Fungal Diversity (2010) 41:41–51
DOI 10.1007/s13225-010-0017-5

Endophytes from the pharmaceutical plant, *Annona squamosa*: isolation, bioactivity, identification and diversity of its polyketide synthase gene

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Received: 24 December 2009 / Accepted: 2 January 2010 / Published online: 5 February 2010

Abstract Endophytic fungi were isolated from the pharmaceutical plant *Annona squamosa* during different seasons and 131 strains were isolated. Fermentation extracts of each strain was assayed using six different screening programs for bioactive compounds. Sixty-three of the strains producing bioactive compounds were identified by ITS rDNA sequence data. The diversity of the polyketide synthase (PKS) genes of these 63 strains was investigated using three pairs of primers, LC1-LC2c, LC3-LC5c and KS3-KS4c. Phylogenetic analysis showed that these fungi are distributed in 10 orders, in 19 genera, mostly in the orders *Diapothales* and *Hypocreales*. The groups with the highest bioactivity were from *Diapothales*, *Clavicipitales* and *Xylariales*. The relationship between the taxa producing bioactive compounds and the PKS genes indicates that 11 out of the 63 strains analysed contained all three KS domains. Eight of these belonged to the order *Diapothales*. Twenty mostly *hypocrealean* and *phyllachoralean* strains lacked a single KS domain in all three pairs of primers amplified. Two important conclusions can be drawn from this investigation. The endophytic fungi of *A. squamosa* contain a community with a high degree of bioactivity: Secondly, compared with other fungi, those taxa from the order *Diapothales* have a rich species diversity and rich PKS gene domains. These findings suggest that they have a high potential as producers of natural bioactive compounds.

Keywords *Annona squamosa* · Bioactivity · Endophytic fungi · Identification · PKS gene

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Introduction

Biodiversity of endophytic fungi in tropical hosts

Endophytic fungi can be found in virtually all terrestrial plants (Hyde and Soytong 2008) and are viewed as an outstanding source of natural bioactive products. This is because endophytes are abundant and occupy a plethora of unique biological niches, e.g higher plants (Strobel and Daisy 2003), pteridophytes (Sati et al. 2009) and lichens (Li et al. 2007). They are also apparent in many unusual environments, such as orchid roots, terrestrial and marine grasses and algae (Strobel and Daisy 2003; Raghukumar 2008; Sánchez Márquez et al. 2008; Tao et al. 2008). Until recently most research on endophytes has been carried out using plants from temperate regions (Azevedo et al. 2000). However tropical plants also support a great diversity of endophytic microorganisms, thus forming an important component of the overall global fungal biodiversity (Hawksworth and Rossman 1997; Fröhlich and Hyde 1999; Hyde et al. 2006). Many of the endophytes have not yet classified and possibly belong to new genera and species (Azevedo et al. 2000; Duong et al. 2006; Mitchell et

al. 2008). On the basis of these observations, Dreyfuss and Chapela (1994) estimated that 1.3 millions species of endophytes might await discovery.

Tropical plants that have been studied for endophytes include tropical palms (Frölhich et al. 2000; Taylor and Crous 1999; Rungjindamai et al. 2008), tropical fruit trees (Azevedo et al. 2000), bananas (Photita et al. 2001), *Amomum siamense* (Bussaban et al. 2001) and teak trees (Chareprasert et al.2006). Gond et al. (2007) studied the endophytic fungi from different parts of *Aegle marmelos* (*Rutaceae*) from Varanasi (India) and Lin et al. (2005) Xu et al. (2009) on mangrove plants. Recent studies have included medicinal plants: *Rhizophora apiculata* (Kumaresan and Suryanarayanan 2002); and *Camptotheca acuminata* (Lin et al. 2007), three *Artemisia* species (Huang et al. 2009) and 29 traditional Chinese medicinal plant species) Huang et al. (2008).

In temperate as well as in tropical climate, endophytes form an almost untapped reservoir of fungal diversity. In all taxonomic and ecological groups examined to date a comparable degree fungal diversity was found to be equal in temperate and tropical regions or was higher in the tropics (Fröhlich and Hyde 1999).

Nature products and PKS-genes as a indicator of chemical diversity

Infectious diseases remain a global problem because of the development and spread of drug-resistant pathogens (Pillay and Zambon 1998). As a result the study of endophytic fungi as potential sources of novel natural bioactive products has intensified (Huang et al. 2001; Strobel 2002; Stinson et al. 2003).

Fungal PKSs are defined as iterative type I synthases and always contain regions highly homologous to sequences encoding β-ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) (Amnuaykanjanasin et al. 2005).

Bingle et al. (1999) designed LC primers based on the highly conserved KS sequence and amplified KS sequence with the LC primers in order to serve as homology probes to KS domains and to be able to investigate new fungal PKSs. This LC primer method had limitations, as Nicholson et al. (2001) found an isolation method for fungal PKSs based on the LC primer method, which was named KS and MT primer method. The method classified the fungal PKSs into three groups based on the extent of reduction of the polyketide ring; namely non-reduced (NR), partially reduced (PR) and highly reduced PKSs (HR).

