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Endophytic Fungi from *Cathranthus roseus*: A Potential Resource for the Discovery of Antimicrobial Polyketides

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Abstract

Endophytic fungi from the stems, roots, leaves and flowers of *Catharanthus roseus* were isolated using routine isolation methods, yielding 25 strains. The crude ethyl acetate extracts of endophyte culture broths were assayed for antimicrobial properties against seven bacterial and two fungal test strains. Thirteen strains producing antimicrobial compounds were taxonomically characterized by 18S rRNA gene sequencing. The majority of these fungi were distributed among the orders *Pleosporales*, *Botryosphaeriales* and *Capnodiales* (class *Dothideomycetes*). Degenerate PCR-based screening was used to target polyketide synthase (PKS) biosynthesis genes in these bioactive strains, resulting in detection of 12 PKS gene fragments from eight strains. The differential antimicrobial activities and unique PKS gene sequences detected in the isolates reflected the potential for these endophytes to produce a range of chemically diverse compounds. Also, this can prove to be another useful dereplication tool for natural product discovery.

Keywords: *Catharanthus roseus*; Endophytic fungi; Polyketide synthase; Antimicrobial activity

Introduction

Terrestrial plants are known to harbor endophytic microbes, which in many cases produce bioactive molecules [1]. Endophytes are microbes that live in the internal tissues of the host plant without showing any symptoms of disease [2], however, recent studies have shown that they may become pathogenic during host plant senescence [3]. Whilst living symbiotically with plants, many endophytes have developed the ability to mimic host chemical diversity and are also believed to be responsible for the production of biologically active metabolites once attributed to the plant host [4]. Some of the chemically complex structures that are of dual endophyte/plant origin and which have been investigated in last few decades include paclitaxel [5], vinca alkaloids [6], camptothecin [7], podophyllotoxin [8] and hypercin [9]. Therefore, endophytes are potential sustainable sources of plant-associated natural products.

Catharanthus roseus (*Apocynaceae*) is a plant well known for the production of the anticancer alkaloids vincristine and vinblastine [10]. The antimitotic effect of these alkaloids is due to their ability to arrest cell division during metaphase through binding tubulin and inhibiting spindle fiber formation [11]. The crude extracts of *C. roseus* are known for their cytotoxic activity against an array of tumour cell lines, including human fibro sarcoma cells (HT 1080), human cervix adenocarcinoma, human lung adenocarcinoma (A549), murine colon carcinoma (26-L5), murine Lewis lung carcinoma and murine melanoma cells (B16-BL6) [12]. In addition to *C. roseus*, an endophytic *Fusarium oxzysporum* strain isolated from *C. roseus* was also reported to produce vinca alkaloids [13]. However, despite the pharmacological importance of endophytic compounds very few investigations have focused on isolating endophytes from *C. roseus* and exploring their chemical diversity and bioactivities [14,15].

Molecular screening for functional genes involved in the biosynthesis of complex chemical structures is a robust strategy used to identify potential candidate microorganisms for natural product discovery. Polyketide synthases (PKS) are involved in the microbial biosynthesis of natural products that possess a variety of biological activities including antibiotics (e.g., erythromycin, tetracycline and vancomycin) [16,17], immunosuppressants (e.g., cyclosporin A) [18], toxins [19] and siderophores [20,21]. The study of PKS genes in endophytes may provide important leads for drug discovery investigations. In our search for novel bioactive molecules we have investigated the endophytic fungal population of *C. roseus*, relating the antimicrobial activities of endophyte crude extracts with PKS gene diversity.

Materials and Methods

Isolation of endophytic fungi

Endophytic fungi were isolated from the mature stem, young stem, mature leaves, tender leaves, roots and flowers of *C. roseus* obtained from Sydney, Australia. These samples were processed within 24 h of collection. During this process samples were cut into small pieces, approximately 0.5 × 0.5 cm, surface sterilized with 0.01% mercuric chloride ($HgCl_2$) solution for 1 min and washed thoroughly with sterile distilled water [22-24]. Residual water was removed from the sample surface by drying on sterile blotting paper. The sterilized plant pieces were placed on the surface of potato dextrose agar (PDA). After 5 to 10 days fungal growth appeared around the plant segments and hyphal tips were transferred to new PDA plates and the fungi cultured were checked for purity. Stock cultures were maintained by sub-culturing at monthly intervals. Seed cultures were grown from an actively growing stock culture and used as the starting material for fermentation experiments.

Fermentation and solvent extraction of fungal cultures

Each isolate of endophytic fungi was cultured in 100 mL malt extract broth for 5~15 days stationary, at 28°C. Fungal matt were harvested by filtering through 4-fold muslin cloth and the culture broths extracted exhaustively with ethyl acetate. Fungal mycelium was extracted with DCM, Methanol (9:1 v/v) and the extracts were pooled

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with the ethyl acetate extracts. The extracts were dried over anhydrous sodium sulphate and concentrated to dryness *in vacuo*. The dry weights of extracted metabolites were determined and a small amount was dissolved in DMSO (25 mg/mL) for use in the antimicrobial assays.

