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PLANT MICROBE INTERACTIONS

Rational Approaches to Improving the Isolation of Endophytic Actinobacteria from Australian Native Trees

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Abstract In recent years, new actinobacterial species have been isolated as endophytes of plants and shrubs and are sought after both for their role as potential producers of new drug candidates for the pharmaceutical industry and as biocontrol inoculants for sustainable agriculture. Molecular-based approaches to the study of microbial ecology generally reveal a broader microbial diversity than can be obtained by cultivation methods. This study aimed to improve the success of isolating individual members of the actinobacterial population as pure cultures as well as improving the ability to characterise the large numbers obtained in pure culture. To achieve this objective, our study successfully employed rational and holistic approaches including the use of isolation media with low concentrations of nutrients normally available to the microorganism in the plant, plating larger quantities of plant sample, incubating isolation plates for up to 16 weeks, excising colonies when they are visible and choosing Australian endemic trees as the source of the actinobacteria. A hierarchy of polyphasic methods based on culture morphology, amplified 16S rRNA gene restriction analysis and limited sequencing was used to classify all 576 actinobacterial isolates from leaf, stem and root samples of two eucalypts: a Grey Box and Red Gum, a native apricot tree and a native pine tree. The classification revealed that, in addition to 413 *Streptomyces* spp., isolates belonged to 16 other actinobacterial genera: *Actinomadura* (two strains), *Actinomycetospora* (six), *Actinopolymorpha* (two), *Amycolatopsis* (six), *Gordonia* (one), *Kribbella* (25), *Micromonospora* (six), *Nocardia* (ten), *Nocardioides* (11), *Nocardiopsis* (one), *Nonomuraea* (one), *Polymorphospora* (two), *Promicromonospora* (51), *Pseudonocardia* (36), *Williamsia* (two) and a novel genus *Flindersiella* (one). In order to prove novelty, 12 strains were characterised fully to the species level based on polyphasic taxonomy. One strain represented a novel genus in the family *Nocardioides*, and the other 11 strains were accepted as novel species. In summary, the holistic isolation strategies were successful in obtaining significant culturable actinobacterial diversity within Australian native trees that includes rare and novel species.

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Introduction

Actinobacteria are widely recognised as having a major role in environmental processes due to degradation of plant material and being the most important sources of bioactive secondary metabolites. They have generally been found in soil and environmental samples [2], but in recent years, another ecological system, the endophytic environments of plants, has proven to be a rich source of these biosynthetically prolific bacteria [9]. In order to ascertain their diversity in plants, actinobacterial endophytes have been studied in a variety of crops and plants using molecular- [7] and cultivation-based techniques [9, 34]. There is usually a discrepancy between the results of the two techniques as molecular methods normally reveal a broader diversity than can be found with culture-based methods [29]. A major reason for this is because ubiquitous genera, such *Streptomyces*, usually grow faster or have a higher abundance of spores, thereby increasing their colony-forming units. Members of genera that grow at slower rates or do not sporulate readily may not emerge to form colonies because the early colonisers can strip nutrients or produce acids or antibiotics. Therefore, if emerging colonies were excised completely from isolation plates and subcultured, the less abundant or slower growers can form colonies. Nutrient composition of the media can be modified to reflect the habitat of the target

actinobacteria. Endophytes have adapted to utilise available nutrients in plants such as plant carbohydrate polymers and their sugar moieties, peptides and amino acids that are present at low concentrations [34]. Burns et al. [3] also used low-nutrient media, but with long incubation times of more than 8 weeks to isolate a high number and diversity of rare groups of haloarchael bacteria from an Australian crystalliser pond. Moreover, gellan gum as a solidifying agent instead of agar was found to support a higher number of colonies and novel isolates from a soil sample [17]. Greater genetic diversity and novel and rare species were obtained as the range of host plants was widened [43, 44], and in some studies, a high proportion of rare genera (41.6 %) was realised [12]. Therefore, the selection of source plants is crucial for a programme targeting the isolation of rare and novel actinobacterial endophytes. It is expected that plants, which are endemic and unique to specific areas, are likely to yield a high diversity of endophytes [34, 42].

The identification of large numbers of isolates to the genus level requires rapid, yet inexpensive identification methods. Amplified 16S rRNA gene restriction analysis (ARDRA) is a molecular approach which presents a rapid means of discriminating homologous genes. Marshall et al. [30] showed that it could be applied to identify *Campylobacter*, *Arcobacter* and *Helicobacter* species in a relatively simple and highly discriminatory manner. With actinobacteria, ARDRA was used for the rapid identification of clinically significant species of aerobic actinobacteria [41] and to categorise strains of *Saccharomonospora* into four validly described species [47]. The combination of ARDRA fragment patterns and the ability to use differences in colony morphology were applied to distinguish different genera in the same subgroups of filamentous actinobacteria isolated from soil to the genus level [8]. This combination of techniques can be used to classify large numbers of isolates to the genus level.