The modular polyketide synthase (PKS) gene has been found to be involved in natural product syntheses in many microorganisms (Zhao et al. 2008). The study of PKS gene diversity in natural environments may provide important ecological insights, in addition to opportunities for antibacterial drug development (Zhao et al. 2008).

Annona squamosa

Many members of the *Annonaceae* are used in folk medicine for anti-parasitic or anti-tumoral treatment of intestinal diseases. However, the phytochemical properties have not been studied extensively due to its distribution in tropical or subtropical areas. In recent years, many novel structures and a wide range of bioactivities were discovered (Araya 2004).

Annona squamosa (custard apple) is a native species of the West Indies and was introduced to Taiwan during the 17th century. This plant is reputed to possess several medicinal properties (Asolkar et al. 1992). The young leaves of *A. squamosa* along with five grains of black pepper are used extensively for its anti-diabetic activity by tribal men in and around the villages of the Aligarh district, India (Atique et al. 1985). The aqueous leaf extract has also been reported to ameliorate hyperthyroidism (Sunanda and Anand 2003). The ethanolic leaf and stem extracts are reported to have an anti-cancerous effect (Bhakuni et al. 1969). Their seeds are reported to have insecticidal and abortifacient properties (Chopra et al. 1956). The bark of *Annona squamosa* yielded three new mono-tetrahydrofuran (THF) rings called acetogenins (Hopp et al. 1997). Annonaceous tetrahydrofuranic acetogenins have attracted much interest due to their broad range of biological activities (Alali et al. 1999).

It is therefore important to study the endophytic fungi isolated from *Annona squamosa*, because they may take part in the synthesis or transformation of metabolic products (Araya et al. 1994a, b, 2002; Araya 2004). In this paper, the isolation, bioassays for six types of bioactivity, and the identification and diversity of the PKS genes of endophytic fungi from *Annona squamosa* are investigated.

Materials and methods

Collection of plant material

Leaves, twigs, bark and roots of three *Annona squamosa* trees, two from Xiamen University, and one from the Xiamen Wanshi Arboretum, all originating from Fujian Province in southeast China were collected in four different months: June, October and December of 2005 and May 2006 in order to increase endophyte isolate biodiversity. The leaves (6~8×4~4.5 cm, length × width), twigs (8~10×0.3~0.5 cm, length × diameter), bark (6~7×2~3×0.5~1.2 cm, length × width × thickness) and

roots (5~6×0.2~0.4 cm, length × diameter) were tagged, placed in separate plastic bags and brought back to the laboratory.

Isolation, identification and phylogenetic analyses of the endophytic fungi

The isolation of the endophytic fungi was performed by the method described by Huang et al. (2001) within 24 h. ITS rDNA sequence data was used to characterize some bioactive fungi. The ITS1-5.8S-ITS2 region of fungi genomic DNA were amplified by primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAG TAAAAGTCGTAACAAGG-3′) then the PCR products were sequenced by Invitrogen Co., Shanghai and sequences were compared with those in GenBank. The data were analysed by DNAMAN software with *Tube aestivum* (GenBank Accession No: AJ888127) as the outgroup.

Fermentation and preparation of the fungi extraction

The endophytic fungi were cultured in PDA solid medium for 7~14 days at 28°C. The cultures were extracted using mixtures of 80% ethyl acetate, 15% methanol and 5% acetic acid. The liquid fungal extraction was evaporated to yield a crude solid extraction. This was dissolved by methanol and filtrated through a filter paper to remove residues that are not dissolved in methanol: then the filtrate was diluted into 10 mg/mL with methanol.

Determination of anti-microbial and anti-oxidation activities

The determination of anti-microbial activity was based on the disk diffusion method (Kronvall and Ringertz 1991). Each disc (filter paper, diam. 6 mm) contained 100 μg of endophytic fungi extraction (10 mg/mL). The indicator organisms included *Bacillus subtilis* ATCC 9372 (BS), *Escherichia coli* ATCC 25922 (EC), *Staphylococcus aureus* ATCC 25923 (SA), *Candida albicans* As 2.538 (CA) and *Aspergillus niger* ACCC 30005 (AN).

The determination of anti-oxidation activity was carried out according to the method of Chen et al. (2006) but the absorbance was measured at 517 nm.

Determinations of trypsin inhibitory and acetylcholinesterase inhibitory activities

The determination of trypsin inhibitory activity was performed by the method described by Lin et al. (2007).

The reaction system of the determination of acetylcholinesterase inhibitory activity containing 40 μL Tris-HCl buffer (pH 8.5, 0.1%), 20 μL enzyme (AchE, activity: 0.025 U/mL, Sigama Co.) and 20 μL endophytic fungal extraction and incubated at 37°C for 15 min. Then 20 μL of 2.5 mM Acetylthiochoine iodide(ACTI) and 100 μL of 0.5 mM 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) were added. The absorbance of the mixtures was measured at 405 nm immediately with a microplated reader (M+3550, Bio-rad), and incubated at 37°C for 30 min. Fifty μL of anhydrous alcohol was added to stop the reaction and its absorbance was measured at 405 nm. The reaction system without enzyme and endophytic fungal extraction was regard as blank control, while the system without endophytic fungal extraction was regarded as the negative control. Glatamin was used as the positive control. The inhibition rate was calculated by the same equation as that of the trypsin inhibitory activity (Lin et al. 2007).