Inhibitory activities of crude extracts against pathogenic microbes

The antimicrobial activity of crude fungal extracts was determined using the disk diffusion method [25]. Luria Bertani and malt extract agar plates were prepared and inoculated with bacterial and fungal target organisms, respectively. Sterile Whatman filter discs (3 mm diameter) were loaded with 125 µg crude extract and placed on the prepared test plates. Microorganisms used for testing were *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* NCTC 6571, *Streptococcus pyogenes, Enterococcus facealis, Corynebacterium* sp., *Pseudomonas aeruginosa* NCTC10490, *Escherichia coli* NTCT10418, *Candida albicans* ATCC10231 and *Aspergillus niger*. The zones of inhibition were measured and recorded after the respective incubation times (24 h for bacteria and 48 h for fungi). Ampicillin (100 µg/mL) and cyclohexamide (100 µg/mL) were used as positive controls for antibacterial and antifungal assays, respectively.

Identification and phylogentic analysis of fungal endophytes

The XS buffer method was used to extract genomic DNA from the fungal isolates which demonstrated antimicrobial activities in the bioactivity assays [26]. The universal primers, SS3 [5' GATCCTTCCGCAGGTTCACCTACGGAAACC 3'] and SS5 [5' GGTGATCCTGCCAGTAGTCATATGCTTG 3'], were used to amplify fungal the 18S rRNA gene by PCR [27]. DNA sequencing was performed using the PRISM BigDye cycle sequencing system v3.1 and an ABI PRISM 373 DNA sequencer (Life Technologies). The sequences were checked against the non-redundant database maintained at National Center for Biotechnology Information using BLASTN algorithm [28]. The 18S rRNA gene sequences of the fungal endophytes were aligned with reference sequences using the CLUSTALW tool available from the European Bioinformatics Institute and alignments manually checked. The accession numbers of the reference strains used for the study are listed in Table 1. Maximum Likelihood phylogenetic trees were constructed using *MEGA* version 5 [29] with 500 bootstrap repetitions. The 18S rRNA gene sequence of *Rhizopus oryzae* TY.GF1 was used as out-group.

Amplification of polyketide synthase genes from endophytic fungi

PKS gene diversity was investigated in the endophytic fungi that demonstrated antimicrobial activity. Three pairs of degenerate primers, DKF and DKR [19], LC1 and LC2c (non-reducing class of KS domains) and LC3 and LC5c (partially reducing class of KS domains) [30], were used to target the ketosynthase (KS) domain in PKS gene pathways (Table 2). All PCR reactions were set up in a 20 µL volume consisting of 40 ng of genomic DNA, 1 X buffer (Bioline), 2.5 µM $MgCl_2$ (Bioline), 0.2 mM dNTPs (Bioline), 25 p mol each primer (Sigma Aldrich) and 0.2 U *Taq* DNA polymerase (Bioline) and sterile double-distilled water. PCR amplicons were sequenced and data was analyzed as described above. Nucleotide sequences were translated into amino acid sequences using the translate tool available in ExPASy (http://web.expasy.org/translate). The appropriate frame of the amino acid sequences was chosen based on sequence similarity to the corresponding NCBI database structures. A phylogenetic tree was constructed using *MEGA* 5 and the maximum likelihood method with 500 bootstrap replicates. BLASTX similarity results and the reference sequences used for construction of the PKS phylogeny are given in Table 3. All the 18S rRNA sequences and polyketide synthase sequences are submitted at NCBI gene data bank.

Results

Endophytic fungi and their tissue specific diversity

Twenty-five endophytic fungi were isolated from the stems, roots, leaves and flowers of *Catharanthus roseus*. Most of the fungi were isolated from leaves (48%), 44% from mature stems and 4% each from the roots and flowers (Table 4). The widespread occurrence of fungal endophytes in leaves was supported by the isolation of fungi from very young leaves.

Antimicrobial assays

The majority of fungal isolates (52%) showed activity against one or more pathogenic test microorganisms (Table 5). Most endophytes were active against the *B. subtilis*, *S. aureus*, *S. pyogenes*, *E. faecalis* and *C. sp* test strains. No extracts showed activity against *C. albicans*, *A. niger* and *P. aeuriginosa*. The zones of inhibition demonstrated by different fungal extracts are listed in Table 6. It is interesting to note that most of the extracts were active against most Gram-positive bacteria but not active against the tested fungi and Gram-negative bacteria, except against *E. coli*. Endophytic fungi which showed inhibitory activity against these test microbes were genetically screened for PKS genes.

