Host Selection of Symbiotic Cyanobacteria in 31 Species of the Australian Cycad Genus: *Macrozamia* (Zamiaceae)

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The nitrogen-fixing cyanobacterium *Nostoc* is a commonly occurring terrestrial and aquatic cyanobacterium often found in symbiosis with a wide range of plant, algal, and fungal species. We investigated the diversity of cyanobacterial species occurring within the coralloid roots of different *Macrozamia* cycad species at diverse locations throughout Australia. In all, 74 coralloid root samples were processed and 56 endosymbiotic cyanobacteria were cultured. DNA was isolated from unialgal cultures and a segment of the 16S rRNA gene was amplified and sequenced. Microscopic analysis was performed on representative isolates. Twenty-two cyanobacterial species were identified, comprising mostly *Nostoc* spp. and a *Calothrix* sp. No correlation was observed between a cycad species and its resident cyanobiont species. The predominant cyanobacterium isolated from 18 root samples occurred over a diverse range of environmental conditions and within 14 different *Macrozamia* spp. Phylogenetic analysis indicated that endosymbionts were not restricted to previously described terrestrial species. An isolate clustering with *Nostoc* PCC7120, an aquatic strain, was identified. This is the first comprehensive study to identify the endosymbionts within a cycad genus using samples obtained from their natural habitats. These results indicate that there is negligible host specialization of cyanobacterial endosymbionts within the cycad genus *Macrozamia* in the wild.

Many species of nitrogen-fixing cyanobacteria can form symbiotic associations with prokaryotes and eukaryotes, providing the host with nitrogen-rich compounds. Hosts include diatoms, fungi (*Geosiphon* and lichens), Bryophytes (liverworts and hornworts), Pteridophytes (*Azolla*), Gymnosperms (Cycads), and Angiosperms (*Gunnera*) (Rai et al. 2000; Bergman et al. 2007). All endosymbionts are heterocystous cyanobacteria, predominantly from the genus *Nostoc*, though species of *Scytonema*, *Calothrix*, and *Richelia* have been reported (Rai et al. 2000; Bergman et al. 2007).

The cycad family Zamiaceae contains three genera endemic to the Australian continent: *Bowenia*, *Lepidozamia*, and *Macrozamia*. The genera *Bowenia* and *Lepidozamia* both contain two species that are found in restricted habitats on the eastern sea-

board of Australia (Hill and Osborne 2001). The genus *Macrozamia* comprises over 40 named species, with the majority of specimens occurring in the eastern states of Queensland and New South Wales (Forster 2004). Some species such as *Macrozamia communis*, *M. macleayi*, and *M. riedlei* have a broad distribution; however, most are limited to small areas of restricted habitats, usually in impoverished soil (Jones 2002).

Cycads form coralloid roots, which are highly specialized structures that accommodate endosymbiotic cyanobacteria (Rai et al. 2000). The plant initially produces apogeotropic precorallous roots with a papillose sheath from a lateral root or directly off the taproot (Ahern and Staff 1994). Colonization by a cyanobacterium results in irreversible changes in the root, such as a change to geotrophic growth, loss of the papillose sheath, and a change in the subcortical layer hosting the cyanobiont (Rai et al. 2000; Costa and Lindblad 2002). Endosymbionts are restricted to the intercellular regions of the subcortical root zone where they grow in a microaerobic, dark environment. Although photosynthetically inactive, the cyanobionts maintain their pigmentation and are obvious as a clear green ring in coralloid root transverse sections (Costa and Lindblad 2002). *Nostoc* spp. are the most commonly found cyanobionts, although a species of *Calothrix* (Grobelaar et al. 1987) has been reported in *Encephalartos hildebrandtii* and *Anabaena* endosymbionts have been reported in *Cycas revoluta* (Obukowicz et al. 1981; Zhu 1982). All known cycad cyanobionts are within the botanical order Nostocales (Komarek and Anagnostidis 1989) or, bacteriologically, subsection IV of the cyanobacteria (Rippka et al. 2001).

Nostoclean cyanobacteria have a vegetative life cycle of two main stages. In the first stage, environmentally induced differentiation of mature filaments or the germination of akinetes produces hormogonia (Komarek and Anagnostidis 1989). Hormogonia are characterized by the absence of exopolysaccharide sheathing, smaller cell size, and the absence of specialized cells such as heterocysts and akinetes. In all three cyanobiont genera reported from cycads, the hormogonia are motile. Plant hosts, including cycads, have been shown to produce a hormogonia induction factor to ensure colonization by motile hormogonia (Bergman et al. 1996). Once established within the roots of the cycad plant, hormogonia undergo differentiation into vegetative and heterocystous (nitrogen-fixing) cells (Costa and Lindblad 2002). This differentiation of cellular function along the filament of heterocystous cyanobacteria is a key feature in their success as symbionts. It is interesting to note that, despite the formation of heterocysts, nitrogen fixation in cycads requires microaerobic conditions (Lindblad et al. 1991). Increased heterocyst frequencies were observed in the roots of *Zamia skinneri* from 16 to 86% over the length of the coralloid root (Lindblad et al. 1985).

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*The e-Xtra logo stands for “electronic extra” and indicates that Figure 3 appears in color online.

Different strains of *Nostoc* cyanobacteria have been identified in host cycads from different geographical areas. Zimmerman and Rosen (1992) collected aseptic soil samples surrounding the coralloid roots of a cultivated population of *Z. integrifolia* and a *Dioon* plant and plated them on nitrogen-free BG11 medium. The filamentous soil cyanobacteria surrounding the cycad root system consisted of *Oscillatoria* and *Calothrix* spp. and a *Nostoc* sp. This *Nostoc* isolate was unable to colonize coralloid roots in infection studies, despite being the predominant *Nostoc* sp. in the soil. A single morphological cyanobiont was identified in all 11 root nodules investigated. The authors suggested that there could be a correlation between the presence of *Nostoc* strains able to infect cycads, overall cyanobacterial diversity within the soil, and host selectivity. The most extensive investigation into the diversity of symbiotic cyanobacteria in specimens collected in their natural habitats was conducted on 31 species of *Encephalartos* indigenous to South Africa (Grobelaar et al. 1987). Of the 41 isolates obtained, *Nostoc commune* was identified in 28 cases, with 5 isolates of *N. punctiforme*, 2 isolates each of *N. ellipsosporum*, *N. paludosum*, and *N. sphaericum*, a single isolate of *N. muscorum*, and a *Calothrix* sp. obtained. Identification was based on microscopic analysis of morphological characteristics in the absence of molecular characterization of these isolates.

Little is known about the genetic diversity of cyanobionts from naturally grown cycads. Lindblad and associates (1989) studied the cyanobionts of five species of cycad growing in their natural habitats from the genera *Ceratozamia*, *Zamia*, and *Dioon*. Unfortunately, they had to pool coralloid root samples from different plants to obtain sufficient material to do restriction fragment length polymorphism (RFLP) analysis. Their work indicated a high diversity of cyanobacteria both within and between different cycad species. Lotti and associates (1996) discovered some diversity in cultured *Nostoc* isolates from cycad plants of the genera *Cycas*, *Encephalartos*, *Macrozamia*, *Lepidozamia*, and *Dioon* from different botanical gardens and greenhouses around Italy. Analysis of tRNA^{Leu} intron sequences indicated that, although diversity of *Nostoc* symbionts existed within a single plant, they were restricted to individual coralloid roots (Costa et al. 1999). The 11 coralloid root samples used in their study were obtained from botanical garden specimens of the genera *Cycas*, *Encephalartos*, and *Zamia*. Zheng and associates (2002) demonstrated a large variety of cyanobacteria in a single coralloid root using polymerase chain reaction (PCR)–short tandemly repeated repetitive (STRR) and PCR using the highly iterated palindromic (HIP) sequences found in cyanobacterial genomes. They also noted diversity between the apical, middle, and basal regions of a single coral-