Determination of brine shrimp inhibitory activity

The eggs of brine shrimps were incubated in seawater at 25°C for 48 h. Endophytic fungi extractions were added into the wells with 100 μL seawater containing 20 brine shrimps [methanol (*v/v*) <1%]. The reaction system was incubated at 25°C and the number of dead brine shrimps was recorded after 24 h. The seawater containing 20 brine shrimps served as negative control. Avermectins were used as positive control. The inhibition rate was calculated and IC_{50} was defined as concentration of crude extract that led to the death of at least 50% of the brine shrimps.

Determination of anti-tumor activity

Human tumor cell lines Raji and HepG2 were obtained from the Cell Line Bank of the the Chinese Academy of Science. The determination of anti-cancer activity was based on the MTT assay which was first described by Mosmann to quantitate mitochondrial dehydrogenase activity (Mosmann 1983). Cisplatin was used as the positive control.

Amplification of ketosynthase domain sequence from endophytic fungal PKS gene

Three pairs of primers, LC1 and LC2c (Bingle et al. 1999), LC3 and LC5c (Bingle et al. 1999), KS3 and KS4c (Nicholson et al. 2001), which are ketosynthase domain specific primers were used to amplify the KS domain sequence of the PKS genes of endophytic fungi DNA by PCR. PCR reactions (50 μL) contained approximately 4 μL genomic DNA template, 5 μL 10 × PCR buffer, 4 μL 2.5 mM of each dNTP, 3 μL of each primer, 1 μL of 5 U/μL rTaq DNA polymerase and 30 μL distilled water. The thermal cycling program was as follows: 5 min at 94°C; 34 cycles of 1 min at 94°C, 1.5 min at 55°C, 3 min at 72°C and 10 min at 72°C.

Results

Endophytic fungi isolation from Annona squamosa

Leaves, twigs, bark and roots of *Annona squamosa* yielded 131 endophytic isolates from the different seasons (Table 1). Most (36.6%) of the endophytic fungi were from leaves, 27.5% from twigs, 16% from bark and 19.9% from roots, numbering 45 (34.4%), 43 (32.8%), 14 (10.7%) and 29 (22.1%) isolates from each plant organ, collected in June, October, December and May respectively.

Anti-microbial activity of endophytic fungi

The result of the anti-microbial activity test (Table 2) indicates that 50 strains (38.2%) displayed anti-microbial activity against at least one indicator organism. Fort-nine strains (37.4%) displayed anti-microbial activity against *Candida albicans,* 4 strains (3.1%) against *Aspergillus niger*, 22 strains (16.8%) against *Staphylococcus aureus*, 6 strains (4.6%) against *Bacillus subtilis* and 9 strains (6.9%) against *Escherichia coli*. Ten of these anti-microbial endophytic fungi isolated from different tissues of *Annona squamosa* originated from leaves, 11 from twigs, 11 from bark and 18 from roots. The 50 strains with anti-microorganism activity are distributed in at least 17 genera, most of them in *Phomopsis*, *Aspergillus*, *Fusarium* and *Diaporthe*.

Anti-oxidation, trypsin inhibition and acetylcholinesterase inhibitory activities

The results of the anti-oxidation, trypsin inhibitory and acetylcholinesterase inhibitory activities tests show that 15 strains (11.5%) display anti-oxidation (IC_{50} ≦100 μg/mL) (Fig. 1) and 4 strains (3.1%) trypsin inhibitory activities (IC_{50} ≦100 μg/mL) (Fig. 1), whereas acetylcholinesterase inhibitory activity was lacking. Endophytic fungi with anti-oxidation activity were mainly isolated from leaves (9 strains, 60%) with 2 from twigs, 3 from bark and 1 from roots, but endophytic fungi which had trypsin inhibitory activity were not tissue specific. The strains with anti-oxidation and trypsin inhibitory activities are distributed in at least 10 genera and 3 genera respectively.

Brine shrimp inhibitory activity and anti-tumor activity of endophytic fungi

Twenty-two (16.8%) of endophytic fungi displayed brine shrimp inhibitory activity (IC_{50} ≦500 μg/mL) (Fig. 1). Seven isolates were from leaves, 6 from twigs, 6 from bark and 3 from roots and were distributed in at least 10 fungal genera.

In the anti-tumor test (Table 3), 29 (22.1%) of the fungi displayed cytotoxic avtivity against tumor cells, 28 (21.4%) against Raji cells (IC_{50} ≦50 μg/mL), 10 (7.6%) against HEP-G2 cells (IC_{50} ≦100 μg/mL) and 9 (6.9%) against both Raji and HEP-G2 cells. These fungi with cytotoxic activity were mostly isolated from twigs (32.3%) and roots (34.4%). The anti-tumor positive strains were found in more than 10 genera and especially *Phomopsis*, *Fusarium* and *Diaporthe*.