Phylogenetic analysis of fungal endophytes with antimicrobial activity

The 18S rRNA gene sequences of fungal endophytes with antimicrobial activities were aligned with reference sequences and the phylogenies reconstructed. Of the isolates that demonstrated antimicrobial activity, the most abundant genera were *Alternaria* and *Phoma* which were mostly isolated from mature stems, while endophytes belonging to the genera *Guignardia* and *Botryospheria* were mainly isolated from the flowers. All of the fungal endophytes belonged to the class Dothideomycetes (Figure 1) indicating a possible host-specific interaction between these fungal species and *C. roseus*. The Dothideomycetes isolates were classified as members of the orders Pleosporales (clade I), Capnodiales (clade II) and Botryosphaeriales (clade III). Fungal endophyte RCCRALF12 formed a well-supported clade with two reference sequences from genera, *Cladosporium* and *Davidiella* with sequence coverage of 87% and identity of 96% (clade II, Capnodiales). While isolates RCCRMSF8 and RCCRMLF20 were grouped with reference sequences from the genera *Guignardia* and *Botryosphaeria,* respectively (clade III, Botryosphaeriales). Fungal endophytes RCCRFF23 and RCCRFF22 grouped with reference sequences from the genus *Phoma*. Endophyte RCCRFF22 was most closely related to *Phoma macrostoma,* showing 98% sequence identity with 97% coverage. The phylogenetic tree revealed that sequences from endophytic fungi RCCRMSF9, RCCRF16, RCCRF21, RCCRF14, RCCR7, RCCRMSF6, RCCRSF15 and RCCRALF13 grouped in clade I with reference sequences from the genera *Alternaria*, *Ulocladium*, *Pyrenophora*, *Cochliobolus* and *Setosphaeria*. In the BLAST results all of the endophyte sequences in this clade showed high similarity to *Alternaria* sp. with an average sequence identity of 92% and 90% sequence coverage.

Diversity of polyketide synthase genes

Focusing on the subset of thirteen bioactive isolates, twelve fragments of PKS biosynthesis genes were amplified from eight fungal

Strain	Accession numbers	Strain	Accession numbers
Alternaria alternate S-f6	HM165489.1	*Ulocladium alternariae* BMP 31-41-05	AF229516.1
Alternaria alternate AFTOL-ID 1610	DQ678031.1	*Phoma macrostoma* var. incolorata	AB454231.1
Selosphaeria sp.	GU190183.1	*Phoma exigua* var. exigua	EU342948.1
Cochliobolus sp.	GU190186.1	*Cladosporium cladosporioides* strain STE-U 3682	AY251093.2
Alternaria sp.	GU190188.1	*Botryosphaeria* sp. MUCC0097	AB454224.1
Alternaria sp.	ABU05197	*Guignardia mangiferae* MUCC0215	AB454259.1
Phoma herbrarum	AY337712.1	*Pyrenophora tritici-repentis* AFTOL-ID 173	AY544716.1
Davidiella tassiana	EU343113.1	*Guignardia alliacea*	AB454248.1
Botryosphaeria dothidea	AB454201.1	*Rhizopus oryzae*	JN003654.1

Table 1: Reference fungal strains with Genbank accession numbers from NCBI used in the phylogenetic analysis of the fungal endophytic isolates.

Target	Primer	Primer Sequence (5'-3')
Ketosynthase domain, Bacterial PKSs (Type 1)	DKF	gtgccggtnccrtgngyytc
	DKR	gcgatggayccncarcarmg
Non reducing Ketosynthase domain, Fungal PKSs (Type 1)	LC1	gayccimgittyttyaayatg
	LC2c	gticcigticcrtgcatytc
Reducing Ketosynthase domain, Fungal PKSs (Type 1)	LC3	gcigarcaratggayccica
	LC5c	gtigaigticrtgigcytc

Table 2: Primer sets used for the study.

isolates (Table 2), 61% of the fungal endophyte population which showed antimicrobial activity possessed potential PKS genes. This supports the possible role of PKS products in the observed activity. Specifically, PKS fragments were amplified from seven endophytic fungi (58.3%) with the DFK and DFR primer set, three fragments with LC1 and LC2c (25%) primer set and two fragments with LC3 and LC5 (16%) primer set.

The phylogenetic analysis of the putative KS domain fragments resulted in a tree with five clades, which was in agreement with previous KS domain structural studies [31] (Figure 2). The KS fragments involved in the production of non-reduced polyketides formed a well-supported clade (clade I) that included sequences from fungal endophytes RCCRALF12, RCCRMSF8 and RCCRSF15 and PKS references from Ascochyta pinodes and Alternaria alternata. Within the group of non-reducing PKS sequences, RCCRMSF8 showed considerable sequence divergence from the other PKS sequences in this group. Two identical PKS fragments were obtained from isolate RCCRSF15, using the DKF/DKR and LC1/LC2c primer sets, indicating that the same gene may have been detected by the different primer sets. The partially reducing PKSs included 6-methlysalicylic acid synthases (6-MSAS) of Aspergillus terreus and Byssochlamys nivea reference sequences grouped with the PKS fragments from RCCRFF21 and RCCRF22 (clade II). The reduced PKS sequences obtained from fungal endophytes RCCRALF12 and RCCRF21 formed a clade with Paracoccidioides brasiliensis, lovastatin nonaketide synthase and acetolactate synthase reference sequences (clade IV). Three more sequences from endophytic fungi RCCRF16, RCCRMSF6 and RCCRALF13 formed a sub clade aligning with PKS sequences from Alternaria solani and Cochliocolus heterstrophus (clade V). The PKS fragment obtained from RCCRMSF8 showed sequence similarity to a PKS-NRPS hybrid sequence from *Metarhizium anisopliae* and grouped together in the resulting phylogenetic tree (clade III).