Therefore, our holistic improvement strategy for isolation and identification of endophytic actinobacteria was applied to native Australian trees which were between 20 and 200 years old and expected to have greater actinobacterial diversity that has developed over long periods of time.

Material and Methods

Collection of Plant Material

Four native Australian trees, *Callitris preissii* (native pine tree), *Eucalyptus camaldulensis* (Red gum), *Eucalyptus microcarpa* (Grey Box) and *Pittosporum phylliraeoides* (native apricot tree), on the Flinders University campus at Bedford Park, Adelaide, South Australia, were used as the source plant material. Twenty leaf samples and 5–10-g stem and 5–10-g root samples were collected from each of the plants during the summer period (December 2007), placed in paper bags and processed within 4 h. The leaf samples were collected from branches between 3 and 6 m from the ground. The stem samples were obtained by removing the bark and using a sterile corer to gain samples up to 10-cm deep, between 3 and 6 m above ground. The roots samples were taken from a depth of 10 to 25 cm below ground. A corer was used to obtain samples up to 10-cm deep after removing the bark.

Surface Sterilisation of Plants

The samples were washed thoroughly with tap water and sonicated for 20 s to dislodge any soil and organic matter, then washed once in sterilised reverse osmosis (RO)-treated water. Barks of the stems and roots were removed, and the 1–2-g samples were washed in sterile RO water and dried on a paper towel. The samples were immersed in sterile 0.1 % Tween-20 for 5 min, in 70 % ethanol for 5 min and in sodium hypochlorite solution (6 % available chloride, freshly prepared) for 5 min, followed by washing in sterile RO water five times to remove the chemicals. The samples were soaked in sterile 10 % (*w*/*v*) $NaHCO_3$ for 10 min to retard the growth of endophytic fungi, followed by washing twice in sterile RO water. The surface sterilisation was checked by aseptically rolling surface-sterilised plant tissues onto each of the isolation media and tryptone soy agar (TSA, Difco). Each sample was cut into small pieces with a sterile scalpel and then crushed aseptically in 1–2-g lots using a sterile mortar and pestle in 5 ml sterile phosphate buffer, pH 7.2

Isolation Method

Approximately 5–10 g of each of the surface-sterilised and crushed root, stem and leaf tissue samples of each plant were plated onto ten isolation media in triplicate for each medium. The isolation media were:

1. Mannitol mung bean yeast extract mineral salt agar (MMY; mannitol 1.0 g, ground mung bean 15.0 g, yeast extract 0.5 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $CaCl_2 \cdot 2H_2O$ 0.1 g, HCl (25 %, 7.7 M) 10 ml, $FeCl_2 \cdot 4H_2O$ 1.5 g, $ZnCl_2$ 70 mg, $MnCl_2 \cdot 4H_2O$ 100 mg, H_3BO_3 6 mg, $CoCl_2 \cdot 6H_2O$ 190 mg, $CuCl_2 \cdot 2H_2O$ 2 mg, $NiCl_2 \cdot 6H_2O$ 24 mg, $Na_2MoO_4 \cdot 2H_2O$ 36 mg, per 990 ml water), 1 ml, Agar 15.0 g, (RO water 1 l)
2. Yeast extract casamino acid glucose agar (YECG, 0.1 % (*w*/*v*) nutrients) [9]
3. Humic acid vitamin B agar (HVA) [15]
4. Humic acid vitamin B medium, solidified with gellan gum (HVG)

5. VL70 gellan gum with a mixture of D-galacturonate, D-glucuronate, L-ascorbate and D-gluconate (GGAG, 0.5 mM of each substrate)
6. VL70 gellan gum with a mixture of D-glucose, D-galactose, D-xylose and L-arabinose (GGXA, 0.5 mM of each sugar)
7. VL70 gellan gum with amino acid mixture (AA, containing 17 amino acids; combined 0.06 % (*w*/*v*)) [16]
8. VL70 gellan gum with pectin (Pec, 0.05 % (*w*/*v*) pectin)
9. VL70 gellan gum with xylan (Xyl, 0.05 % (*w*/*v*) xylan)
10. VL70 gellan gum with carboxymethyl cellulose (CMC, 0.05 % (*w*/*v*))

The composition of VL70 medium was taken from Joseph et al. [18] and Schoenborn et al. [37]. The pH of all media was adjusted to 7.2. Each medium was supplemented with 20 μg/ml nalidixic acid and 100 IU/ml nystatin as antibacterial and antifungal agents, respectively. Plates were kept in small sealable plastic boxes which were lined with wet paper towels to maintain the moisture levels during the long incubation times and incubated at 27 °C for up to 16 weeks.

Purification of Isolates

Isolation plates were examined weekly. Emergence time of each colony was recorded, and whole colonies of at least 1-mm diameter were removed completely from the isolation plates every week for 12 weeks and purified. However, plates, which still had colonies that were minute (pinpoint size), were incubated for a further 4 weeks, until the size of the colony was large enough to transfer to another medium. Colonies were purified by streaking onto half-strength potato dextrose agar (HPDA, Difco) plates. The slow-growing isolates were purified on 0.1× strength TSA or cultured in 0.1× strength TSB for 3–5 days before further purification on 0.1× strength TSA. Pure cultures were maintained on HPDA slants at 4 °C and in 20 % glycerol at −80 °C, for further study.