Main bioactive strains

Sixty-three strains with bioactivity were chosen for ITS rDNA sequencing and phylogenetic analysis. The results are shown in Table 4 and Fig. 1 where the most related strains (96% to 100% similarity) in GenBank are shown. Very similar matches indicated that strain D7 was close to *Phomopsis phyllanthicola*, strain A41 to *Pestalotiopsis microspora*, strain B15 to *Glomerella cingulata* (100% similarity) and the other strains to corresponding genera (Fig. 1). Bioactive endophytic fungi isolated from *Annona squamosa*, which can be identified belonged to 18 taxa, while several of them can't be identified certainly. *Fusarium*, *Diaporthe*, *Phomopsis* species and *Aspergillus* species are the dominant bioactive genera comprising 23.8%, 15.8%, 11.1% and 8% of the bioactive endophytic fungi respectively.

Phylogenetic analysis of the endophytic fungi (Fig. 1) shows that their dominance is mostly confined to the endophytic orders Diapothales and Hypocreales.

Table 1 Number (N) and percentage (%) of endophytic fungi isolated from different tissues of *Annona squamosa* in different seasons

Time	Tissues				
	Leaves (%)	Twigs (%)	Barks (%)	Roots (%)	Total (%)
June, 2005	20(15.3)	9(6.9)	16(12.2)	0(0)	45(34.4)
October, 2005	21(16)	7(5.3)	0(0)	15(11.5)	43(32.8)
December, 2005	2(1.5)	8(6.1)	2(1.5)	2(1.5)	14(10.7)
May, 2006	5(3.8)	12(9.2)	3(2.3)	9(6.9)	29(22.1)
Total N (%)	48(36.6)	36(27.5)	21(16)	26(19.9)	131(100)

Table 2 The anti-microbial activities of the endophytic fungi isolated from *Annona squamosa* leaves, twigs, barks and roots

Taxa	Number of activity strains (%)	Anti-microbial activity (%)				
		CA	AN	SA	BS	EC
Punctularia sp.	1(0.8)	1(0.8)	0(0)	0(0)	0(0)	0(0)
Peniophora sp.	1(0.8)	1(0.8)	0(0)	0(0)	0(0)	0(0)
Guignardia sp.	2(1.5)	2(1.5)	0(0)	1(0.8)	0(0)	1(0.8)
Phomopsis sp.	6(4.6)	6(4.6)	1(0.8)	1(0.8)	1(0.8)	1(0.8)
Diaporthe sp.	4(3.1)	4(3.1)	0(0)	2(1.5)	2(1.5)	0(0)
Cordyceps sp.	1(0.8)	1(0.8)	0(0)	1(0.8)	1(0.8)	0(0)
Fusarium sp.	15(11.5)	15(11.5)	0(0)	10(7.6)	0(0)	7(5.3)
Nectria sp.	1(0.8)	1(0.8)	0(0)	0(0)	0(0)	0(0)
Paecilomyces sp.	1(0.8)	1(0.8)	1(0.8)	1(0.8)	0(0)	0(0)
Codinaeopsis sp.	1(0.8)	0(0)	1(0.8)	0(0)	0(0)	0(0)
Pestalotiopsis sp.	1(0.8)	1(0.8)	0(0)	1(0.8)	0(0)	0(0)
Xylaria sp.	1(0.8)	1(0.8)	0(0)	1(0.8)	1(0.8)	0(0)
Annulohypoxylon sp.	1(0.8)	1(0.8)	0(0)	1(0.8)	0(0)	0(0)
Eupenicillium sp.	1(0.8)	1(0.8)	0(0)	0(0)	0(0)	0(0)
Penicillium sp.	1(0.8)	1(0.8)	0(0)	1(0.8)	0(0)	0(0)
Aspergillus sp.	5(3.8)	5(3.8)	0(0)	0(0)	0(0)	0(0)
Pyrenochaeta sp.	1(0.8)	1(0.8)	1(0.8)	1(0.8)	0(0)	0(0)
The others	6(4.6)	6(4.6)	0(0)	1(0.8)	1(0.8)	0(0)
Total N (%)	50(38.2)	49(37.4)	4(3.1)	22(16.8)	6(4.6)	9(6.9)

CA *Candida albicans* As 2.538, AN *Aspergillus niger* ACCC 30005, SA *Staphylococcus aureus* ATCC 25923, BS *Bacillus subtilis* ATCC 9372, EC *Escherichia coli* ATCC 25922

PCR amplification of KS domain by three pairs of ketosynthase domain specific primers

The DNA of 63 bioactive strains were amplified by three ketosynthase domain specific primers pairs, LC1-LC2c, LC3-LC5c and KS3-KS4c (Fig. 1). PCR products were amplified by the LC1-LC2c primers from 17 (27%) strains, 0 strains by the LC3-LC5c primers, 1 strain (1.6%) by the KS3-KS4c primers, 6 strains (9.5%) by the LC1-LC2c and LC3-LC5c primers, 5 strains (7.9%) by the LC1-LC2c and KS3-KS4c primers, and 2 strains (3.2%) by the LC3-LC5c and KS3-KS4c primers. DNA was amplified from (17.5%) 11 strains by the three primer pairs, while in 21strains (33.3%) amplification failed.