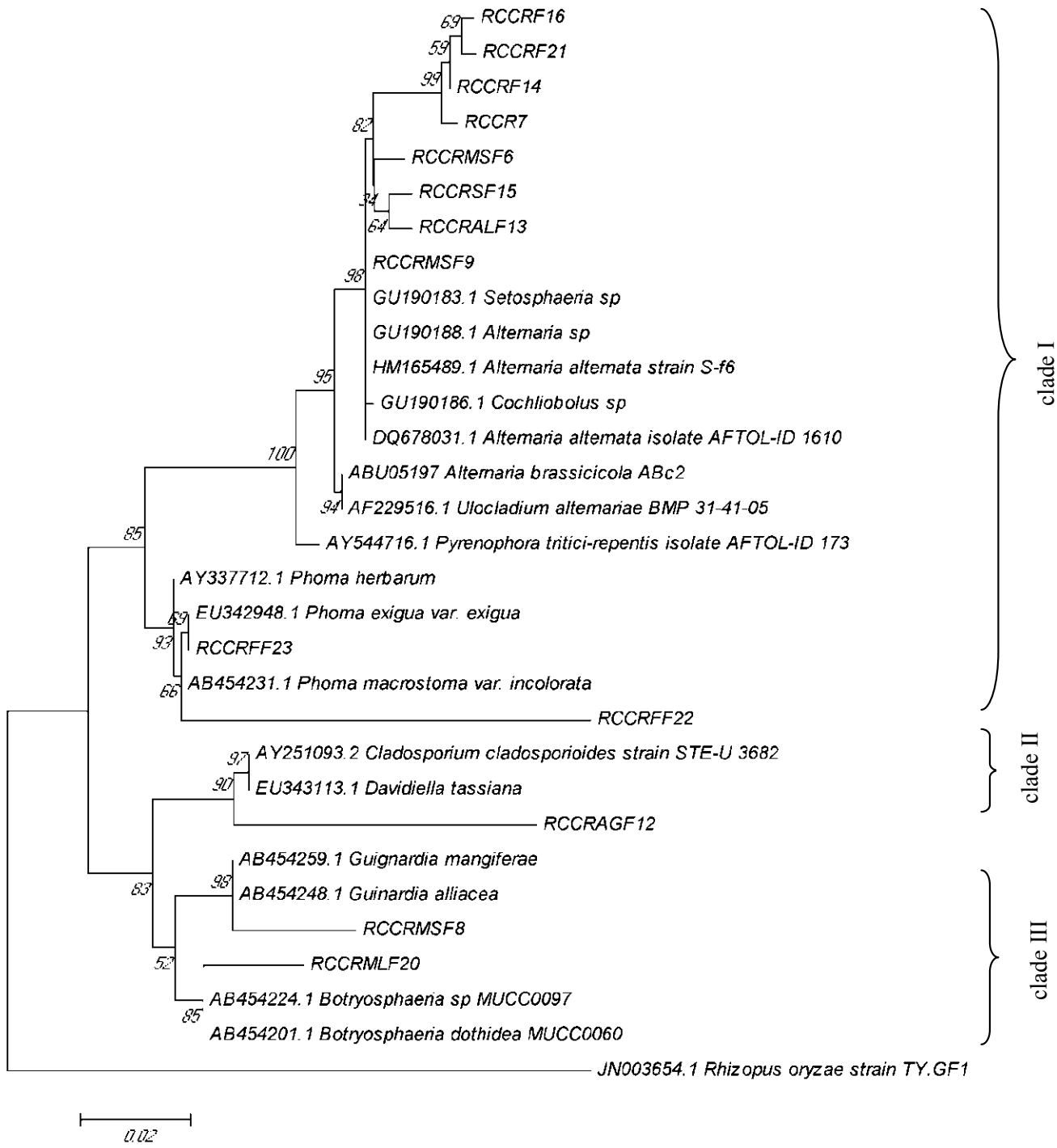
Discussion

In recent years, it has been established that terrestrial plants are host to a range of endophytic microorganisms [32,33]. Fungal endophytes have been found to produce an array of chemically complex structures which have pharmaceutical and ecological importance [34]. Interest in exploring the chemical diversity of endophytes has been fuelled by their demonstrated ability to mimic host plant chemistry [4].