Table 1. Cycad communities sampled in this study^a

Sample no.	Cycad species	Location of sample site	Voucher no.	Collection	Cyanobionts
B1	<i>Bowenia serrulata</i>	Byfield	PIF32324	BRI	Y
C1	<i>Cycas media</i> subsp. <i>media</i>	Royal Botanic Gardens	C1	NSW	Y
C2	<i>C. ophiolitica</i>	Beserker Range	PIF12258	BRI	Y
C3	<i>C. megacarpa</i>	Mt. Colosseum	PIF12247	BRI	No
C4	<i>C. ophiolitica</i>	Princhester	PIF29717	BRI	Y
C5	<i>C. megacarpa</i>	Dee Range	PIF29707	BRI	Y
LZ1	<i>Lepidozamia peroffskyana</i>	Dinosaur's Playground	PIF25985	BRI	Y
LZ2	<i>L. peroffskyana</i>	Breezy Hill	PIF25986	BRI	Y
LZ3	<i>L. peroffskyana</i>	Girralong	LZ3	NSW	Y
LZ4	<i>L. peroffskyana</i>	Girralong	LZ4	NSW	No
MZ5	<i>Macrozamia lucida</i>	D'Aguilar Range	PM15	BRI	No
MZ6	<i>M. macleayi</i>	Brisbane Forest Park	PIF9886A	BRI	Y
MZ7	<i>M. reducta</i>	Royal Botanic Gardens	MZ7	NSW	Y
MZ8	<i>M. lucida</i>	Royal Botanic Gardens	MZ8	NSW	Y
MZ9	<i>M. miquelii</i>	Royal Botanic Gardens	MZ9	NSW	Y
MZ12	<i>M. communis</i>	Myola	MZ12	NSW	Y
MZ13	<i>M. spiralis</i>	Berkshire Park	MZ13	NSW	Y
MZ14	<i>M. spiralis</i>	Landilo Road	MZ14	NSW	Y
MZ15	<i>M. elegans</i>	Comleroy	MZ15	NSW	Y
MZ16	<i>M. reducta</i>	Aberdare State Forest	MZ16	NSW	Y
MZ17	<i>M. reducta</i> vt	Aberdare State Forest	MZ17	NSW	Y
MZ18	<i>M. flexuosa</i>	Pelaw Main	MZ18	NSW	Y
MZ19	<i>M. flexuosa</i>	Mulwee	MZ19	NSW	No
MZ20	<i>M. reducta</i>	Mt. Sugarloaf	MZ20	NSW	Y
MZ21	<i>M. moorei</i>	Springsure	PIF30133	BRI	Y
MZ22	<i>M. reducta</i>	Gardens of Stone National Park	MZ22	NSW	Y
MZ23	<i>M. reducta</i>	Gardens of Stone National Park	MZ23	NSW	No
MZ24	<i>M. secunda</i>	Gardens of Stone National Park	MZ24	NSW	Y
MZ25	<i>M. reducta</i>	Munghorn Gap Nature Reserve	MZ25	NSW	Y
MZ26	<i>M. secunda</i>	Munghorn Gap Nature Reserve	MZ26	NSW	No
MZ27	<i>M. heteromera</i>	Goonoo State Forest	MZ27	NSW	Y
MZ28	<i>M. heteromera</i> vt	Goonoo State Forest	MZ28	NSW	Y
MZ29	<i>M. heteromera</i>	Wurrumbungle National Park	MZ29	NSW	Y
MZ30	<i>M. polymorpha</i>	Pilliga Nature Reserve	MZ30	NSW	Y
MZ31	<i>M. diplomera</i>	Pilliga Nature Reserve	MZ31	NSW	No
MZ32	<i>M. heteromera</i>	Wurrumbungle National Park	MZ32	NSW	No
MZ33	<i>M. diplomera</i>	Premar	MZ33	NSW	No
MZ34	<i>M. diplomera</i>	Spring Ridge	MZ34	NSW	Y
MZ35	<i>M. glaucophylla</i>	Pilliga East State Forest	MZ35	NSW	Y

(continued on next page)

^a Cycad identification was confirmed at the following herbaria: BRI = Queensland Herbarium, Brisbane and NSW = National Herbarium of New South Wales, Sydney. Voucher numbers and their present location are provided. Cyanobiont: y indicates a cyanobacterium was isolated from the coralloid root; no indicates no endosymbiont was isolated; leaf only indicates that no coralloid root was found; no-ring indicates that the coralloid root was either immature and uncolonized or dead without the characteristic green ring indicating cyanobacterial growth.

loid root. Their study made use of indigenously growing samples, predominantly of the genus *Cycas*, from a National Park in China and greenhouse specimens from Stockholm. The latter showed reduced diversity in endosymbiont species. They concluded that little is known about the genetic diversity of cyanobacteria from cycads growing in the wild or the cyanobacteria associated with the cycads in the rhizosphere. Shortcomings of this study were the potential of contamination with plant DNA in samples and the lack of plant DNA controls. The diversity of cyanobacterial endosymbionts within a single coralloid root was contested by Costa and associates (2004), who studied specimens of *M. riedlei* growing in their natural habitat and in greenhouses in Perth, Australia. They identified only a single strain of *Nostoc* within an individual coralloid root and in a single plant using the tRNA^{Leu} intron as a genetic marker. Plants nearby each other were very similar with regard to their endosymbiont *Nostoc* strain.

A low specificity in the Angiosperm–*Nostoc* symbiosis was found during an investigation into cyanobacterial diversity in geographically related and distant host plants of the angiosperm genus *Gunnera* (Nilsson et al. 2000). The authors compared cyanobacterial symbionts isolated from *Gunnera* plants in New Zealand and Chile by PCR fingerprinting using STRR sequences as primers (Nilsson et al. 2000). More than one species of *Nostoc* was found capable of infecting a single plant or even a

single stem gland. Their data supported a previous study indicating that closely related *Nostoc* strains could act as endosymbionts of different *Gunnera* spp. and that there was heterogeneity among *Nostoc* isolates of the same *Gunnera* sp. (Rasmussen and Svernnig 1998). A more in-depth study of the diversity of *Gunnera* endosymbionts using RFLP analysis of amplified 16S rRNA genes, sequencing of the 16S–23S intergenic region, and denaturing gradient gel electrophoresis of *hetR* PCR products indicated that there was no correlation between clustering of the endosymbiotic *Nostoc* spp. and their *Gunnera* host plants (Rasmussen and Svernnig 2001). Guevara and associates (2002) used PCR–STRR directly on tissues from the stem glands of *Gunnera* specimens from Chile. They found genetic diversity in *Nostoc* endosymbionts among host plants both within and between *Gunnera* populations. The authors stressed that further studies using molecular techniques on more extensive samples of symbiotic tissue from *Gunnera* spp. from more geographic locations should reveal a clearer picture of the cyanobacterial diversity present in natural populations. Papaefthimiou and associates (2008) investigated the phylogeny and morphology of cyanobiont isolates from the genera *Gunnera*, *Cycas*, *Dioon*, *Encephalartos*, *Macrozamia*, *Azolla*, and *Anthoceros* and compared them with those of free-living isolates. They suggested that the ability to form symbioses may have evolved twice. The first led to the ability of *Nostoc* spp. to form symbioses with a

Table 1. (continued from preceding page)

Sample no.	Cycad species	Location of sample site	Voucher no.	Collection	Cyanobionts
MZ36	<i>M. stenomera</i>	Mt. Coryah	MZ36	NSW	Y
MZ37	<i>M. stenomera</i>	Deriah State Forest	MZ37	NSW	Y
MZ38	<i>M. concinna</i>	Nundle State Forest	MZ38	NSW	Y
MZ40	<i>M. communis</i>	Currambene State Forest	MZ40	NSW	Y
MZ41	<i>M. communis</i>	Myola	MZ41	NSW	Y
MZ42	<i>M. communis</i>	Seven Miles Beach National Park	MZ42	NSW	Y
MZ43	<i>M. crassifolia</i>	SSW of Mundubbera	PIF31081	BRI	No ring
MZ44	<i>M. fawcettii</i>	Royal Camp	PIF31372	BRI	Y
MZ45	<i>M. johnsonii</i>	Dalmorton	PIF31373	BRI	Y
MZ46	<i>M. montana</i>	Big Hill	PIF31375	BRI	Y
MZ47	<i>Macrozamia</i> sp. Tamworth	Watsons Creek	PIF31379	BRI	Y
MZ48	<i>M. humilis</i>	Inverell	PIF31380	BRI	No ring
MZ49	<i>Macrozamia</i> sp. Bundarra	Munro	PIF31384	BRI	No ring
MZ50	<i>M. plurinervia</i>	Torrington	PIF31385	BRI	Y
MZ51	<i>M. fawcettii</i>	Tabulum	PIF31368	BRI	Y
MZ52	<i>Macrozamia</i> sp. Bundarra	Sepoy	PIF31381	BRI	Leaf only
MZ53	<i>M. conferta</i>	near Warwick	PIF31387	BRI	Leaf only
MZ54	<i>M. occidua</i>	Sundown	PIF31386	BRI	Leaf only
MZ55	<i>M. viridis</i>	Girraween	PIF31804	BRI	No ring
MZ56	<i>M. spiralis</i>	Royal National Park	MZ56	NSW	Y
MZ57	<i>M. cardiacensis</i>	Mt. Walsh	PIF26495	BRI	Y
MZ58	<i>M. douglasii</i>	Fraser Island	PM86	BRI	No
MZ59	<i>M. longispina</i>	Mt. Widgee	PIF19138	BRI	No
MZ60	<i>M. moutperriensis</i>	Seaview Range	PIF13372	BRI	No
MZ61	<i>M. parcifolia</i>	Seaview Range	PIF13107	BRI	Y
MZ62	<i>M. moutperriensis</i>	Brooweena	PIF9343	BRI	Y
MZ63	<i>M. pauli-guilielmi</i>	Cooloolo	PIF30817	BRI	No
MZ64	<i>M. machinii</i>	Inglewood	PIF16430	BRI	Y
MZ65	<i>M. riedlei</i>	NW Narragin	PIF30395	BRI	Y
MZ66	<i>M. riedlei</i>	NW Narragin	PIF30395	BRI	Y
MZ67	<i>M. riedlei</i>	NW Narragin	PIF30395	BRI	Y
MZ68	<i>M. macdonnellii</i>	Standley Chasm	I. Terry	BRI	Leaf only
MZ69	<i>M. pauli-guilielmi</i>	Isis River	PIF29655	BRI	Y
MZ70	<i>M. miquelii</i>	Mt. Archer	PIF12252B	BRI	No
MZ71	<i>M. miquelii</i>	Byfield	PIF32323	BRI	No
MZ72	<i>M. serpentina</i>	Glen Geddes	PIF17365	BRI	No
MZ73	<i>M. serpentina</i>	Mt. Slope way	PIF12273A	BRI	Y
MZ74	<i>M. macleayi</i>	Mt. Colosseum	PIF12248	BRI	Y
MZ75	<i>M. lomandroides</i>	Bundaberg	PIF32320	BRI	No
MZ76	<i>M. platyrhachis</i>	Blackdown Tableland	PIF29637	BRI	No
MZ77	<i>M. fearnsidei</i>	Glenhaughton	PIF32351	BRI	Y
MZ78	<i>M. miquelii</i>	Dee Range	PIF29713	BRI	Y
MZ79	<i>M. communis</i>	Murramarang National Park	MZ79	NSW	No ring
MZ80	<i>M. communis</i>	Currowan State Forest	MZ80	NSW	Y