Figure 1 illustrates the relationship between the bioactive taxa and presence of PKS genes. All 3 KS domains were present in 11 strains of which 8 belong to the Diaporthales. On the other hand 20 strains mainly attributed to the Hypocreales and Phyllachorales did not contain a single KS domain at least in all three pairs of primers amplified.

Discussion

The biodiversity of the endophytic fungi isolated from Annona squamosa

"Of the myriad of ecosystems on earth, those having the greatest biodiversity seem also to be those with the most endophytes and biodiverse microorganisms" (Strobel and Daisy 2003). *Annona squamosa* with its high endophyte biodiversity is a good example.

When blasted in GenBank, strain A23 was dissimilar to most fungi, being 71% similar to *Phialemonium sp.* (CBS 111658). This low similarity indicates that strain A23 may be a new genus, or alternatively if already described, the genus has yet to be sequenced. For this reason strain A23 does is not represented in Fig. 1, however, it does produce natural bioactive products.

Another peculiar specimen is *Cordyceps sinensis*, "Winter worm and summern grass" which is one of the most valued traditional Chinese medicines that reputedly improves immunity, increases longevity and is an anti-cancer agent (Paterson 2008). The taxon is an entomopathogenic fungus and its natural lepidopteran host is *Hepialus armoricanus* (Li et al. 2008) and it has rarely been isolated as an endophyte (Wang 2008). Most endophytic fungi are ascomycetes (Rungjindamai et al. 2008), and their association with plants has been extensively researched. Murali et al. (2007) worked on endophytic communities of 15 different hosts, Photita et al. (2001) on endophytes of wild banana, Frohlich et al. (2000) on endophytes of palms and Vujanovic and Brisson (2002) on endophytes of *Acer saccharum* and most fungi were ascomycetes or their anamorphs. Endophytic ascomycetes were also isolated by Espinosa-Garcia and Langenheim (1990) from *Sequoia semperoirens* in California and Suryanarayanan et al. (2005) from Arizona

cactus as well as Murali et al. (2007) from two tropical forests of southern India.

Endophytes do not appear to be very specific as far as tropical hosts are concerned. Kumaresan and Suryanarayanan (2001) for instance found that among the ascomycetes from mangroves, only a single Basidiumycetes species, *Trimmastroma* was found. Lin et al. (2007) isolated *Blastomycetes* from *Camptotheca acuminate*. Rubini et al. (2005) isolated two Basidiomycete species, *Blastomyces* sp. and *Pleurotus* sp. among 23 genera of endophytic fungi from *Theobroma cacao*.

The present paper also confirms the existence of two Basidiomycete species *Peniophora* sp. and. *Punctularia* sp. as endophytes of *Annona squamosa*. The results of endophytic studies have generally shown that diversity of endophytic fungi is greater in the tropics than in temperate regions (Cannon and Hawksworth 1995).

In this paper 10 orders of endophytic fungi, comprising 19 genera have been identified and phylogenetically analysed. Most of the bioactive endophytic strains analysed in this paper belong to the orders of *Diapothales*, *Clavicipitales* and *Xylariales*.

The biodiversity of the endophytic fungi isolated from *Annona squamosa* also depends on season. In June and October when *Annona squamosa* grows very well in Xiamen, the fungi of the plant investigated displayed great biodiversity containing at least 14 genera. In December, when the tree begins to shed its leaves and in May, when it buds, the biodiversity of its endophytic fungi, distributed among several common genera, such as *Penicillium* species, *Fusarium* species, *Aspergillus* species decreased. This may be because endophytes occur in leaves (Fisher et al. 1995; Carrol 1998). Compared with the endophytic fungi isolated from leaves, twigs, bark and roots of *Annona squamosa*, the fungi from leaves showed a greater biodiversity than the others. *Diaporthe* species were mostly distributed in leaves and twigs, those of the *Fusarium* species in roots.

Bioactive diversity of the endophytic fungi isolated from Annona squamosa

Anona squamosa owes its pharmarcological reputation to the fact that it produces bioactive compounds in its leaves (Bhakuni et al. 1969; Kotkar et al. 2002; Kaleem et al. 2006), twigs (Bhakuni et al. 1969), seeds (Hopp et al. 1996) and fruits (Wu et al. 1996). It was most interesting to observe that the endophytic fungi from this plant also exhibited bioactivity. The fungal bioactivity assays showed that the endophytes displayed broad anti-microbial, anti-oxidation, anti-tumor, trypsin inhibitory and brine shrimp inhibitory activities (Fig. 1).

The endophytic fungi isolated from leaves for instance displayed good anti-microbial activity; the leaves too were the main hosts to fungi displaying anti-oxidantion activity; most strains with this activity however were from the roots. Four strains with a high brine shrimp inhibitory activity were isolated from bark. The endophytes with anti-tumor activity originated from leaves, bark and roots, but mainly from the latter two. Four strains out of seven which had trypsin inhibitory activity were isolated from twigs. In general fungi with bioactivity were mostly distributed in roots.