Morphological Characterisation

Morphological differences on ISP 2 [39], HPDA and mannitol soybean agar (MS; mannitol 20 g, defatted soy flour 20 g, agar 20 g/l of RO water) were recorded with reference to pigment or melanin production, presence or absence of sporulation and mycelium colour and growth characteristics, following the general guidelines of the International *Streptomyces* Project [39]. Micromorphology of cultures on agar was studied by using the slide culture method and spore chain morphology, and the presence of spore structures was recorded.

16S rRNA Gene Amplification and Sequencing Analysis

Genomic DNA was extracted from actinobacterial cells by applying the method of Kieser et al. [25]. The 16S rRNA gene was amplified separately in two segments using the primer pairs 27f and 765r (R1 amplicon) and the pair 704f and 1492r (R2 amplicon). PCR amplification and sequencing of the 16S rRNA gene was achieved as described previously by Coombs and Franco [9]. The resultant sequences were compared to an online database using BLAST [1] at the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov). The standard blastn (nucleotide–nucleotide) algorithm was used with the default settings.

ARDRA

The PCR products of the R1 amplicon of the 16S rRNA gene were digested with the restriction endonuclease *Hha*I (Promega). The digestion reaction of 8 μl PCR product, 2 μl restriction enzyme and 1 μl 10× buffer (Promega) was incubated at 37 °C until complete digestion was achieved (12–18 h). The PCR product of *Streptomyces griseus* DSM 40855 was digested under similar conditions as a positive control. The digestion products were separated by 1.8 % agarose gel electrophoresis. A low molecular weight DNA ladder (Bio Labs, 25–766 bp) was loaded on both sides and the middle of the gel. The gel was run in 0.5× TBE buffer at 90 V for 2.45 h. The Gene tools program (SYNGENE product version 3.08, Synoptic Ltd., England) was used to compare the band sizes (in base pairs) from each digestion product with the low molecular weight DNA ladder. Digestion with the second enzymes, *Pst*I or *Rsa*I, was carried out singly using the same conditions as for *Hha*I.

Polyphasic Taxonomy

Twelve strains CAP 94, CAP 215, CAP 261, CAP 290^T, EUM 221^T, EUM 273^T, EUM 374^T, EUM 378^T, PIP 118^T, PIP 143^T, PIP 158 and PIP 175 were identified to the species level based on polyphasic taxonomy as required for classification of actinobacteria [40]. Six of these strains have been accepted as novel, and full phenotypic and genotypic details are described by Kaewkla and Franco [19–24].

Results

Actinobacterial Diversity and Isolation Media

Numbers of isolates from each isolation medium, as well as the number of non-*Streptomyces* genera obtained, are listed in Table 1. The VL70 medium with CMC yielded the highest combined number of *Streptomyces* and non-

Table 1 Number of *Streptomyces* and non-*Streptomyces* isolates and genera obtained from each isolation medium and number isolated each week

Isolation medium[a]	Number of *Streptomyces* isolates	Number of non-*Streptomyces* isolates	Total number	Number of non-*Streptomyces* genera
MMY	27	5	32	3
YECG	25	9	34	6
HVA	37	17	54	9
Xyl	40	18	58	6
CMC	57	26	83	7
GGXA	51	17	68	8
Pec	34	15	49	5
GGAG	53	15	68	5
AA	46	21	67	8
HVG	43	20	63	6
Week of subculture				
Week 1	153	43	196	4
Week 2	80	16	96	6
Week 3	36	13	49	4
Week 4	24	6	30	3
Week 5	24	9	33	5
Week 6	17	9	26	4
Week 7	19	8	27	4
Week 8	11	17	28	6
Week 9	8	5	13	2
Week 10	8	4	12	3
Week 11	18	7	25	2
Week 12[b]	15	26	41	6

[a]The full name of the isolation medium is presented in the "Material and Methods"

[b]Colonies observed at week 12 but allowed to grow until week 16

Streptomyces isolates and gave the highest diversity of seven non-*Streptomyces* genera. VL70 with GGXA and with AA media yielded the next highest numbers of isolates and had the same number of non-*Streptomyces* genera. HVG supported more isolates than HVA, but HVA gave a highest diversity of non-*Streptomyces* with nine different genera including two unique genera, *Gordonia* and *Nonomuraea*, which were not isolated on the other media (Tables 1, 2 and 4). The two nutrient rich media, MMY and YECG, yielded the lowest number of both *Streptomyces* and non-*Streptomyces* genera.