diverse variety of plants while the second led to the unique association of specific cyanobacteria with the water fern *Azolla* (Papaefthimiou et al. 2008).

Host specialization in symbiotic cyanobacteria has been assessed in several species of the lichenized fungus *Peltigera*. By conducting phylogenetic analysis of the 16S rRNA, *rbcL*, and *trnL* gene sequences, O'Brien and associates (2005) determined that the genus *Nostoc* comprised two lineages and that the symbionts of the lichen *Peltigera* belonged predominantly to a single lineage which included other free-living *Nostoc* spp. The fact that some lichen endosymbionts clustered with symbiont *Nostoc* spp. isolated from other plants and lichens or free-living strains suggested that host specialization is not significant for *Nostoc* spp. and that co-evolution with its lichen hosts was unlikely (O'Brien et al. 2005). Contrasting studies have reported that mycobionts are highly selective in their choice of cyanobacterial symbiont species (Paulsrud and Lindblad 1998; Paulsrud et al. 1998; Rikkinen et al. 2002; Myllys et al. 2007). The last study collected lichen specimens from the same aspen stand and

determined that most lichens were particular with their choice of cyanobiont, except for *Lobaria pulmonaria* (Myllys et al. 2007).

Endosymbiont diversity studies involving samples from botanical gardens, although insightful, pose problems for diversity studies because they may either retain their original endosymbiont or become infected with a local *Nostoc* sp. This investigation aimed to isolate and identify, at the molecular level, the diversity of cyanobacteria occurring in the coralloid roots of *Macrozamia* spp. in their natural habitat. By studying multiple samples of the same cycad species at different locations, we proposed to identify whether a specific relationship existed between individual *Macrozamia* spp. and their endosymbiont cyanobacterium.

RESULTS

Cyanobiont isolation.

Coralloid root samples were obtained from 83 cycad plants, of which 73 belonged to the genus *Macrozamia* (Table

Table 2. Cyanobacterial species isolated from cycads in this study^a

GenBank acc. no.	Cyanobiont name	Seq. sim. (%)	Closest Blastn isolate match	Reference GenBank acc. no.	Origin ^b
GU254514	<i>Nostoc</i> sp. "Cycas media 1.8"	100	<i>Anabaena flos aquae</i> UTEX LB2391	DQ234824	Fresh water; Unknown
...	...	...	<i>Nostoc</i> sp. 8964:3	AM711541	<i>Gunnera</i> , New Zealand ^c
...	...	...	<i>Nostoc entophyllum</i> IAM M-267	AB093490	Unknown
GU254515	<i>Nostoc</i> sp. "Cycas ophiolitica 2.5"	99	<i>Nostoc</i> sp. Cc2	AM711532	<i>Cycas</i> , Italy ^c
	<i>Nostoc</i> sp. "Macrozamia reducta 16.7"	99	<i>Nostoc calcicola</i> VI	AJ630448	Unknown
	<i>Nostoc</i> sp. "Macrozamia reducta 17.9"	99	<i>Nostoc calcicola</i> VI	...	...
	<i>Nostoc</i> sp. "Macrozamia reducta 7.14"	98	<i>Nostoc calcicola</i> VI	...	...
	<i>Nostoc</i> sp. "Macrozamia stenomera 36.5"	99	<i>Nostoc calcicola</i> VI	...	...
GU254533	<i>Nostoc</i> sp. "Macrozamia communis 42.2"	100	<i>Nostoc calcicola</i> VI	...	...
	<i>Nostoc</i> sp. "Macrozamia riedlei 67.8"	98	<i>Nostoc calcicola</i> VI	...	...
	<i>Nostoc</i> sp. "Macrozamia communis 80.1"	99	<i>Nostoc calcicola</i> VI	...	...
GU254519	<i>Nostoc</i> sp. "Macrozamia spiralis 14.13"	97	<i>Nostoc linckia</i>	AB074503	Fresh Water, Unknown
...	...	...	<i>Nostoc</i> sp. 8964:3	AM711541	<i>Gunnera</i> , New Zealand ^c
...	...	...	<i>Anabaena flos aquae</i> UTEX LB2391	DQ234824	Fresh water; Unknown
GU254517	<i>Nostoc</i> sp. "Cycas megacarpa 5.1"	98	<i>N. linckia</i>	AB074503	Fresh Water, Unknown
...	...	...	<i>Nostoc</i> sp. 8964:3	AM711541	<i>Gunnera</i> , New Zealand ^c
...	...	...	<i>Anabaena flos aquae</i> UTEX LB2391	DQ234824	Fresh water; Unknown
GU254516	<i>Nostoc</i> sp. "Macrozamia macleayi 74.2"	99	<i>N. linckia</i>	AB074503	Fresh Water, Unknown
GU254531	<i>Nostoc</i> sp. "Macrozamia communis 40.5"	100	<i>Nostoc</i> PCC73102	AF027655	<i>Macrozamia</i> , Australia ^d
GU254532	<i>Nostoc</i> sp. "Macrozamia fawcettii 51.1"	100	<i>Nostoc</i> PCC73102	...	...
GU254525	<i>Nostoc</i> sp. "Macrozamia concinna 38.2"	98	<i>Nostoc</i> sp. IO-102-I	AY566855	<i>Pannaria</i> , Finland ^e
GU254520	<i>Nostoc</i> sp. "Lepidozamia peroffskyana 3.1"	98	<i>Nostoc</i> sp. Mollenhauer 1:1-088	DQ185208	<i>Geosiphon</i> , Germany
GU254534	<i>Nostoc</i> sp. "Macrozamia spiralis 13.14"	99	<i>Nostoc</i> sp. Mollenhauer 1:1-108	DQ185210	<i>Encephalartos</i> , Italy ^d
	<i>Nostoc</i> sp. "Macrozamia flexuosa 18.2"	99	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia reducta 20.2"	98	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia reducta 22.16"	99	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia secunda 24.11"	98	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia reducta 25.2"	99	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia heteromera 27.5"	98	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia heteromera vt 28.5"	99	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia heteromera 29.8"	99	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia polymorpha 30.1"	99	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia glaucophylla 35.11"	99	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia stenomera 37.6"	98	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...

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^a GenBank accession numbers (acc. no.) are for sequences generated in this study (approximately 770 bp of the 16S rRNA gene). The entries in bold were used for phylogenetic analysis. Seq. sim. = sequence similarity. ... = same as above.

^b References citing the 16S rDNA sequence and locality of the reference sequences.

^c Papaefthimiou et al. 2008.

^d O'Brien et al. 2005.

^e Oksanen et al. 2004.

^f Elvebakk et al. 2008.

^g These two 16S rDNA sequences were obtained directly from DNA extracted from the green endosymbiont layer in the coralloid root samples of MZ61 and MZ77.

^h Beltran and Neilan 2000.

ⁱ Lyra et al. 2001.

^j Pereira et al. 2004.

^k Sihvonen et al. 2007.