Fig. 1 Phylogenetic relationship of bioactive fungi isolated from *Annona squamosa* to other fungi from GenBank, deduced from ITS rDNA sequences. And the bioactivity of bioactive fungi, the amplification of the KS domain of bioactive fungi DNA using three pairs of primers, LC1-LC2c, LC3-LC5c, KS3-KS4c. The numbers at branches indicate the percentages of trees from 1,000 bootstrap replication in which the branch occurs. ■ display bioactivity. □ didn't display bioactivity. ● PCR amplification products displayed 700 bp DNA bands. ○ PCR amplification products didn't display 700 bp DNA bands

Some strains displayed especially high bioactivity, for example strain B27 displayed antagonism against all indicator organisms, and thus is a candidate for studying the structures of the active substances. Strain A45 whose IC_{50} for anti-oxidation activity is 9 μg ml^{-1} is worthy of further study for many of the complications of diabetes mellitus that have been linked to oxidative stress and could be treated by antioxidants (Cunningham 1998). Four strains with trypsin inhibitory activity whose IC_{50} are between 25.4 μg ml^{-1} to 81.4 μg ml^{-1} are also worthy of further study, because the trypsin inhibitor plays a crucial role related to physiological functions connected with blood coagulation, the complementing of pathways, viral maturation and cancer. This approach may lead to the development of innovative therapies for life-threatening diseases (Yang and Craik 1998).

Cheema et al. (1985) reported on the use of *Annona squamosa* extracts as insecticidal agents. Therefore the natural products of the four high activity strains (IC_{50} ≦100 μg/mL) with brine shrimp inhibitory activity might be useful as insecticidal agents. Among all the strains with cytotoxity, B27 (*Phomopsis* sp.), B36 (*Pyrenochaeta* sp.), B37 (*Eupenicillium* sp.) and C6 (*Paecilomyces* sp.) display high activities having been tested with the Raji cell line, their IC_{50} were lower than 5 μg/mL. When tested with the HEP-G2 cell line the IC_{50} of B36 and B37 reached about 20 μg/mL. Most of the bioactive endophytic strains analysed in this paper belong to the orders *Diapothales*, *Clavicipitales* and *Xylariales*. They are promising organisms for research on bioactive nature products.

The findings of this study extend Strobel's and Daisy's (2003) general assumption regarding endophytic fungi as a source of bioactive nature products in plants. The tissues and fungi of this outstanding plant may very well harbour a

