data. Since the divergence of *Ceuthostoma* and *Casuarina* + *Allocasuarina* there has been a major radiation of species, especially in *Allocasuarina*. The xeromorphic characters developed in the 'cryptostomes' allowed *Casuarina* and especially *Allocasuarina* to diversify and exploit the increasing variety of niches that arose with the gradual desiccation of Australia over the past 30 million years. The dark, shiny samaras in *Allocasuarina*, for example, are unique in the family. The inflated cells of their mesocarp layer have walls that are spirally thickened; these thickenings expand through the weak exocarp and hold water around the fruit when moistened (Ladd, 1989). The spirals are also present in *Casuarina* and water is held by them, but these species have a stronger exocarp so that the spirals do not break through the exocarp and trap less water than in *Allocasuarina*. The composition of the spirals is suggested to be cellulosic by Ladd (1989) rather than hygroscopic polysaccharides as thought by Torrey (1983). The end result is a moist, mucilaginous-looking samara; as suggested by Turnbull and Martensz (1983) and Torrey (1983), this could be considered an adaptation for rapid germination and establishment in habitats with erratic water supply, as found in so many parts of Australia.

Johnson and Wilson (1989) recognised 14 sections in *Allocasuarina*. Although the *mat*K data do not provide enough information to resolve fully the relationships among the sections, they do indicate that section 11, *Cylindropitys*, is monophyletic, while the two other large sections (*Ceropitys* and *Oxypitys*) appear to be polyphyletic. The species in Clade A3, while sufficiently morphologically different to be placed by Johnson and Wilson (1989) into separate sections, have overlapping distributions in Western Australia. This raises the possibility that, as for *Gymnostoma* and *Casuarina*, the species phylogeny within *Allocasuarina* is more closely

aligned with biogeography than with morphology. However, while the geographic partitioning within *Gymnostoma* and *Casuarina* is most likely due to ancient biogeography (e.g., vicariant evolution, long distance dispersal), the events leading to the biogeographic patterns seen in *Allocasuarina* are possibly more recent and may not reflect species phylogeny per se. It is possible that within *Allocasuarina*, reproductive isolation between some species is incomplete, and interspecific hybridisation may occur among some sympatric species from different sections (e.g., the Western Australian species in clade A3), a phenomenon that could result in the sharing of chloroplast genomes among morphologically distinct taxa. Extensive sharing of chloroplast haplotypes—attributed to some form of horizontal transfer, such as hybridisation—between species has been observed among Tasmanian species of *Eucalyptus* (Steane et al., 1998; McKinnon et al., 2001), as well as northern hemisphere *Armeria* (Gutiérrez Larena et al., 2002), *Quercus* (Belahbib et al., 2001) and *Pinus* (Matos and Schaal, 2000). Some Western Australian eucalypts also demonstrate extensive sharing of chloroplast haplotypes, but in this case lineage sorting, rather than hybridisation, has been proposed as the most likely mechanism [Dean Nicolle, (Flinders University, South Australia), pers. comm.]. We are undertaking further work using more variable DNA sequences [e.g., the *psb*A–*trn*H spacer region of the chloroplast DNA and the nuclear ribosomal internal transcribed spacer (ITS) regions] that may help to clarify the intersectional relationships within *Allocasuarina*.

Acknowledgments

We would like to thank the following individuals and institutions for providing us with plant material for the study: David Albrecht (Alice Springs Herbarium, Northern Territory); Australian National Botanic Gardens, Canberra; Bill Baker and Mark Chase (RBG Kew, England); Tim Brodribb (Harvard University, USA); Richard Chung (Forest Research Institute, Kuala Lumpur, Malaysia); Andrew Ford and Bernie Hyland (CSIRO, Atherton, Queensland); Greg Jordan (University of Tasmania, Australia); Kings Park and Botanic Garden, Perth, Western Australia; Tanya Livshultz (LH Bailey Hortorium, USA); Domingo Madulid (National Museum, Manila, Philippines); Kay Morgan (Sydney, NSW); Royal Botanic Gardens Sydney, New South Wales (especially Peter Jobson, Joy Everett, Ken Hill, Natasha Leist, Steve Skinner, and Gillian Towler); Royal Tasmanian Botanical Garden, Hobart, Tasmania; Philip Sharpe (Coolum, Queensland); Runi Sylvester, Pusat Penyelidikan Hutan, Sarawak; Luke Vanzino, Hobart, Tasmania. Our thanks also go to Annette Blanchfield, Peter Bundock and Ashley Edwards for technical assistance. This research was funded by the Australian Biological Resources Study (ABRS), 1999–2000 and the Australian Research Council.

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