1). In total, 150 cyanobacterial isolates were cultured from 56 coralloid roots and characterized by their 16S rRNA sequence. A summary of which cycad species were successfully sampled, their location, voucher numbers, and place of lodgment is provided in Table 1. No coralloid roots could be located for four cycad plant communities. The sampling season was particularly dry and the number of coralloid roots per plant was reduced and restricted to the central root as opposed to lateral root systems. Typically, five to eight plants were investigated before a suitable coralloid root sample was obtained. Five samples were immature and had not been colonized by a cyanobacterium, not having the characteristic green ring visible in a transverse section of their coralloid root sample. There was a 76% recovery rate of endosymbionts from coralloid root samples exhibiting a healthy green ring. Those samples from which endosymbionts could not be cultured either had excessive fungal growth or a high tannin content, as indicated by a dark brown ring leaching into the media around the root section.

Multiple isolates from both BG11 and BG11₀ plates for the plant samples LZ2 (three samples), MZ8 (three samples), MZ21 (four samples), MZ24 (four samples), and MZ34 (four samples) were sequenced. Two 16S rDNA sequences were obtained for an isolate from the BG11 and BG11₀ plates for each of the 21 other samples and were shown to be identical. Therefore, a single isolate from the remaining samples was processed for genotyping on the assumption that the coralloid roots carried a single species of cyanobiont. A breakdown of cyanobionts isolated and their respective host species is provided in Table 2. Each endosymbiotic cyanobacterium was assigned a provisional species name based on its sequence similarity to existing sequences in GenBank. A single endosymbiont was identified from a single coralloid root in all but two cases. Two different *Nostoc* spp. were isolated from each of the coralloid root samples of *M. mountperriensis* (MZ62) and *M. moorei* (MZ21). Inspection of the original root sample indicated that these coralloid root samples were fragmented, suggesting a potential mixing of different coralloid root clusters at sampling.

Table 2. (continued from preceding page)

GenBank acc. no.	Cyanobiont name	Seq. sim. (%)	Closest Blastn isolate match	Reference GenBank acc. no.	Origin ^b
GU254534 (continued)					
	<i>Nostoc</i> sp. "Macrozamia johnsonii 45.1"	98	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia montana 46.2"	99	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia plurinervia 50.5"	99	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia cardiacensis 57.1"	99	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
	<i>Nostoc</i> sp. "Macrozamia mountperriensis 62.2"	99	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
GU254535	<i>Nostoc</i> sp. "Macrozamia miquelii 78.2"	98	<i>Nostoc</i> sp. Mollenhauer 1:1-108	...	...
GU254528	<i>Nostoc</i> sp. "Bowenia serrulata 1.3"	98	<i>Nostoc</i> sp. Mollenhauer 1:1-125	DQ185212	<i>Blasia</i> , Germany ^d
GU254537	<i>Nostoc</i> sp. "Macrozamia sp. Tamworth 47.1"	98	<i>Nostoc</i> sp. Mollenhauer 1:1-150b	DQ185213	<i>Anthoceros</i> , Germany ^d
	<i>Nostoc</i> sp. "Macrozamia moorei 21.1"	99	<i>Nostoc</i> sp. Mollenhauer 94.1	DQ185214	<i>Peltigera</i> , Unknown ^d
	<i>Nostoc</i> sp. "Macrozamia diplomera 34.1"	99	<i>Nostoc</i> sp. Mollenhauer 94.1	...	...
GU254526	<i>Nostoc</i> sp. "Macrozamia mountperriensis 62.1"	98	<i>Nostoc</i> sp. Mollenhauer 94.1	...	...
GU254527	<i>Nostoc</i> sp. "Macrozamia serpentina 73.1"	98	<i>Nostoc</i> sp. "Pannaria obscura cyanobiont"	EF174232	<i>Pannaria</i> , Australia ^f
	<i>Nostoc</i> sp. "Macrozamia macleayi 6.17"	97	<i>Nostoc</i> sp. "Pannaria obscura cyanobiont"	...	...
GU254518	<i>Nostoc</i> sp. "Macrozamia moorei 21.6"	98	<i>Nostoc</i> sp. PCC 6720	DQ185240	Soil, Indonesia ^d
	<i>Nostoc</i> sp. "Lepidozamia peroffskyana 1.17"	98	<i>Nostoc</i> sp. "Peltigera canina 2 cyanobiont"	DQ185230	<i>Peltigera</i> , U.S.A. ^e
GU254529	<i>Nostoc</i> sp. "Lepidozamia peroffskyana 2.9"	98	<i>Nostoc</i> sp. "Peltigera canina 2 cyanobiont"	...	...
	<i>Nostoc</i> sp. "Macrozamia lucida 8.2"	99	<i>Nostoc</i> sp. "Peltigera canina 2 cyanobiont"	...	...
	<i>Nostoc</i> sp. "Macrozamia machinii 64.2"	99	<i>Nostoc</i> sp. "Peltigera degeni cyanobiont"	DQ185244	<i>Peltigera</i> , Canada ^d
GU254524	<i>Nostoc</i> sp. "Macrozamia riedlei 65.1"	100	<i>Nostoc</i> sp. "Peltigera degeni cyanobiont"	...	...
GU254522	<i>Nostoc</i> sp. "Macrozamia fawcettii 44.1"	99	<i>Nostoc</i> sp. "Peltigera membranacea 5 cyanobiont"	DQ185247	<i>Peltigera</i> , Russia ^d
	<i>Nostoc</i> sp. "Macrozamia riedlei 66.1"	98	<i>Nostoc</i> sp. "Peltigera membranacea 5 cyanobiont"	...	...
GU254521	<i>Nostoc</i> sp. "Macrozamia communis 12.13"	99	<i>Nostoc</i> sp. "Peltigera rufescens 2 cyanobiont"	DQ185218	<i>Peltigera</i> , Germany ^d
	<i>Nostoc</i> sp. "Macrozamia miquelii 9.2"	99	<i>Nostoc</i> sp. "Peltigera rufescens 2 cyanobiont"	...	...
GU254536	<i>Nostoc</i> sp. "Macrozamia elegans 15.9"	98	<i>Nostoc</i> sp. SAG 29.90	DQ185250	<i>Cycas</i> , Brazil ^d
	<i>Nostoc</i> sp. "Macrozamia communis 41.8"	98	<i>Nostoc</i> sp. SAG 29.90	...	...
GU254523	<i>Nostoc</i> sp. "Macrozamia spiralis 56.4"	98	<i>Nostoc</i> sp. SAG 41.87	DQ185253	<i>Peltigera</i> , Switzerland ^d
GU254509	<i>Calothrix</i> sp. "Macrozamia parcifolia 61.4"	98	<i>Nostoc carneum</i> IAM M-35/NIES2107	AB325906	Unknown
		97	<i>Calothrix</i> PCC7101	AB325535	Unknown
GU254512	<i>Calothrix</i> sp. "Macrozamia pauli-guilielmi 69.1"	...	...	...	...
GU254513	<i>Calothrix</i> sp. "Macrozamia fearnsidei 77.1"	...	...	...	...
GU254511	<i>Calothrix</i> sp. "Macrozamia parcifolia_Root" ^g	97-98	<i>N. carneum</i> IAM M-35 / NIES2107	AB325906	Unknown
		97	<i>Calothrix</i> PCC7101	AB325535	Unknown
GU254510	<i>Calothrix</i> sp. "Macrozamia fearnsidei_Root" ^g	...	...	...	...
		...	<i>N. commune</i>	DQ185223	Soil, U.S.A. ^d
		...	<i>Nostoc</i> sp. PCC7120	AF317631	Fresh water, Australia
		...	<i>N. punctiforme</i> SAG 71.79	DQ185258	Soil, France ^d
		...	<i>N. muscorum</i> SAG 57.79	DQ185254	Soil, France ^d
		...	<i>Anabaena cylindrica</i> PCC7122	AF091150	Freshwater, Japan ^b
		...	<i>Anabaena flos aquae</i> strain AWQC112D	AF247590	Freshwater, Australia ^b
		...	<i>Anabaena</i> sp. 90	AJ133156	Lake Vesijarvi, Finland ⁱ
		...	<i>Aphanizomenon gracile</i> LMECYA40	AY354194	Fresh water ^j
		...	<i>Calothrix desertica</i> PCC7102	AM230699	Sand, Chile ^k
		...	<i>Tolypothrix</i> sp. PCC 7415	AM230668	Soil, Sweden ^k
		...	<i>Chroococcidiopsis thermalis</i> PCC 7203	AF132791	...