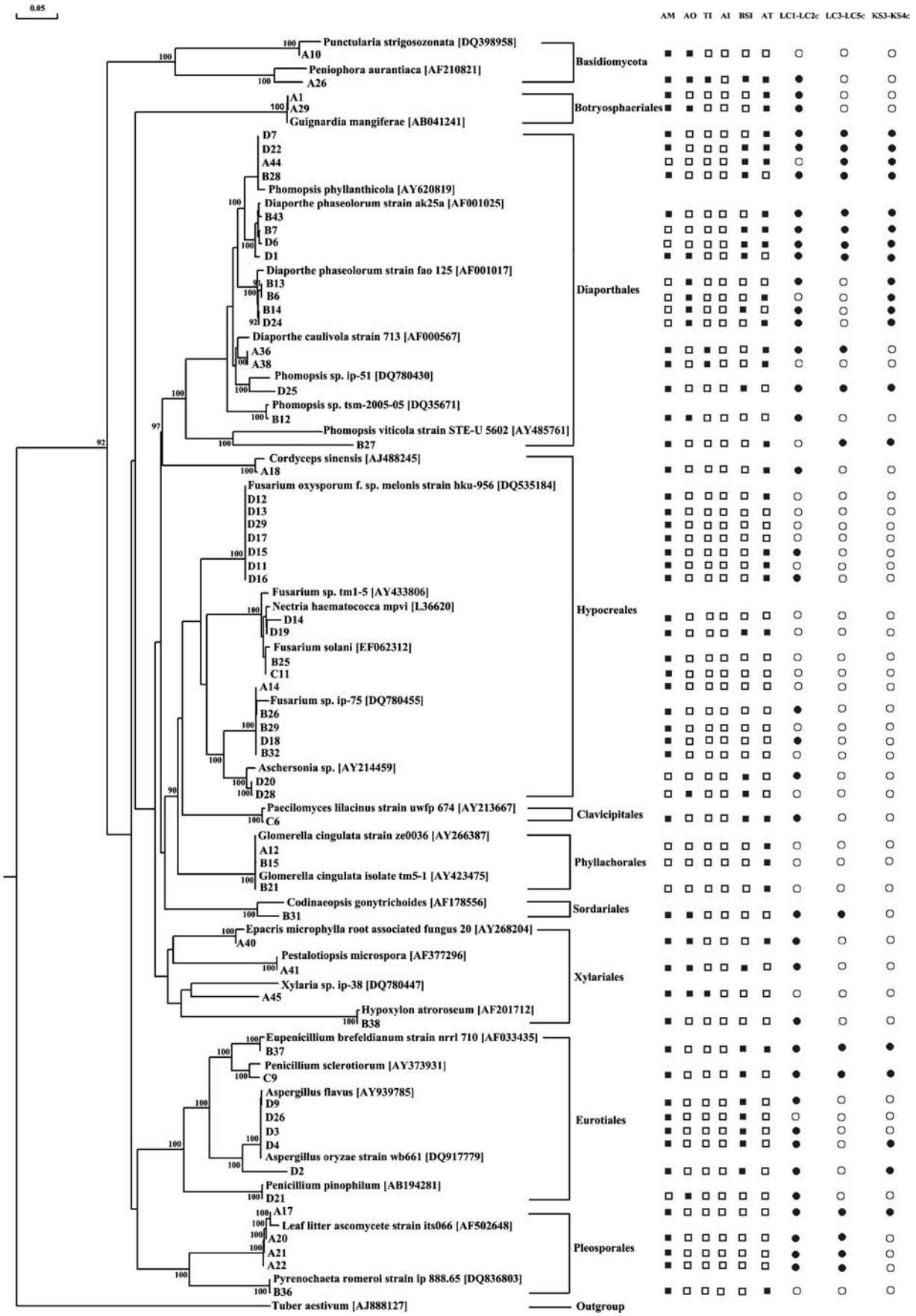
0.05
AM AO TI AI BSI AT LC1-LC2c LC3-LC5c KS3-KS4c
Punctularia strigosozonata [DQ398958]
A10
Peniophora aurantiaca [AF210821]
A26
Basidiomycota
A1
A29
Guignardia mangiferae [AB041241]
Botryosphaeriales
D7
D22
A44
B28
Phomopsis phyllanthicola [AY620819]
Diaporthe phaseolorum strain ak25a [AF001025]
B43
B7
D6
D1
Diaporthe phaseolorum strain fao 125 [AF001017]
B13
B6
B14
D24
Diaporthales
Diaporthe caulivola strain 713 [AF000567]
A36
A38
Phomopsis sp. ip-51 [DQ780430]
D25
Phomopsis sp. tsm-2005-05 [DQ35671]
B12
Phomopsis viticola strain STE-U 5602 [AY485761]
B27
Cordyceps sinensis [AJ488245]
A18
Fusarium oxysporum f. sp. melonis strain hku-956 [DQ535184]
D12
D13
D29
D17
D15
D11
D16
Fusarium sp. tm1-5 [AY433806]
Nectria haematococca mpvi [L36620]
D14
D19
Fusarium solani [EF062312]
B25
C11
A14
Fusarium sp. ip-75 [DQ780455]
B26
B29
D18
B32
Aschersonia sp. [AY214459]
D20
D28
Hypocreales
Paecilomyces lilacinus strain uwfp 674 [AY213667]
C6
Clavicipitales
Glomerella cingulata strain ze0036 [AY266387]
A12
B15
Glomerella cingulata isolate tm5-1 [AY423475]
B21
Phyllachorales
Codinaeopsis gonytrichoides [AF178556]
B31
Sordariales
Epacris microphylla root associated fungus 20 [AY268204]
A40
Pestalotiopsis microspora [AF377296]
A41
Xylaria sp. ip-38 [DQ780447]
A45
Hypoxylon atroroseum [AF201712]
B38
Xylariales
Eupenicillium brefeldianum strain nrrl 710 [AF033435]
B37
Penicillium sclerotiorum [AY373931]
C9
Aspergillus flavus [AY939785]
D9
D26
D3
D4
Aspergillus oryzae strain wb661 [DQ917779]
D2
Penicillium pinophilum [AB194281]
D21
Eurotiales
A17
Leaf litter ascomycete strain its066 [AF502648]
A20
A21
A22
Pyrenochaeta romeroi strain ip 888.65 [DQ836803]
B36
Pleosporales
Tuber aestivum [AJ888127]
Outgroup

Table 3 The anti-tumor activity of the endophytic fungi isolated from *Annona squamosa* leaves, twigs, bark and roots

Taxa	Number of activity strains (%)	Anti Raji activity IC_{50} (μg ml^{-1})			Anti HEP-G2 activity IC_{50} (μg ml^{-1})			
		50~25	25~10	≤10	100~70	70~40	40~10	≤10
Peniophora sp.	1(0.8)	1(0.8)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Guignardia sp.	2(1.5)	2(1.5)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Phomopsis sp.	4(3.1)	1(0.8)	2(1.5)	1(0.8)	0(0)	0(0)	1(0.8)	0(0)
Diaporthe sp.	7(5.3)	4(3.1)	0(0)	2(1.5)	2(1.5)	0(0)	0(0)	0(0)
Cordyceps sp.	1(0.8)	0(0)	1(0.8)	0(0)	0(0)	0(0)	0(0)	0(0)
Fusarium sp.	6(4.6)	6(4.6)	0(0)	0(0)	1(0.8)	1(0.8)	0(0)	0(0)
Paecilomyces sp.	1(0.8)	0(0)	0(0)	1(0.8)	0(0)	0(0)	0(0)	0(0)
Glomerella sp.	3(2.3)	1(0.8)	2(1.5)	0(0)	0(0)	1(0.8)	0(0)	0(0)
Eupenicillium sp.	1(0.8)	0(0)	0(0)	1(0.8)	0(0)	0(0)	1(0.8)	0(0)
Pyrenochaeta sp.	1(0.8)	0(0)	0(0)	1(0.8)	0(0)	0(0)	1(0.8)	0(0)
The others	2(1.5)	1(0.8)	0(0)	1(0.8)	1(0.8)	1(0.8)	0(0)	0(0)
Total N (%)	29 (22.1)	16(12.2)	5(3.8)	7(5.3)	4(3.1)	3(2.3)	3(2.3)	0(0)
		28(21.4)			10(7.6)			

process of interaction enhancing the generation of such bioactive nature substances of utmost pharmacological importance (Asolkar et al. 1992). This neglected aspect of bioactivity of an endophytic fungi population prompts further study.