When analysed for isolation time, the majority of strains (>60 %) emerging within the first 3 weeks were *Streptomyces* spp., while most (>55 %) non-*Streptomyces* genera emerged after 3 weeks. Also, the majority of strains of two uncommon genera, *Pseudonocardia* (>65 %) and *Kribbella* (>90 %), emerged after 6 weeks of incubation (Fig. 1). Therefore, incubation time appeared to be more influential than the composition of isolation medium in yielding rare genera of actinobacteria.

Diversity of Actinobacteria from Each Plant

The numbers of isolates from leaves, stems and roots from each plant is shown in Table 2. Isolates from the native pine tree, Grey Box, native apricot tree and red gum made up 33.7, 33.1, 26.4 and 6.8 % of the total, respectively. The red gum samples had fungi, possibly fungal endophytes, which prevented the growth of actinobacteria. Most isolates from the other three plants were from root samples, while the highest number of isolates from red gum was from leaves. Leaves of Grey Box and native pine tree yielded one and two isolates, respectively, whereas the leaves of the native apricot tree provided 33 isolates. The low numbers of isolates from the leaves suggest that the hypochlorite may have penetrated the leaf tissue and killed the endophytic bacteria within. Non-*Streptomyces* spp. constituted 42.3, 28.8, 27.6 and 1.2 % of the isolates from Grey Box, native pine, native apricot tree and red gum, respectively, and most were obtained from root samples. The native apricot tree yielded the highest diversity, with 12 non-*Streptomyces* genera (Table 2) including four genera, *Actinopolymorpha*, *Amycolatopsis*, *Nocardiopsis* and *Polymorphospora*, which were not isolated from other plants. The native pine tree yielded nine different genera including *Actinomadura*, *Actinomycetospora* and *Nonomuraea*, which were only recovered from this plant. Grey Box and red gum tree samples contained eight and two genera, respectively. Although very few isolates were obtained from the red gum samples, it contained *Gordonia* which is relatively rare.

Table 2 Tree samples and type of medium employed to isolate actinobacterial endophytes belonging to different genera

Tree Samples / Genus	Number of groups[a]	Isolation medium[b]	Grey Box			Native apricot			Red Gum			Native pine			Total
			L	S	R	L	S	R	L	S	R	L	S	R	
Actinomadura	1	CMC, HVA	–	–	–	–	–	–	–	–	–	–	–	2	2
Actinopolymorpha	2	CMC, GGXA, HVA, HVG, Xyl	–	–	–	2	–	–	–	–	–	–	–	–	2
Actinomycetospora	1	GGAG	–	–	–		–	–	–	–	–	–	–	6	6
Amycolatopsis	1	CMC, GGXA, Pec, YECG	–	–	–	1	–	5	–	–	–	–	–	–	6
Flindersiella	1	AA	–	–	1	–	–	–	–	–	–	–	–	–	1
Gordonia	1	HVA	–	–		–	–	–	–	–	1	–	–	–	1
Kribbella	5	AA, CMC, GGAG GGXA, HVA, MMY, Pec, Xyl, YECG	–	–	14	1	2	3	–	–	–	–	–	5	25
Micromonospora	5	AA, GGXA, HVG, Xyl,	–	2	1		1	–	–	1	–	–	–	1	6
Nocardia	7	AA, CMC, GGXA, HVA, Pec, Xyl, YE	–	–	1	1	–	–	–	–	–	–	–	8	10
Nocardioides	1	AA, CMC, GGAG, GGXA, HVA, HVG, Xyl, YE	–	–	4	–	–	1	–	–	–	–	–	6	11
Nocardiopsis	1	HVG	–	–	–	–	1	–	–	–	–	–	–	–	1
Nonomuraea	1	HVA	–	–	–	–	–	–	–	–	–	–	–	1	1
Polymorphospora	1	AA, HVA	–	–	–	–	1	1	–	–	–	–	–	–	2
Promicromonospora	2	AA, CMC, GGAG, GGXA, HVA, HVG, MMY, Pec, Xyl, YECG	–	1	33	–	2	3	–	–	–	–	–	12	51
Pseudonocardia	9	AA, CMC, GGAG, GGXA, HVA, HVG, MMY, Pec, Xyl, YECG	1	5	5	5	7	7	–	–	–	–	–	6	36
Streptomyces	163	AA, CMC, GGAG, GGXA, HVA, HVG, MMY, Pec, Xyl YECG	–	13	109	23	14	70	22	7	8	2	3	142	413
Williamsia	1	GXA, HVG	–	–	1	–	–	1	–	–	–	–	–	–	2
Total for each part			1	21	169	33	28	91	22	8	9	2	3	189	576
Total for each tree			191			152			39			194			576

L leaf, *S* stem, *R* root

[a] Number of groups based on morphological characterisation

[b] The name of the isolation medium is presented in the "Material and Methods"

Similarly, *Williamsia* strains are reported as endophytes for the first time. *Streptomyces*, *Kribbella* and *Pseudonocardia* were distributed across most parts of plants. However, the majority of isolates were from roots of plants, and for native pine, it was the source of 97.5 % of all isolates (Table 2).