The most commonly isolated cyanobiont species was similar to *Nostoc* sp. strain Mollenhauer 1:1-108 and was found in 18 separate cycad plants representing 14 different species (Table 2). These plants were spread over a wide range of localities along the eastern seaboard of Australia. Four species of *Macrozamia*

were also sampled multiple times at different locations. *M. communis* yielded four different cyanobiont isolates at five locations, *M. reducta* revealed four at five locations, while *M. heteromera* had one endosymbiont species over three locations (Table 2). *M. riedlei* was sampled three times within

Table 3. Key morphological characteristics of cyanobionts^a

Isolate	Clade	Filament		Cell		Heterocyst		
		Polarity	Cell No.	Width	Length	Shape	Width	Length
<i>Nostoc</i> sp. “ <i>Bowenia serrulata</i> 1.3”	Ia	Isopolar	57 (20, 105)	2.88 (2.41, 3.18)	2.82 (1.86, 3.6)	Square	4.01 (3.67, 4.46)	3.77 (3.27, 4.52)
<i>Nostoc</i> sp. “ <i>Lepidozamia peroffskyana</i> 2.9”	Ia	Isopolar	48 (24, 86)	2.73 (2.37, 3)	2.11 (1.89, 2.28)	Square	3.8 (3.37, 4.6)	3.44 (2.74, 4.33)
<i>Nostoc</i> sp. “ <i>Macrozamia communis</i> 12.13”	Ia	Isopolar	45 (26, 62)	1.83 (1.6, 2.17)	2.05 (1.72, 2.39)	Spherical, ovoid	5.28 (4.19, 5.73)	5.96 (4.31, 7.31)
<i>Nostoc</i> sp. “ <i>Macrozamia concinna</i> 38.2”	Ia	Isopolar	ND	ND	ND	Spherical	ND	ND
<i>Nostoc</i> sp. “ <i>Macrozamia communis</i> 40.5”	Ia	Isopolar	28 (18, 47)	2.32 (1.82, 3.11)	2.47 (1.96, 2.85)	Ovoid	3.9 (3.54, 4.65)	4.37 (3.53, 5.18)
<i>Nostoc</i> sp. “ <i>Macrozamia communis</i> 42.2”	Ia	Isopolar	ND	4.26 (3.69, 4.71)	3.17 (2.25, 3.81)	Spherical	6.03 (5.48, 6.89)	5.36 (4.99, 5.98)
<i>Nostoc</i> sp. “ <i>Macrozamia fawcettii</i> 44.1”	Ia	ND	ND	ND	ND	ND	ND	ND
<i>Nostoc</i> sp. “ <i>Macrozamia fawcettii</i> 51.1”	Ia	Isopolar	>100	2.66 (2.31, 2.87)	2.35 (1.84, 2.84)	Spherical, ovoid	4.34 (3.4, 5.2)	3.89 (3.22, 4.58)
<i>Nostoc</i> sp. “ <i>Macrozamia spiralis</i> 56.4”	Ia	Isopolar	74 (37, 113)	1.46 (1.32, 1.7)	1.6 (1.19, 2.0)	Spherical, ovoid	3.52 (2.82, 4.33)	3.67 (2.96, 4.43)
<i>Nostoc</i> sp. “ <i>Macrozamia mountperriensis</i> 62.1”	Ia	Isopolar	>150	2.88 (2.54, 3.35)	3.19 (2.76, 3.66)	Ovoid	4.24 (2.88, 4.98)	5.7 (3.5, 5.63)
<i>Nostoc</i> sp. “ <i>Macrozamia riedlei</i> 65.1”	Ia	Isopolar	87 (50, 179)	2.54 (2.21, 2.83)	2.35 (2.09, 2.67)	Spherical	3.2 (2.64, 3.61)	3.28 (2.55, 4.12)
<i>Nostoc</i> sp. “ <i>Macrozamia serpentina</i> 73.1”	Ia	Isopolar	31 (20, 64)	3.91 (3.29, 4.4)	3.86 (3.3, 4.98)	Ovoid	3.43 (2.0, 4.16)	3.42 (2.84, 4.4)
<i>Nostoc</i> sp. “ <i>Macrozamia spiralis</i> 13.14”	Ib	Isopolar	47 (18, 74)	3.03 (2.77, 3.29)	2.34 (2.02, 2.77)	Spherical	4.62 (3.81, 4.97)	4.32 (3.53, 5.41)
<i>Nostoc</i> sp. “ <i>Macrozamia elegans</i> 15.9”	Ib	Isopolar	70 (44, 95)	3.52 (3.25, 3.99)	2.67 (2.23, 3.08)	Spherical, ovoid	4.48 (3.84, 4.75)	4.46 (3.6, 5.6)
<i>Nostoc</i> sp. “ <i>Macrozamia</i> sp. Tamworth 47.1”	Ib	Isopolar	36 (28, 53)	1.39 (1.09, 1.6)	1.58 (1.08, 1.93)	Ovoid	3.81 (3.25, 4.45)	4.43 (3.52, 4.98)
<i>Nostoc</i> sp. “ <i>Macrozamia miquelii</i> 78.2”	Ib	Isopolar	73 (37, 141)	3.19 (2.59, 3.76)	3.19 (2.52, 3.98)	Spherical, ovoid	3.82 (3.4, 4.28)	4.04 (3.43, 4.63)
<i>Nostoc</i> sp. “ <i>Cycas media</i> 1.8”	Ila	Isopolar	58 (34, 82)	4.95 (4.32, 5.63)	4.73 (3.9, 5.59)	Spherical, ovoid	5.17 (4.71, 5.75)	5.49 (4.55, 6.5)
<i>Nostoc</i> sp. “ <i>Cycas ophiolitica</i> 2.5”	Ila	Isopolar	29 (26, 36)	2.7 (2.3, 3.0)	3.0 (2.64, 3.74)	Spherical	2.82 (2.54, 3.54)	3.18 (2.69, 4.14)
<i>Nostoc</i> sp. “ <i>Cycas megacarpa</i> 5.1”	Ila	Isopolar	34 (24, 49)	3.53 (3.3, 3.86)	3.39 (2.76, 3.97)	Spherical	4.26 (3.56, 4.74)	3.7 (3.25, 4.31)
<i>Nostoc</i> sp. “ <i>Macrozamia spiralis</i> 14.13”	Ila	Isopolar	79 (41, 125)	3.63 (2.95, 4.26)	2.99 (2.37, 3.86)	Spherical	4.48 (3.87, 5.15)	4.06 (3.35, 4.59)
<i>Nostoc</i> sp. “ <i>Macrozamia moorei</i> 21.6”	Ila	Isopolar	ND	3.4 (3.17, 3.59)	2.46 (2.0, 3.18)	Spherical, ovoid	5.11 (4.44, 6.12)	5.2 (4.09, 5.9)
<i>Nostoc</i> sp. “ <i>Macrozamia macleayi</i> 74.2”	Ia	Isopolar	41 (30, 52)	3.49 (2.76, 4.18)	3.66 (2.95, 4.23)	Spherical, square, ellipsoid	4.98 (4.16, 6.78)	4.79 (3.45, 6.86)
<i>Nostoc</i> sp. “ <i>Lepidozamia peroffskyana</i> 3.1”	Ia	ND	ND	ND	ND	ND	ND	ND
<i>Calothrix</i> sp. “ <i>Macrozamia parcifolia</i> 61.4”	Iib	Heteropolar	16 (7, 24)	2.1 (1.68, 2.79) ^b	2.36 (1.59, 2.98) ^b	Conical	2.09 (1.57, 2.73)	2.48 (2.02, 3.17)
...	...	...	...	1.86 (1.78, 2.35) ^c	1.68 (0.89, 2.27) ^c	...	...	...
...	...	...	...	1.54 (1.13, 1.85) ^d	1.96 (1.19, 2.69) ^d	...	...	...
<i>Calothrix</i> sp. “ <i>Macrozamia pauli-guilielmi</i> 69.1”	Iib	Heteropolar	14 (10, 20)	4.67 (3.68, 5.46) ^b	3.14 (2.25, 3.68) ^b	Conical	3.16 (2.32, 4.63)	3.43 (2.23, 5.21)
...	...	...	...	3.05 (2.84, 3.52) ^c	2.57 (1.59, 3.18) ^c	...	...	...
...	...	...	...	2.21 (1.65, 2.88) ^d	2.69 (1.62, 3.67) ^d	...	...	...
<i>Calothrix</i> sp. “ <i>Macrozamia fearnsidei</i> 77.1”	Iib	Heteropolar	ND	ND	ND	ND	ND	ND

^a Measurements are the average of 10 random filaments (numbers in parentheses indicated minimum and maximum values, respectively). ND = not determined.

^b Basal cells.

^c Vegetative cells.

^d Apical cells.

a single community and yielded three different endosymbiont species.