The diversity of the polyketide synthase genes

Polyketides represent a large group of structurally diverse secondary metabolites, exhibiting a wide array of pharmacologically important activities. Fungal PKSs are defined as

Table 4 The taxa with anti-microbial, anti-oxidation, trypsin inhibitory, brine shrimp inhibitory and anti-tumor activities

Taxa	Number of activity strains (%)	Bioactivity						
		AM	AO	TH	AH	BSH	AT	
							Raji	HEP-G2
Punctularia sp.	1(1.6)	1	1	0	0	0	0	0
Peniophora sp.	1(1.6)	1	1	1	0	1	1	0
Guignardia sp.	2(3.2)	2	1	0	0	0	2	0
Phomopsis sp.	7(11.1)	6	1	0	0	4	4	1
Diaporthe sp.	10(15.8)	4	5	2	0	4	6	2
Cordyceps sp.	1(1.6)	1	0	0	0	0	1	0
Fusarium sp.	15(23.8)	15	0	0	0	1	6	2
Nectria sp.	1(1.6)	1	0	0	0	0	0	0
Aschersonia sp.	2(3.2)	0	1	0	0	2	0	0
Paecilomyces sp.	1(1.6)	1	0	0	0	1	1	0
Glomerella sp.	3(4.8)	0	0	0	0	0	3	1
Codinaeopsis sp.	1(1.6)	1	1	0	0	0	0	0
Pestalotiopsis sp.	1(1.6)	1	1	0	0	1	0	0
Xylaria sp.	1(1.6)	1	1	1	0	0	0	0
Annulohypoxylon sp.	1(1.6)	1	0	0	0	0	0	0
Eupenicillium sp.	1(1.6)	1	0	0	0	1	1	1
Penicillium sp.	2(3.2)	1	1	0	0	1	0	0
Aspergillus sp.	5(8)	5	0	0	0	5	0	0
Pyrenochaeta sp.	1(1.6)	1	0	0	0	0	1	1
The others	6(9.5)	5	1	0	0	1	2	2
Total	63(100)	49	15	4	0	22	28	10

AM anti-microbial activity, *AO* anti-oxidation activity, *TI* trypsin inhibitory activity, *AI* acetylcholinesterase inhibitory activity, *BSI* brine shrimp inhibitory activity, *AT* anti-tumor activity

iterative type I synthases and are classified into three groups based on the extent of reduction of the polyketide ring produced, namely non-reduced (NR), partially reduced (PR) and highly reduced PKSs (HR) (Nicholson et al. 2001).

The bioactive endophytic fungi isolated from *Annona squamosa* are all distributed in the subphylum *Pezizomycotina.* In order to screen the bioactive fungi carrying iterative type I PKS, we used three pairs of primers, LC1-LC2c, LC3-LC5c and KS3-KS4c which are specific for the particular types of the, non-reduced, partially reduced and highly reduced KS domains of endophytic fungal polyketide. The result showed that 63 bioactive endophytic fungi displayed a good diversity of KS domain (Fig. 1). When compared with each other the non-reduced KS domain had the largest number of strains. In addition, the analysis of the relationship between the taxa and the diversity of the fungal PKS genes revealed that in the eleven strains which carried NR, PR and HR KS domains, eight belonged in the fungi order *Diaporthales,* whereas the strains without the three kinds of KS domain mentioned mostly belonged to the *Hypocreales* and *Phyllachorales*. We also clarified for example that D3, D4, D9 and D6 are all *Aspergillus oryzae*, but their PKS genes are different. This result further confirms the opinion that the PKS dendrograms do not reflect the phylogeny of the organisms, although PKS genes from related species occasionally form a clade and a single species may contain several different PKS-like sequences with disparate evolutionary histories (Lee et al. 2001).

This investigation however was not an exhaustive analysis since only a few primer pairs were tested. With small changes in the primer structure, those isolates that were negative might also contain KS domains when reanalysed (Lee et al. 2001).

The important conclusion PKS genes are common among the endophytic fungi of *Annona squamosa*. This confirms that fungal species have many *PKS* genes (Kroken et al. 2003). Further research on the PKS diversities in natural environments may in addition to opportunities for drug developments provide important ecological insights (Zhao et al. 2008), because fungal genomes predict that an astonishingly large number of PKs are produced by the subphylum of *Pezizomycotina* (Kroken et al. 2003).

Acknowledgements This work was supported by grands from project (F) 2007DFA30970 of the Ministry of Science and Technology.

We are grateful for the support of The National High Technology Research and Development Program of China (2007AA091503).

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