In this investigation, we have explored the fungal community living within in the host plant *Catharanthus roseus*. Twenty-five endophytic fungi were isolated from different tissues of *C. roseus,* 48% from leaves, 44% from stems and 4% from each the roots and flowers. A more in-depth analysis of the fungal species distribution revealed that using the isolation methods of this study, most of the fungal communities were distributed within the stem and midrib region of leaves. Endophytes were not readily isolated from flower specimens, a reflection of low endophyte colonization rates in this organ. Additionally, the lack of endophytes in the flowers supports the theory that these endophytes of *C. roseus* are transferred to hosts by horizontal transmission [35]. The endophyte community observed in this study differs from that previously reported from *C. roseus* grown in India [15]. The variation in reported endophyte community could be because of the geographical variation.

Phylogenetic analysis of the 18S rRNA gene sequence of the bioactive *C. roseus* endophytes indicated that all investigated strains belonged to the Ascomycetes. This finding supports previous investigations in which plant-Ascomycetes associations were studied [36]. Many of the isolates which showed antimicrobial activity were classified to genus level, with the genera *Alternaria* (61%), *Cladosporium* (7.6%), and *Phoma* (15.3%) most frequently isolated. These genera are often reported as endophytes in terrestrial plants [37] and strains of *Alternaria* have been previously isolated from *C. roseus* [15]. The regular occurrence of the *Alternaria* genera within *C. roseus* indicates that certain strains belonging to this genus may exhibit host specificity towards *C. roseus*. Additionally, *Alternaria* strains may have been selectively targeted due to their established production of antimicrobial compounds [38]. The other fungal genera represented in the endophyte community included *Botryosphaeria* sp. and *Guignardia* sp., one isolate from each genus. Although common to endophytes of other terrestrial plants [33], this study is the first to report *Botryosphaeria* sp., *Guignardia* sp. and *Phoma* sp. as endophytes of *C. roseus.* It is also interesting to note that although similar, none of the endophyte 18S rRNA gene sequences from *Alternaria* isolates were completely identical to any sequences in the NCBI database. This finding indicates that there may be high sequence diversity among these endophytes of the same species contributing to the unique 18S rRNA sequences possessed by the endophytes in clade I (Figure 1).

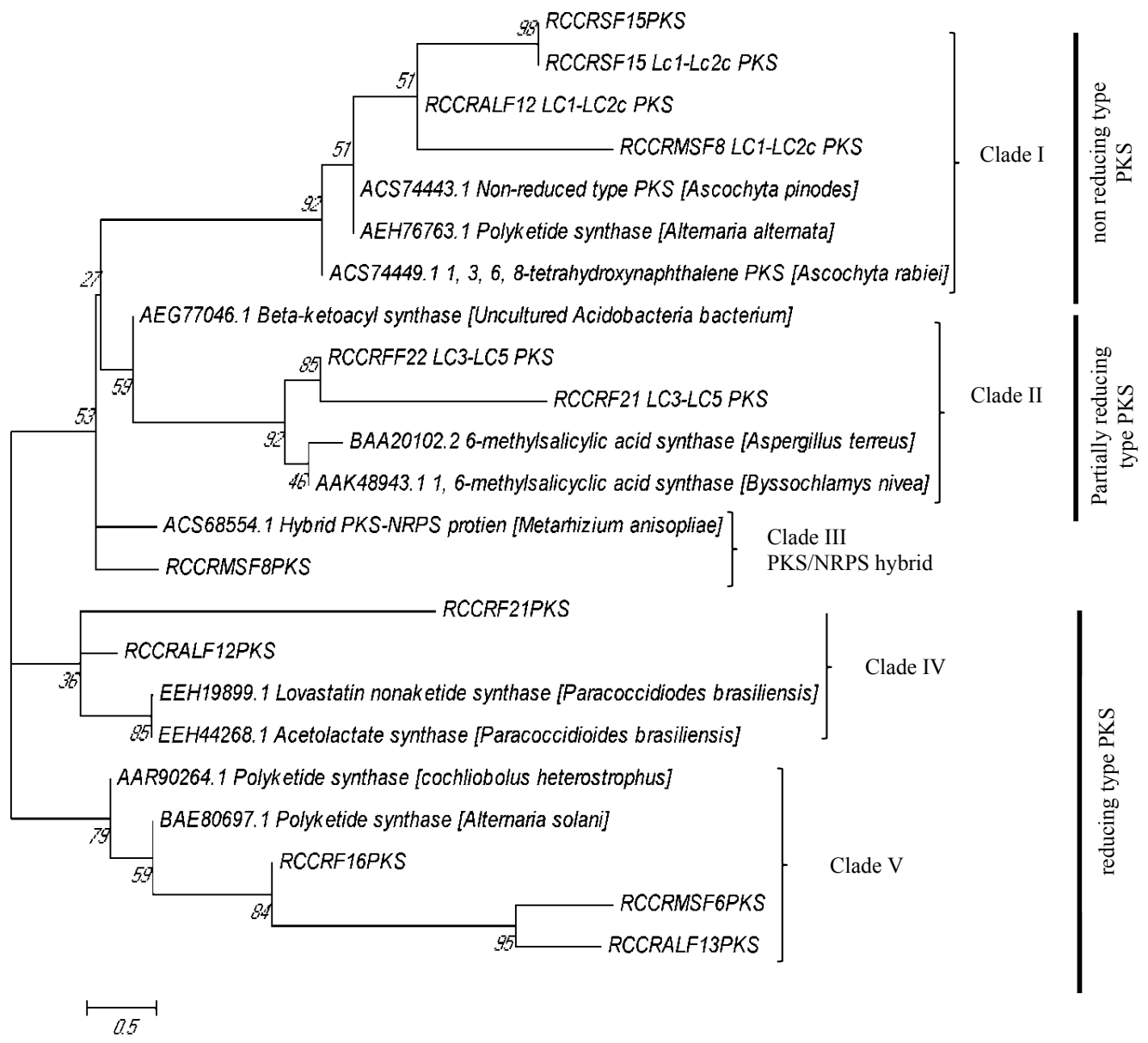
The prospect of endophytes mimicking host plant chemistry, such as for the production of vinca alkaloids, means that they are attractive targets for investigating the production of other classes of bioactive compounds including polyketides and non-ribosomal peptides. There are no reports of endophytes showing antimicrobial properties isolated from *C. roseus*. Considering this, we investigated the presence of polyketide synthase genes in the isolates which demonstrated antimicrobial activity. Chemical analysis of some crude extracts identified alternariol, alternariol *O* methyl ether, altechromone,