The *Macrozamia* spp. were clustered into regional localities with similar climatic and soil conditions and assessed for endosymbiont diversity. The Shoalhaven region south of Sydney, encompassing all the *M. communis* (MZ12, MZ40, MZ41, MZ42, and MZ80) populations, yielded four endosymbiont species from five locations. The region northwest of Narrogin in Western Australia provided three endosymbiont species over a single community of *M. riedlei* (MZ65 to MZ67). The Blue Mountains area west of Sydney was the origin of three samples of *M. reducta* (MZ20, MZ22, and MZ25) from separate communities and a single sample of *M. secunda* (MZ24), all possessing *Nostoc* sp. strain Mollenhauer 1:1-108 as an endosymbiont. Communities of *Macrozamia* sampled in the eucalypt forests of inland New South Wales carried the same endosymbiont species, namely *Nostoc* sp. strain Mollenhauer 1:1-108, from single samples of *M. polymorpha* (MZ30) and *M. glaucophylla* (MZ35) and three samples of *M. heteromera* (MZ27, MZ28, and MZ29). Endosymbionts were isolated from four species of cycad in the Royal Botanic Gardens, Sydney; namely, *C. media* (C1), *M. reducta* (MZ7), *M. lucida* (MZ8), and *M. miquelii* (MZ9) that had four different species of cyanobacteria.

The majority of bacterial endosymbiont species isolated belonged to the genus *Nostoc*. Two new isolates of *Nostoc* PCC73102 were obtained; namely, *Nostoc* sp. strain *Macrozamia communis* 40.5 and *Nostoc* sp. strain *Macrozamia fawcettii* 51.1. In addition, three cycad species yielded endosymbiotic cyanobacteria belonging to the genus *Calothrix*; namely, *Calothrix* sp. strain *Macrozamia parcifolia* 61.4, *Calothrix* sp. strain *Macrozamia pauli-guilielmi* 69.1, and *Calothrix* sp. strain *Macrozamia fearnsidei* 77.1. In summary, 22 separate isolates of cyanobacteria were identified from endosymbiont

cultures of the coralloid roots of Australian cycad species based on sequence similarities to the available database sequences.

Microscopy.

Microscopic analysis confirmed that all cultures were unialgal. A summary of the features of endosymbiont isolates is provided in Table 3. The majority of these were confirmed to be *Nostoc* sensu Rippka and associates (2001) with obvious, differentiable hormogonia. Regions of akinete differentiation were observed both distally and proximal to heterocysts. All measured morphological characters were highly variable among the *Nostoc* isolates, including filament length, vegetative cell size, and heterocyst shape and size. *Nostoc* heterocysts were square, ellipsoid, spherical, or ovoid. The *Calothrix* spp. *Macrozamia parcifolia* 61.4, *Macrozamia pauli-guilielmi* 69.1, and *Macrozamia fearnsidei* 77.1 isolated were confirmed as *Calothrix* spp. with heteropolar filaments and terminal heterocysts (Rippka et al. 2001). The degree of filament polarity varied between *Calothrix* isolates and within the culture samples for each isolate. *Calothrix* sp. strain *Macrozamia parcifolia* 61.4 had a polarity of 1.29 to 2 (mean of 1.62) while *Calothrix* sp. strain *Macrozamia pauli-guilielmi* 69.1 had a filament polarity of 1.72 to 3.17 (mean of 2.18). Examples of the *Nostoc* and *Calothrix* isolates identified are illustrated in Figure 1. *Nostoc* isolates had a life cycle of hormogonia (Fig. 1A) maturing to heterocystous trichomes (Fig. 1B and C) before forming aseriate masses in exopolysaccharide envelopes (Fig. 1D) and akinetes. *Nostoc* sp. strain *Cycas media* 1.8 (Fig. 1C) displayed typical *Nostoc* morphology with clearly differentiable hormogonia and ellipsoid, ovoid, and spherical vegetative cells and heterocysts. Chains of akinetes, characteristic of *Nostoc* spp., were observed for this isolate. For *Calothrix*, filament heteropolarity and conical terminal heterocysts were observed in *Calothrix* sp. strain *Macrozamia pauli-guilielmi*

Fig. 1. Photomicrographs of *Nostoc* and *Calothrix* cyanobionts. *Nostoc* morphology showing **A**, hormogonia of *Nostoc* sp. “*Macrozamia macleayi* 74.2”; **B**, filaments with heterocysts of *Nostoc* sp. “*Macrozamia miquelii* 78.2”; and **C**, *Nostoc* sp. “*Cycas media* 1.8” and **D**, spherical aseriate masses of *Nostoc* sp. “*Macrozamia miquelii* 78.2”. *Calothrix* morphology showing heteropolar filaments with terminal heterocysts in **E**, *Calothrix* sp. “*Macrozamia pauli-guilielmi* 69.1” and **F**, *Calothrix* sp. “*Macrozamia parcifolia* 61.4”. Arrows indicate heterocysts. Scale bar is 10 µm except in d, where it is 50 µm.

69.1 (Fig. 1E) and *Calothrix* sp. strain *Macrozamia parcifolia* 61.4 (Fig. 1F). Occasional square intercalary heterocysts were also observed in *Calothrix* sp. strain *Macrozamia pauli-guilielmi* 69.1 (data not presented).

Phylogenetics.

Representative sequences of each cyanobacterial species isolate as well as related reference sequences obtained from cultured isolates were used to reconstruct a phylogeny. Those sequences used for analysis, together with their GenBank accession numbers, are highlighted in Table 2.

The *Anabaena*, *Calothrix*, and *Aphanizomenon* reference species all clustered outside the *Nostoc* clade containing the endosymbionts obtained in this study. The *Nostoc* endosymbiont isolates were split between two clades (Fig. 2, I and II). Clade I contained 15 cyanobiont species isolates as well as the free-living terrestrial reference species *N. punctiforme* SAG 71.79, *N. muscorum* SAG57.79, *N. calcicola* VI, and *N. commune*. The majority ($n = 11$) of *Nostoc* spp. isolated clustered within group I of the order Nostocales (O'Brien et al. 2005)

containing terrestrial *Nostoc* spp., the reference strain being *Nostoc* sp. strain PCC73102, located within clade Ia.

Of the 22 endosymbiont species obtained in this study, 15 were other symbiotic strains of cyanobacteria, including the reference *Nostoc* sp. strain PCC73102, which was originally isolated from a *Macrozamia* sp.; *Nostoc* sp. strain Mollenhauer 1:1-108, isolated from an *Encephalartos* cycad sp.; and *Nostoc* sp. strain SAG 29.90, obtained from *C. circinalis*. Sequences from other *Nostoc* symbioses were also located within clade I; namely, the *Anthoceros* symbiont, *Nostoc* sp. strain Mollenhauer 1:1-150b; the *Blasia* symbiont, *Nostoc* sp. strain Mollenhauer 1:1-125; the *Pannaria* endosymbionts, *Nostoc* IO-102-I and *Nostoc* sp. strain *Pannaria obscura*; and the *Peltigera* symbionts, *Nostoc* sp. strain Mollenhauer 94.1, *Nostoc* sp. strain *Peltigera canina* 2, *Nostoc* sp. strain SAG 41.87, *Nostoc* sp. strain *Peltigera membranacea* 5, *Nostoc* sp. strain *Peltigera rufescens* 2, and *Nostoc* sp. strain *Peltigera degeni*. Interestingly, clade Ib contained only symbionts of cycad species whereas clade Ia contained symbionts from cycads, hornworts, liverworts, and lichens.

Fig. 2. Maximum likelihood phylogenetic tree based on the 16S rDNA sequences obtained from cyanobiont isolates of Australian cycad species. Sequences obtained in this study are highlighted in bold. Naming reflects the cyanobacterial genus and the cycad host species, as well as the isolate number. Numbers at nodes indicate the bootstrap values (1,000 bootstrap events). Values $\geq 50\%$ are provided.

Clade II contained an early branching for reference strain *Nostoc* sp. strain Mollenhauer 1:1-088, a *Geosiphon* symbiont, and the endosymbiont *Nostoc* sp. strain *Lepidozamia peroffskyana* 3.1. The rest of Clade II contained 18 sequences divided into two groups with low statistical support for clade IIa and clade IIb. *Nostoc* sp. strain PCC7120 is the type species of aquatic *Nostoc* isolates and was found within clade IIa, together with two other aquatic species (*N. linckia* and *Anabaena flos aquae* UTEX LB2391) and the terrestrial species *Nostoc* strain PCC6720. Six endosymbionts obtained in this study were in clade IIa together with symbionts isolated from the genera *Cycas* (*Nostoc* sp. strain Cc2) and *Gunnera* (*Nostoc* sp. strain 8964:3). Clade IIb also contained the three endosymbiont isolates identified as a *Calothrix* sp. from three different species of *Macrozamia*. Their 16S rRNA gene sequences clustered with the reference strain for *Tolypothrix* isolated from soil (namely *Tolypothrix* sp. strain PCC7415) and *Calothrix* PCC7101.