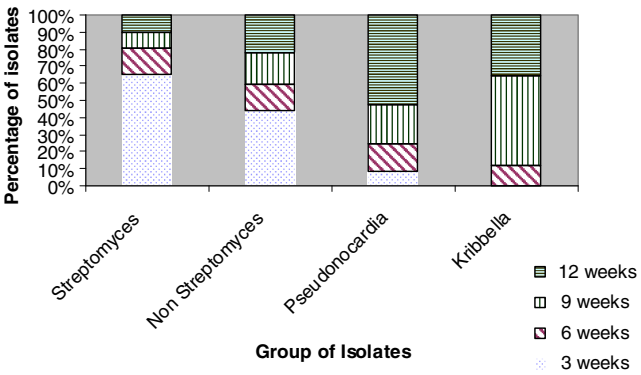

Figure 1 Percentage of isolates that emerged from isolation plates at 3, 6, 9 and 12 weeks. Data are presented as *Streptomyces* and non-*Streptomyces* with additional analysis of two uncommon genera

Isolates and Their Identification to the Genus Level

Five hundred and seventy-six isolates were obtained over the 16 weeks of incubation. The prefixes CAP, EUM, EUC and PIP refer to isolates from samples of *C. preissii*, *E. microcarpa*, *E. camaldulensis* and *P. phylliraeoides*, respectively. Two hundred and three groups were distinguished based on cultural and micromorphological characteristics on the three different media. Of these, 163 groups were classified as *Streptomyces* or *Streptomyces*-like (n=413 isolates; 71.7 %), while 40 morphological groups were non-*Streptomyces* (n=163; 28.3 %).

Based on morphological characterisation, representatives of different genera from each of the 38 non-*Streptomyces* groups and 41 strains from the ambiguous *Streptomyces*/*Streptomyces*-like isolates were classified using ARDRA. *Hha*I digestion yielded 13 ARDRA patterns (Table 3). Even though some of these ARDRA patterns contained more than one genus, it was possible to distinguish the different constituent genera on the basis of their distinctive morphological properties. For example, pattern 1 contained five genera

Table 3 Clustering of isolates based on their ARDRA profiles using *Hha*I

Pattern	Genera	Number of isolates	Fragment sizes
1	*Streptomyces*	25	475, 160, 90
	Micromonospora	2	
	Polymorphospora	1	
	Actinopolymorpha	1	
	Promicromonospora	1	
2	*Amycolatopsis*	1	443, 240
	Nocardia	1	
	Nocardioides	2	
3	*Actinomadura*	1	387, 166, 126
	Nonomuraea	1	
	Streptomyces	1	
4	*Micromonospora*	1	>650
	Nocardia	4	
	Streptomyces	7	
5	*Streptomyces*	1	470, 250
	Nocardia	1	
6	*Micromonospora*	1	266, 195, 163, 97
7	*Actinomycetospora*	1	375, 330
	Kribbella	4	
	Nocardiopsis	1	
	Pseudonocardia	5	
8	*Streptomyces*	1	187, 155, 105, 54
9	*Nocardia*	1	353, 228, 81
	Pseudonocardia	2	
10	*Gordonia*	1	390, 320
	Williamsia	1	
11	*Actinopolymorpha*	1	304, 178, 92
12	*Streptomyces*	6	500, 175, 75
13	*Streptomyces*	1	297, 183, 159, 57
	Promicromonospora	2	

that were readily identified by their distinct cultural morphology. Patterns 2, 3, 9 and 13 also included more than one genus, but with small numbers of isolates that could also be identified by morphological traits. Representative isolates of each morphology group from these ARDRA patterns were sequenced to validate the putative identification. Both strains in patterns 5 and 10 were sequenced. Of note, *Streptomyces* and *Nocardia* strains had a high divergence in their 16S rRNA genes as they presented seven and four different ARDRA patterns, respectively.

With two ARDRA patterns (nos. 4 and 7), however, it was not possible to distinguish some of the isolates on the basis of morphology; hence, a second enzyme digestion was required. In silico restriction analysis was used to select the second enzymes, *Rsa*I and *Pst*I, to distinguish the genera in ARDRA patterns 4 and 7 (Table 4), respectively. *Micromonospora* and *Actinomycetospora* were not included as

Table 4 The differentiation of isolates in patterns 4 and 7 by ARDRA with a second enzyme

Enzyme	Pattern	Genus	Fragment sizes
*Rsa*I[a]	4A	*Nocardia*	595, 145
	4B	*Streptomyces*	440, 305
*Pst*I[b]	7A	*Kribbella*	460, 300
	7B	*Nocardiopsis*	610,130
	7C	*Pseudonocardia*	750

[a] Isolates belonged to pattern 4 with *Hha*I digestion

[b] Isolates belonged to pattern 7 with *Hha*I digestion

their distinct cultural morphologies distinguished them from the other two and three genera clustered in ARDRA patterns 4 and 7, respectively. On the basis of these combined ARDRA and morphological analyses, 47 isolates representing each putative genus and isolates with distinct colony and spore morphologies were selected for 16S rRNA gene sequencing, and the resultant BlastN matches of partial and full 16S rRNA gene sequences are presented in Tables 5 and 6. Eighteen isolates, which showed 16S rRNA gene sequence similarity with validated species at ≤99 %, were chosen for full 16S rRNA gene sequencing (Tables 5 and 6).