Phylogentic tree of 18S rRNA gene sequences of fungal endophytes and reference fungi. The Maximum likelihood tree was generated from a 790 bp multiple sequence alignment with 500 bootstrap replicates, indicated next to the nodes. The accession numbers belonging to reference strains are included and the sequence of Rhizopus oryzae was used as an outgroup. The scale indicates the number of inferred nucleotide changes.

Figure 1: Phylogentic analysis of endophytic fungal isolates from *Cathranthus roseus*.

cerevisterol, dihydroxyergostadien and hydroxyergostadien products of polyketide biosynthetic pathway [39]. Our hypothesis on this is very well supported by the recent report on complete identification of polyketide biosynthetic pathway which synthesizes alternariol and alternariol O methyl ether in *Alternaria alternate* [40]. Identification of PKS genes in uncharacterized isolates is an established method by which microbes with biosynthetic potential can be identified [31,41]. Approximately 61% of the fungal isolates, which demonstrated antimicrobial activity, exhibited PKS gene amplification with at least one of the three sets of primers used. It was interesting to report that the degenerate DKF



Phylogenetic tree of PKS gene sequences of fungal endophytes and reference fungi. The Maximum likelihood tree was generated from a multiple sequence alignment of 198 amino acids in length. Bootstrap analysis (500 replicates) was performed and values >50% are as indicated above nodes. The accession numbers belonging to reference strains are included. The scale indicates the number of inferred nucleotide changes.

Figure 2: Phylogentic analysis of endophytic fungal isolates from *Cathranthus roseus*.

and DKR primers, designed to target bacterial PKS [19], were able to amplify fungal PKS genes. This is likely a result of conservation of core motifs in the KS domain targeted by the primers [42].

Phylogenetic analysis of the PKS gene sequences revealed a well-supported tree. The most striking result was that none of the identified putative endophyte PKS genes possessed high similarity to already characterized PKS genes. Thus they have the potential to be involved in the production of novel bioactive compounds. This proposed chemical diversity is supported by the variable range of activities and levels of inhibition exhibited by the different endophyte extracts. In the phylogenetic tree Clade I represented non-reducing type PKSs involved in production of spore pigments, aflatoxins or melanin like pigments [24]. Non-reduced polyketides are usually aromatic, synthesized by PKSs which lack ER, DH and KR domains, and contain a keto group that is either not reduced or reduced by enzymes other than PKS. Although PKS genes identified from *Alternaria* sp. are commonly involved in the production of reduced polyketides [43]. This study identified a non-reduced PKS gene from RCCRMSF15 (*Alternaria* sp) which could indicate that these genes are involved in production of spore pigment. Two other PKS genes, from RCCRMSF8 (*Guignardia* sp) and RCCRALF12 (*Cladosporium* sp), are related to aromatic polyketide synthases involved in production of toxins or spore pigments. Additionally, it is yet to be determined if the products of any of the identified PKS genes are involved in the observed antimicrobial activity of these endophytes. It would also be interesting to further investigate