DISCUSSION

The ability of cycad species worldwide to survive in nutrient-poor soils is strongly associated with their ability to form a symbiosis with nitrogen-fixing cyanobacteria. The specificity of this interaction has been suggested to be nonspecific between members of different genera of cycads (Lindblad et al. 1989; Lotti et al. 1996; Costa et al. 1999). However, most of the endosymbiont studies to date have involved the analysis of greenhouse specimens and, therefore, often reflect the diversity of the cyanobacteria residing in the local rhizosphere *ex situ* and not the native terrestrial cyanobacteria from the plants' origin. This study constitutes the most extensive survey of cyanobacterial endosymbionts residing in the coralloid roots of cycads in their natural habitats. We have deliberately focused on Australian cycads belonging to the genus *Macrozamia*, thereby excluding potential differences in host plant selectivity between different families of cycads.

No specificity between host cycad species and the resident endosymbiont was identified in this study, thus supporting previous work done on other cycad species (Lindblad et al. 1989; Lotti et al. 1996; Costa et al. 1999; Zheng et al. 2002; Costa et al. 2004). The endosymbiont species within the coralloid roots probably only reflect the predominant potential symbiont species residing within the immediate rhizosphere. The most commonly isolated endosymbiont, characterized with sequence similarities of 98 to 99%, was *Nostoc* sp. strain Mollenhauer 1:1-108, and was obtained from cycad host plants over a diverse range of geographical and environmental locations (Table 2). This endosymbiotic cyanobacterial species was originally isolated from the coralloid roots of *E. natalensis* from Italy (O'Brien et al. 2005). The hardiness of this organism is intriguing because it was identified in the roots of cycad communities living in an environment experiencing a 5-year drought. The predominance of this cyanobiont may reflect its ability to withstand harsh environmental conditions in the soil and its ability to reestablish itself in the soil once conditions are favorable (Potts 2000). In contrast, cycad species occurring in less harsh environments appeared to have been colonized by a higher diversity of endosymbiont species, the best example being the three different endosymbiont species isolated from a single community of *M. riedlei* in Western Australia. Here, *Nostoc* sp. strain *Peltigera degeni* cyanobiont, *Nostoc* sp. strain *Peltigera membranacea* 5 cyanobiont, and *N. calcicola* VI were isolated from the roots of neighboring plants. Two of these isolates have previously been identified in lichen symbioses (O'Brien et al. 2005).

The lichen endosymbionts analyzed in Myllys and associates (2007) all clustered within clade I (Fig. 2) with the endo-

symbionts obtained in this study, with the common reference species being *Nostoc* sp. strain SAG29.20, *N. commune*, *Nostoc* PCC73102, *Nostoc* sp. strain *Peltigera degeni*, and *N. muscorum*. High selectivity of lichens for their particular endosymbiont species was reported; however, this does not appear to be the case for the *Macrozamia* endosymbionts identified in the present study. Our data agreed with that obtained by O'Brien and associates (2005), in that the endosymbionts of Australian cycad species showed greater similarity to the endosymbionts of lichens, other cycad species, and free-living cyanobacteria than the endosymbionts from closely related *Macrozamia* spp.

Symbiotic cyanobacterial species isolated from the *Peltigera* samples demonstrated no geographical restrictions with strains from specimens collected in Europe and North America interspersed throughout the *Nostoc* II cluster (O'Brien et al. 2005). The endosymbiotic cyanobacterial isolates identified in this study were also found associated in symbioses with other hosts (Table 2).

The well-characterized heterocystous cyanobacterial reference strain *Nostoc* sp. strain PCC73102 was originally isolated from *M. riedlei* in Australia (Rippka et al. 1979). Two new isolates were obtained in this study from different *Macrozamia* host species; namely, *M. communis* and *M. fawcetti*.

Lepidozamia peroffskyana hosted *Nostoc* sp. strain Mollenhauer 1:1-088, a symbiont of the fungus *Geosiphon pyriforme* with restricted growth to a single site in Germany (Bergman et al. 2007). *Nostoc* sp. strain Mollenhauer 1:1-125 was originally isolated from a liverwort specimen, *Blasia pusilla*, in Germany while the as-yet-unclassified *Macrozamia* sp. strain Tamworth was host to *Nostoc* sp. strain Mollenhauer 1:1-150, originally isolated from a specimen of the hornwort genus, *Anthoceros*, in Germany.

Finally, the strain *Nostoc* sp. strain IO-102-I was originally isolated from a *Pannaria pezizoides* lichen symbiosis and found to produce microcystin-LR (Okasanen et al. 2004). The *Nostoc* endosymbiont isolated from *M. concinna* in this study showed 98% 16S rDNA sequence similarity to this isolate, and suggests that further investigation is required of its potential to produce toxins.

The deep rooting of the phylogenetic tree in Figure 2 is supported by the literature regarding the taxonomy of the genera *Anabaena*, *Aphanizomenon*, *Microcystis*, and *Planktothrix* (Lyra et al. 2001). *Calothrix* PCC7714 branched from before the *Anabaena* cluster containing *Anabaena* sp. strain 90, followed by *Nostoc* spp., including *Nostoc* strains PCC73102 and PCC 7120. Thus, the physiological characteristics and the geographic origins of the free-living cyanobacterial isolates does not reflect high similarities of the 16S rDNA sequences. They also demonstrated that the classification of heterocystous filamentous cyanobacterial genera overlaps considerably but three discrete groups exist; namely, *Anabaena/Aphanizomenon*, *Anabaena/Cylindrospermum/Nodularia/Nostoc*, and *Calothrix*. Morphologically, there was no distinction between clades Ia, Ib, and IIa. All isolates from these clades were confirmed to be *Nostoc* spp. *Nostoc* spp. are filamentous, heterocystous cyanobacteria capable of forming both spore-like akinetes and vegetative hormogonia (Komarek and Anagnostidis 1989). The site of akinete differentiation was previously used as a diacritical feature between *Nostoc* and *Anabaena* spp. by Komarek and Anagnostidis (1989); however, Rippka and associates (2001) determined that akinete formation is highly variable in both genera and differentiated between the two genera based on hormogonia characteristics. According to Rippka and associates (2001), *Nostoc* spp. are defined to have clearly differentiable hormogonia, whereas no clear size difference exists between hormogonia and mature filaments in *Anabaena* spp. Cellular

division and akinete formation throughout the present clades I and IIa were highly variable, occurring both distally and proximally to intercalary heterocysts (Table 3). Throughout clade I and IIa, hormogonia were distinct from the mature trichomes. Rajaniemi and associates (2005) noted that neither the classifications of Komarek and Anagnostidis (1989) or Rippka and associates (2001) adequately explained their phylogenetic results.

Anabaena spp. have previously been identified as endosymbionts of cycads (Obukowicz et al. 1981; Zhu 1982); however, no *Anabaena* sp. has ever been isolated from a *Macrozamia* host. The *Nostoc* sp. strain *Cycas media* 1.8 isolate in clade IIa (Fig. 1) was distantly related to strains of aquatic *A. flos aquae* species identified in fresh water blooms in Australia represented by *A. flos aquae* AWQC112D (Beltran and Neilan 2000). Microscopy determined that this isolate had clearly differentiable hormogonia and spherical to ovoid cells, confirming its *Nostoc* status. The separation of *Nostoc* spp. PCC 7120 and PCC 6720 from clade I agrees with the analysis of O'Brien and associates (2005). Previous studies have suggested that symbiotic *Nostoc* spp. do not occur in this clade (Rikkinen et al. 2002). Subsequent molecular analyses demonstrated that this clade does contain endosymbiotic cyanobacteria (Papaefthimiou et al. 2008). This was also reflected by the isolates from *M. macleayi*, *C. media*, *C. ophiolitica*, and *M. moorei* (Fig. 2) identified in this study.

An endosymbiotic *Calothrix* sp. belonging to the order Nostocales (family Rivulariaceae) was previously isolated from *E. hildebrandtii* (Grobelaar et al. 1987). Identification was based on morphological characteristics without molecular taxonomy. *Calothrix* spp. are characterized by short filaments with distinct heteropolarity, pronounced tapering, and terminal heterocysts (Rippka et al. 2001). The *Calothrix* isolates obtained in this study had a polar ratio of >1.5 excluding their classification in the genus *Tolypothrix*, as described by Rippka and associates (2001). The rigorous root surface sterilization precludes the *Calothrix* isolates from being epiphytic contaminants. Analysis of the cyanobacterial 16S rDNA from the inner coralloid root also revealed the same *Calothrix* sequence (Table 2), confirming that this species is endosymbiotic with *M. parcifolia*, *M. pauli-guilielmi*, and *M. fearnsidei* and not the surrounding rhizosphere or root surface. No other symbiotic cyanobacterial species were present in clade IIb (Fig. 2).