Overall, identification was based on a combination of morphological characterisation, ARDRA, using more than one restriction enzyme where necessary and judicious selection of candidates for 16S rRNA gene sequencing (Table 5).

There were 17 genera identified, of which the majority of strains belonged to *Streptomyces* (71.7 %), with the rest being *Promicromonospora* (8.9 %), *Pseudonocardia* (6.3 %), *Kribbella* (4.3 %), *Nocardioides* (1.9 %), *Nocardia* (1.7 %), *Amycolatopsis* (1.0 %), *Micromonospora* (1.0 %), *Actinomycetospora* (1.0 %), *Actinopolymorpha* (0.4 %), *Actinomadura* (0.3 %), *Polymorphospora* (0.3 %), *Williamsia* (0.3 %), *Gordonia* (0.2 %), *Nocardiopsis* (0.2 %), *Nonomuraea* (0.2 %) and a new genus called *Flindersiella* (0.2 %).

Characterisation of Novel Species

The analysis of 47 strains (Tables 5 and 6) yielded 29 isolates, including eight *Streptomyces* spp., with a 16S rRNA gene sequence similarity of <99 % to their nearest matching type strain. Of these, 12 isolates were chosen for detailed characterisation by polyphasic taxonomy, and six have been validated as novel (Table 6). Four were isolated from Grey Box, and one isolate each from pine and native apricot, and the isolation time for five of these isolates was at least 8 weeks. The results of the polyphasic taxonomy study confirmed the novelty of six strains: strain EUM 378^T, CAP 290^T, EUM 221^T, EUM 374^T, PIP 143^T and EUM 273^T.

Table 5 Blast matches of partial and near complete 16S rRNA gene sequences for selected endophytic actinobacterial isolates

Isolate[a]	The closest match	Bits	Percent	Isolate[a]	The closest match	Bits	Percent
CAP 33 (GU434235)	*Nocardia salmonicida* DSM 40472^T	987	99	EUM 96 (GU434252)	*Nocardioides luteus* ATCC 43052^T	1,273	99
CAP 47[b] (HQ396156)	*Amycolatopsis orientalis* DSM 40040^T	2,538	99	EUM 208[b] (HQ396153)	*Amycolatopsis orientalis* DSM 40040^T	2,542	99
CAP 48 (GU434236)	*Actinomadura meyerii* A288^T	876	99	EUM 285 (GU434254)	*Streptomyces durmitorensis* MS405^T	1,157	99
CAP 94[b] (GU434237)	*Promicromonospora sukumoe* DSM 44121^T	1,116	98	EUM 354 (GU434255)	*Williamsia muralis* DSM 44343^T	1,122	99
CAP 122 (HQ396154)	*Amycolatopsis keratiniphila* subsp. *nogabecina* DSM 44586^T	1,415	99	EUM 357 (HQ396158)	*Pseudonocardia zijingensis* DSM 44774^T	1,858	95
CAP 124 (GU434238)	*Nocardioides luteus* DSM 43366^T	1,177	99	EUM 377 (GU434256)	*Pseudonocardia benzenivorans* DSM 44703^T	1,044	95
CAP 140 (GU434239)	*Nocardia carnea* DSM 43397^T	1,146	99	PIP 77 (GU434257)	*Streptomyces chattanoogensis* NBRC 12754^T	1,164	99
CAP181[b] (GU434267)	*Micromonospora coerulea* DSM 43143	2,342	99	PIP 79 (GU434258)	*Williamsia muralis* DSM 44343^T	1,046	99
CAP 185 (GU434240)	*Streptomyces sodiiphilus* YIM 80305^T	662	99	PIP 94 (GU434259)	*Streptomyces gelaticus* NRRL B-2928^T	1,221	97
CAP 196 (GU434241)	*Streptomyces lincolnensis* ATCC 25466^T	974	97	PIP 118[b] (HQ396152)	*Kribbella antibiotica* YIM 31530^T	2,523	98
CAP 203 (GU434242)	*Nocardia callitridis* DSM 45353^T	1,033	98	PIP 148[b] (HQ396151)	*Kribbella sandramycini* ATCC 39419^T	2,401	98
CAP 215[b] (GU434243)	*Streptomyces thioluteus* NBRC 3364^T	1,005	96	PIP 153 (GU434260)	*Pseudonocardia carboxydivorans* Y8^T	1,269	98
CAP 221 (GU434244)	*Streptomyces ochraceiscleroticus* NBRC 12394^T	647	99	PIP 172 (GU434261)	*Nocardiopsis alba* DSM 43377^T	1,146	99
CAP 263 (GU434246)	*Streptomyces alboniger* ATCC 12461^T	968	98	PIP 175[b] (GU434262)	*Streptomyces aculeolatus* NBRC 14824^T	1,090	98
CAP 280 (GU434245)	*Streptomyces amakusaensis* NRRL B-3351^T	1,122	96	PIP 194 (GU434263)	*Kribbella hippodromi* S1.4^T	1,249	98
CAP 261[b] (GU434247)	*Streptomyces rishiriensis* strain NRRL B-3239^T	1,177	97	PIP 199 (GU434264)	*Amycolatopsis japonica* DSM 44213^T	1,029	98
CAP 305 (GU434248)	*Pseudonocardia oroxyli* D10^T	1,127	98	PIP 232 (GU434265)	*Polymorphospora rubra* DSM 44947^T	874	99
CAP 329 (GU434249)	*Nonomuraea salmonea* ATCC 33580^T	1,155	97	PIP 250 (GU434266)	*Streptomyces yogyakartensis* NBRC 100779^T	1,249	98
CAP 335[b] (HQ396157)	*Actinomycetospora chiangmaiensis* strain YIM 0006^T	2,287	97	PIP 158[b] (GU434268)	*Kribbella flavida* JCM 10339^T	2,409	98
EUC 70 (GU434250)	*Gordonia terrae* DSM 43249^T	1,136	99	PIP 160[b] (GU434269)	*Micromonospora pattaloongensis* JCM 12833^T	2,167	98
EUM 48 (GU434251)	*Micromonospora rosaria* ATCC 29337^T	1,243	98				