Isolate	Primer used	Similarity %	BLASTX match	*Strain*	Accession number
RCCRMSF15	LC1-LC2c DKF-DKR	76	polyketide synthase	*Alternaria alternata*	AEH76763.1
		76	polyketide synthase	*Cochliobolus heterostrophus*	AAR90272.1
		75	non-reduced type polyketide synthase protein	*[Ascochyta pinodes]*	ACS74443.1
RCCRALF12	LC1-LC2c	80	non-reduced type polyketide synthase	*Peyronellaea pinodella]*	ACS74444.1
		90	non-reducing polyketide synthase	*Epicoccum sp*	ADY75776.1
		78	tetrahydroxynaphthalene polyketide synthase	*Ascochyta rabiei*	ACS74449.1
RCCRMSF8	LC1-LC2c	79	polyketide synthase	*Alternaria alternata*	AEH76763.1
		80	ketoacyl synthase	*Trypethelium eluteriae*	ADB77860.1
		80	polyketide synthase	*Mycosphaerella graminicola*	EGP83620.1
RCCRFF22	LC3-LC5	59	6-MSAS	*Aspergillus terreus*	BAA20102.2
		58	polyketide synthase	*Penicillium freii*	CAA65133.1
		49	beta-keto acyl synthase	*Salinispora arenicola*	ADD84279.1
RCCRF21	LC3-LC5	59	6-MSAS	*Aspergillus terreus*	BAA20102.2
		60	6-MSAS	*Byssochlamys nivea*	AAK48943.1
		67	polyketide synthase	*Cochliobolus heterostrophus*	AAR90279.1
RCCRMSF8	DKF-DKR	75	polyketide synthase	*Metarhizium anisopliae*	EFY98483.1
		75	polyketide synthase	*Metarhizium acridum*	EFY92873.1
		74	LovB-like polyketide synthase, putative	*Arthroderma benhamiae*	XP_003014229.1
RCCRALF12	DKF-DKR	62	polyketide synthase	*Cochliobolus heterostrophus*	AAR90260.1
		48	acetolactate synthase	*Paracoccidioides brasiliensis*	EEH44268.1
		47	lovastatin nonaketide synthase	*Paracoccidioides brasiliensis*	EEH19899.1
RCCRMSF6	DKF-DKR	41	polyketide synthase	*Cochliobolus heterostrophus*	AAR90260.1
		36	polyketide synthase	*Gibberella moniliformis*	AAR92216.1
		34	equisetin synthetase	*Fusarium heterosporum*	AAV66106.2
RCCRF21	DKF-DKR	51	polyketide synthase	*Alternaria solani*	BAE80697.1
		49	phenolpthiocerol synthesis polyketide synthase	*Pyrenophora tritici-repentis*	XP_001930443.1
		48	polyketide synthase	*Cochliobolus heterostrophus*	AAR90264.1
RCCRF16	DKF-DKR	87	polyketide synthase	*Alternaria solani*	BAE80697.1
		85	polyketide synthase	*Cochliobolus heterostrophus*	AAR90264.1
		44	polyketide synthase	*Xylaria sp*	AAY40862.1
RCCRALF13	DKF-DKR	41	polyketide synthase	*Alternaria solani*	BAE80697.1
		38	polyketide synthase	*Cochliobolus heterostrophus*	AAR90264.1
		33	polyketide synthase	*Aspergillus ochraceus*	AAT92022.1

Table 3: BLASTX results of PKS genes sequences from the endophytic fungi.

the PKS genes identified from *Guignardia* sp.. There have been few reports of PKS genes in this genera and it has been reported that the crude extracts of an endophytic *Guignardia* sp showed antimicrobial activity against array of bacteria and fungi [37].

Clade II of the PKS gene tree contained sequences from endophytic fungi RCCRFF22 (*Phoma* sp) and RCCRF21 (*Alternaria* sp) and represented partially reduced PKSs involved in synthesis of 6-methlysalcylic acid (6-MSAS), a compound frequently associated with fungal strains form the Pleosporales [44]. The aromatic backbone of 6-MSAS is a partially reduced intermediate that is the precursor molecule for various toxins [45]. Production of these toxins is well studied in *Aspergillus* sp, *Pencillium* sp. and *Byssochlamys nivea* [7,46,47]. There are no reports of 6-MSAS genes isolated from *Alternaria* sp. [40], however, amplified 6-MSAS type PKSs from *Phoma* sp. using the same set of primers used in this study (LC3-LC5). Also, squalestatins and phomalone polyketides have been reportedly produced by *Phoma* sp. [48,49].