The diversity of Nostocales in the rhizosphere of cycad communities may reveal the range of potential cyanobiont species in soil. Dispersal of cycad seed is limited and populations are restricted, with sharp geographical boundaries. Without a stringent selection for particular *Nostoc* strains, cycads have a greater chance to establish in nutrient-poor environments. In addition, the composition of the rhizosphere cyanobacterial population may also change over time and under different

environmental conditions. Given the long lifespan and infrequent coning of cycads (Hill and Osborne 2001), cyanobacterial species selection would reduce the number of potential endosymbioses and, hence, the survival of the host. The phylogenetic analysis did not suggest any co-evolutionary pattern between cyanobionts and the host *Macrozamia* spp. All isolated cyanobiont species fell within the heterocystous order, Nostocales. The genus *Calothrix* was confirmed as a cyanobacterial genus endosymbiotic in cycads. The distribution of endosymbiont *Nostoc* spp. in the phylogenetic tree indicated the cosmopolitan distribution of symbiont *Nostoc* spp. (O'Brien et al. 2005) at both the intra- (Australian) and inter-continental levels.

MATERIALS AND METHODS

Sampling protocols.

Populations of cycads were selected for sampling from the database of the National Herbarium of New South Wales (NSW), Royal Botanic Garden, Sydney and the Queensland Herbarium, Brisbane, Australia (BRI). Communities were located by GPS and were photographed prior to sampling. A single cycad plant from each community was sampled for a single large coralloid root cluster. This entailed digging 15 to 30 cm below the surface and gently removing a cluster with a sterile blade from near the main root. Only *M. spiralis* (Table 1, MZ13) had coralloid roots protruding from the ground for sampling. A healthy leaf was obtained from each plant sampled as a voucher specimen. Samples were bagged, clearly labeled, and stored at 15°C. Samples obtained further afield were mailed overnight at ambient temperature. Voucher specimens were deposited at the NSW, Sydney, or the BRI as listed in Table 1.

Root processing and isolation of cyanobacterial endosymbionts.

Coralloid roots were washed gently under a stream of running water and scrubbed clean of adherent dirt using a soft brush. A section of the root was placed in 6% hydrogen peroxide solution in a 50-ml tube for two washes of 15 min each. The roots were subsequently rinsed with copious amounts of sterile milliQ water, followed by three 10-min washes with gentle shaking. The surface-sterilized roots were placed in a sterile plastic dish. The tips were cut off and slices (approximately 2 mm thick) were obtained using a sterile scalpel (Fig. 3). Four slices were transferred to a plate each of BG11 and BG11₀ medium containing cyclohexamide at 100 µg ml⁻¹ to control fungal growth, using sterile forceps. Plates were incubated in a light room at 22°C on a cycle of 16 h of light and 8 h of darkness at 2,000 lux. A section of the sterilized sample root was peeled off its outer surface layer and the green, cyano-

Fig. 3. Coralloid roots obtained from *Macrozamia communis* (Currowan State Forest, Shoalhaven region). **A**, Cleaned and sterilized root cut away to reveal the green ring in the subcortical zone containing the endosymbiotic cyanobacteria. The root is 6 mm in diameter. **B**, Sections of coralloid root (approximately 2 mm thick) on a plate of BG11 medium. **C**, Cyanobacterial endosymbionts growing on BG11 medium after 3 weeks.

biont-containing layer scraped off using a sterile blade. A 100-mg portion was transferred to a sterile 2-ml screw cap tube and frozen at -80°C for later DNA extraction.

Individual colonies of cyanobacteria growing on BG11 and BG11₀ were transferred to fresh media containing cyclohexamide at $100\text{ }\mu\text{g ml}^{-1}$ within 4 weeks of processing. These isolates were subsequently subcultured three to five times at 3-month intervals to obtain unialgal cultures. Representative colonies with a combined weight of 100 mg were picked and used for DNA extraction.

DNA extraction and amplification.

Pellets of cyanobacteria and root material were incubated with XS buffer (Tillet and Neilan 2000) at 65°C for 4 h with hourly vortexing. Sterile $1\text{-}\mu\text{m}$ glass beads were added to the mixture and disrupted to beating using a FastPrep FP120 bead-beater (Savant, Scoresby, Australia) for two cycles of 30 s at 4.5 intensity followed by another hour of incubation at 65°C . The mixture was placed on ice for 10 min prior to centrifugation at $12,000 \times g$ for 10 min. The supernatant fluid was transferred to a new 1.5-ml Eppendorf tube and extracted twice with a phenol/chloroform/isoamyl alcohol (25:24:1) solution. The aqueous phase was transferred to a new 1.5-ml Eppendorf tube with $1/10$ volume of 3 M potassium acetate (pH 7.5) and 2 volumes of isopropanol. The mixture was gently inverted prior to centrifugation at $12,000 \times g$ for 20 min. The resultant pellet was washed with 70% ethanol, air dried, and resuspended in $50\text{ }\mu\text{l}$ of sterile milliQ water at 55°C for 1 h. The DNA was quantified using a NanoDrop UV/VIS spectrophotometer (Biosciences, Sydney, Australia). A 782-bp portion of the 16S rDNA gene was amplified using a standard bacterial 16S rDNA primer (27F) (Neilan et al. 1997) and a cyanobacterial-specific reverse primer (809R) as described by Jungblut and associates (2005). Briefly, 50 ng of DNA was used in a $20\text{-}\mu\text{l}$ reaction in 0.2-ml tubes containing the following: $1\times$ F1 *Taq* polymerase buffer, 0.2 U of F1 *Taq* (Fisher Biotech, Wembley, Australia), $200\text{ }\mu\text{M}$ each deoxynucleotide triphosphate, 2.5 mM MgCl_2 , and 5 pmol of each primer. The amplification cycle was initially 2 min at 95°C ; followed by 35 cycles of 30 s at 95°C , 40 s at 55°C , and 50 s at 72°C ; with a final extension of 5 min at 72°C . The resultant product was run on a 1% Tris-acetate-EDTA gel, stained with ethidium bromide ($1\text{ }\mu\text{g ml}^{-1}$), and visualized on a Gel-DOC (Bio-Rad, Gladesville, Australia) system running Quantity One 4.1R software (Bio-Rad) to confirm a single PCR product. The remaining PCR product was precipitated with $80\text{ }\mu\text{l}$ of 80% ethanol and incubated at room temperature for 20 min prior to centrifugation at $12,000 \times g$ for 20 min. The washed and air-dried DNA pellet was resuspended in $15\text{ }\mu\text{l}$ of milliQ water. The PCR product (25 ng) was subjected to automated sequencing using the cyanobacterial-specific primer, 809R, and the PRISM Big Dye Terminator V3.1 cycle sequencing system (Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's instructions and analyzed using the ABI 3730 Capillary Sequencer. Sequences were viewed using the Applied Biosystems Sequence Scanner Software version 1.0 and confirmed to be good sequences that did not indicate mixed cyanobacterial cultures. The edited sequences were converted to FASTA format for subsequent analysis. Sequences used in this study are available under the GenBank accession numbers listed in Table 2. In order to partially identify the isolates, similarity searches were conducted against the GenBank database (National Center for Biotechnology Information) using BLAST (Altschul et al. 1997) and ascribed an identity based on the highest full-length sequence.

Phylogenetic analysis.

The sequences obtained in this study along with representative reference strains were aligned using Clustal W (Thompson

et al. 1997), end gaps removed, and the approximately 770-bp sequences realigned. The program Findmodel (Posada and Crandall 2001) was used to analyze the alignment and identify which phylogenetic model best suited the data; in this case, the GTR gamma substitution model was identified with a gamma shape parameter of 0.141. A DNA distance matrix was obtained using the DNAdist program in Phylip (Felsenstein 1993). Maximum likelihood trees were generated using the PhyML package (Guindon and Gascuel 2003) with 1,000 bootstrap resampling events.

Microscopy.

Cultures were monitored by microscopy to ensure that they remained unialgal. A Zeiss Axioskop light microscope with Axiovision 4.6 software was used to record the morphology of the strains using bright-field microscopy. Isolates were cultured in liquid BG11₀ media containing cyclohexamide at $100\text{ }\mu\text{g ml}^{-1}$. Samples were prepared for microscopy by fixing overnight in either 5% phenol or 5% neutral phosphate-buffered formalin (0.4% [wt/vol] $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.6% [wt/vol] Na_2HPO_4 , and 5% formalin). To allow comparison between isolates, measurements of isopolar filaments were made on filaments with differentiated intercalary heterocysts. For heteropolar filaments, measurements were done on filaments with fully matured terminal heterocysts. All filaments measured had not formed akinetes. The number of cells per filament and the width and length of individual vegetative cells were measured. Intercalary (not terminal) heterocysts were measured in isopolar filaments whereas only terminal heterocysts were measured in the heteropolar filaments.

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