[a] 16S RNA gene was fully sequenced

[b] GenBank accession no. of the 16S rRNA gene sequence is in parentheses

Discussion

The aim of this project was realised with the isolation of 576 endophytic actinobacteria from the Australian native tree samples. The next challenge was to characterise all the strains to the genus level.

Other studies that employed ARDRA for classification of actinobacteria [8, 28, 41, 47] required the application of this molecular method to all the strains in their study and used a larger combination of restriction enzymes. As this is not practical when dealing with over 500 strains, we have shown that a combination of cultural discrimination followed by application of molecular analyses on a smaller number of discrete morphology-based groups can offer a simple, rapid and highly discriminatory characterisation that can be implemented economically.

The majority of isolates obtained were *Streptomyces* as was reported in a number of studies on the isolation of endophytic actinobacteria [4, 5, 9, 27, 36, 43]. New members of this prolific genus were also identified, which is important as these new members are more likely to produce new metabolites. Studies on the presence of secondary metabolite biosynthetic genes, as well as metabolic profiles of secondary metabolite extracts, using HPLC-photodiode

Table 6 The percentage similarity of actinobacteria isolates with the closest related species based on BLASTN analysis of 16S rRNA gene sequence and isolation details

Strains[a]	Source sample of plant	Isolation medium and isolation time (weeks)	The closest phylogenetic type strains	16S rRNA gene sequence similarity (%)
CAP 290^T (FJ805428)	Native pine	VL70 AA	*Nocardia nova* DSM 43843	97.4
	Root	8	*Nocardia terpenica* DSM 44935^T	96.7
EUM 221^T (FJ805427)	Grey Box	VL70 GGAG	*Pseudonocardia zijingensis* DSM 44774 T	98.7
	Stem	8	*Pseudonocardia aurantiaca* DSM 44773^T	97.8
EUM 374^T (FJ805426)	Grey Box	VL70 AA	*Pseudonocardia acaciae* GMKU095T	96.1
	Root	9	*Pseudonocardia spinosispora* LM 141^T	96.3
PIP 143^T (FJ805429)	Apricot	VL70 AA	*Actinopolymorpha cephalotaxi* I06-2230^T	98.7
	Leaf	11	*Actinopolymorpha rutila* DSM 18448^T	98.1
EUM 378^T (FJ805430)	Grey Box	VL70 AA	*Actinopolymorpha cephalotaxi* I06-2230^T	94.5
	Root	12	*Actinopolymorpha alba* YIM 48868	94.2
EUM 273^T (GU434253)	Grey Box	VL70 CMC	*Promicromonospora xylanilytica* YIM61515^T	98.2
	Root	3	*Promicromonospora vindobonensis* V45^T	98.0

[a] GenBank accession no. of the 16S rRNA gene sequence is in parentheses

array analysis of *Streptomyces* and non-streptomycetes from this study will be reported elsewhere. Early results indicate that a number of the streptomycetes are producers of putative new metabolites (Franco, personal communication). In addition, this study provided a significantly high diversity of endophytic actinobacteria with 17 genera from four types of plants, while the next most diverse range of these bacteria was reported to have 32 actinomycete genera isolated from over 90 plants [34, 35].