The PKS genes RCCRAGF12 (*Guignardia* sp), RCCRF21 (*Alternaria* sp), RCCRF16 (*Alternaria* sp), RCCRMSF6 (*Alternaria* sp) and RCCRALF13 (*Alternaria* sp) grouped together in two clades which represented PKSs involved in the production of reduced polyketides. These reduced polyketides generally have CoA thioesterified carboxylic acids as precursors [42]. Two of these sequences (RCCRAGF12, RCCRF21) formed a clade with PKS genes involved in lovastatin biosynthesis, a compound often associated with *Aspergillus* sp. [50]. Lovastatin is a non-aromatic, reduced product of an iterative type I PKS [47]. The remaining reducing-type PKSs did not have any significant similarity to PKSs of known function. Thus, these PKS biosynthesis genes could be interesting to study as they may be involved in the production of novel bioactive compounds. The antimicrobial activity of these endophytic fungal extracts demonstrated the highest zones of inhibition observed in this study, with average zone measuring of 8.7, 7.6 and 7.5 mm for isolates RCCRF16, RCCRMSF6, and RCCRALF13 respectively. It was also interesting to see that all three extracts showed inhibitory activities against the same set of test microbes, suggesting to a shared chemical composition in the extracts.

The PKS gene sequence identified from RCCRMSF8 (*Guignardia*

	Tissue				
	Stem (%)	Root (%)	Leaves (%)	Flower (%)	Total (%)
Number of endophytes	11(44)	1(4)	12(48)	1(4)	25(100)

Table 4: Number of fungal isolates from different plant tissues.

Taxa	Number of active strains	Strains with antimicrobial activity					
		EC	SA	SP	B	EF	C
Alternaria sp	7	0	6	2	6	6	4
Guignardia sp	2	1	2	0	2	1	1
Cladosporium sp	1	1	0	0	0	0	0
Botryosphaeria sp	1	1	0	0	1	0	1
Phoma sp	2	1	2	0	1	1	2

Table 5: The number of isolates from each genera and presence of antimicrobial activity (%).

Fungal Isolate	Zone of inhibition in mm 25 mg/mL crude extract					
	EC	SA	SP	B	EF	C
RCCRMSF6	0.0	9.0	7.0	7.0	8.0	7.0
RCCRF7	0.0	7.0	0.0	8.0	6.0	10.0
RCCRMLF8	0.0	4.0	0.0	4.0	0.0	0.0
RCCRMSF9	0.0	5.0	0.0	7.0	5.0	6.0
RCCRF10	3.0	8.0	0.0	8.0	7.0	10.0
RCCRAGF12	0.0	0.0	0.0	3.0	0.0	0.0
RCCRALF13	0.0	8.0	6.0	8.0	8.0	7.0
RCCRSF15	0.0	7.0	5.0	3.0	5.0	0.0
RCCRF16	0.0	10.0	8.0	7.0	10.0	0.0
RCCRMLF20	4.0	3.0	0.0	5.0	3.0	4.0
RCCRF21	3.0	0.0	0.0	3.0	0.0	7.0
RCCRFF22	0.0	3.0	0.0	5.0	0.0	4.0
RCCRF25	0.0	7.0	0.0	9.0	7.0	8.0

Table 6: Zones of inhibition produced by fungal endophytes against pathogenic bacteria.

sp.) did not fall into any of the groups discussed above and was shown to be most related to a sequence from a hybrid PKS/NRPS biosynthetic gene cluster of *Metarhizium anisopliae* (clade III). PKS/NRPS hybrid genes were first reported in bacteria, and recently have been identified in filamentous fungi. These hybrid enzymes typically contain domains from both PKS and NRPSs, synthesizing compounds containing both peptide and polyketide groups [7]. Another PKS gene from the same *Guignardia* sp. endophyte was phylogenetically grouped with the non-reducing type PKS clade, indicating that this isolate may possess multiple PKS gene clusters. Endophytes which showed antimicrobial activity were also screened for NRPS gene clusters by PCR as described by Neilan [51], however none of the isolates showed the presence of NRPS gene clusters.

PKS derived compounds isolated from *Alternaria* sp. have previously shown antimicrobial activities against Gram positive and Gram negative bacteria, as well as fungi [38,52]. It is not known if the bioactivity from our endophytes is due to the compounds previously reported. Considering that none of the identified endophyte PKS gene sequences showed complete homology with characterized PKS genes, there remains the possibility to explore interesting chemical diversity from these isolates.

In conclusion, the endophytic fungal community that demonstrated antimicrobial activity was dominated by the genera *Alternaria, Phoma*, *Botryosphaeria*, *Guignardia* and *Cladosporium*. The amplified PKS gene sequences represented several functional genes with proposed involvement in synthesis of diverse and complex chemical structures. Thus, we propose screening of microbes for polyketide biosynthetic pathways can help us identify potential candidate microbes for production of novel bioactive molecules. In our study, variety of PKS genes detected supported the range and level of antimicrobial activity produced by the extracts of *C. roseus* endophytes. *C. roseus* endophytes are already of interest for natural product studies due to their potential to biosynthesize vinca alkaloids. However, the detection of novel PKS biosynthesis genes suggests that it would also be interesting to purify and characterize other classes of compounds that may account for the observed antimicrobial activity exhibited by extracts of these endophytes.

Competing Interests

The authors declare that they have are no competing interests.

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