Of particular note, the majority of the streptomycetes emerged within the first 3 weeks, whereas over 50 % of the non-streptomycetes appeared at or after 6 weeks. Therefore, the longer incubation times are critical in obtaining uncommon and novel isolates. Furthermore, there are higher numbers of *Pseudonocardia*, *Nocardioides* and *Kribbella* strains compared to reports of isolation from soil [10, 46]. Therefore, this small selection of four native Australian trees has given an indication that other native trees are potentially rich sources of high diversity and rare genera of endophytic actinobacteria.

Another major finding is that media containing low levels of nutrients and polymers as the growth substrate such as the VL70-based media, especially with carboxymethylcellulose as C-source, yielded the highest number of both *Streptomyces* and non-*Streptomyces* isolates. Tree sap, including sap from *Eucalyptus*, contains cellulose, amino acids [33, 38] and sugars [31] and correlates with the higher number of isolates with media containing these nutrients. These media were able to reduce contamination by fast-growing bacteria and allowed slow-growing or low-abundant actinobacteria to emerge. The long incubation times, coupled with excision of emerging colonies from the isolation plates, were critical for the emergence of the so-called rare genera. We hypothesise that members of these genera would otherwise be outcompeted by the relatively more abundant and faster growing *Streptomyces* strains, and that complete removal of colonies that emerge early allows members of the low-abundant genera to emerge.

Although, HVA [26, 32] gave a moderate number of isolates, it was the medium which yielded the highest diversity of non-*Streptomyces*, including two unique genera, and can still be recommended as a medium of choice for the isolation of a broad range of actinobacteria. In comparison, HVG with gellan gum as the solidifying agent was less effective as it had higher contamination from non-actinobacterial microorganisms (data not shown). Nevertheless, the other low-nutrient media that contained gellan gum yielded significant actinobacterial diversity, similar to the reports by Janssen et al. [17], with these media. Of note, the one genus and three novel species that have been accepted as novel to date were isolated on VL70-AA, with the amino acid mixture.

Another facet worth considering is that isolation of microorganisms uses small volumes (100 to 200 μl) of a very diluted sample onto a few (typically 3–5) media. Therefore, only a small fraction of the total population is placed onto a growth medium. In this study, the crushed plant material containing a small number of microorganisms per gram tissue was added directly to the isolation media. Therefore, actinobacteria that were both released due to the crushing and those still attached to the plant material were exposed to agar medium and had an equal chance of emerging as colonies. The fact that ten different media were used in triplicate meant that a lot more of each of the plant sample (root, leaf and stem) was plated out. Therefore, the success in isolating large numbers of actinobacteria can also be

attributed to the approximately 5–10 g of each sample of source material, generally harbouring a low density of actinobacteria (~$10^1.g^{-1}$) in their tissue, being plated onto at least 30 isolation plates. It is also likely that the original plant material, added as macerates onto the isolation plates, can also provide micronutrients or growth factors that are essential for colonies to form. Based on these observations, we advocate plating out large amounts of plant sample using many more plates per sample to achieve the large numbers required to access the broader diversity and any novel species. Here, we should point out that, in terms of actual numbers, it is among the highest reported in any one study. The 16S rRNA gene sequence analysis showed that at least 29 of the 47 selected strains evaluated had a percentage of maximum sequence similarity with species with valid names at lower than 99 % and were likely to be novel species, with a significant number of novel *Streptomyces* spp. Most other publications rely on differences in this one factor—16S rRNA gene sequence similarity—to indicate novelty, whereas a full polyphasic taxonomy study is required to describe a new genus or species. Therefore, in this study, novelty was validated by subjecting 12 strains to a full polyphasic taxonomical characterisation. The test used was acceptance for publication in a journal that specialises in bacterial systematics to indicate the rigour of our claims of novelty and to strengthen the value of this study. These publications are based only on taxonomic characterisation of each strain and do not reveal the comprehensive approach to isolation and genus level identification described here.

The results confirmed the identification of a novel genus in the family *Nocardioides*, called *Flindersiella endophytica* gen. nov., sp. nov. [23]. *Nocardia callitridis* sp. nov. [19], *Actinopolymorpha pittospori* sp. nov. [22] and *Promicromonospora endophytica* sp. nov. [24] are the first reported endophytic type species of these genera, whereas *Pseudonocardia adelaidensis* sp. nov. [20] and *Pseudonocardia eucalypti* sp. nov. [21] are in a genus containing other type species of endophytic origin [6, 11, 13, 14, 34, 45], indicating that this genus has a strong association with plants.

In conclusion, this study with a limited number of native trees has proven that it is possible to culture the significant diversity that resides within plants, including novel and rare genera by using a rational, holistic approach for their isolation and identification. We recommend the use of isolation media with low concentrations of plant polymers, their constituent sugars and amino acids; plating out larger quantities of the plant samples onto multiple plates and incubating isolation plates for up to 16 weeks while removing emerging colonies every week. Further research is being undertaken with these isolates to determine their functional roles and whether they are indeed a new resource for the discovery of novel bioactive compounds or other biotechnology applications in agriculture.

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