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Identification of Endophytic Fungi Isolated from Licorice, A Traditional Chinese Medicine Plant

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Licorice is the most important herb used in the traditional Chinese (TCM) medicine. It was used to treat individuals with gastric or duodenal ulcers, bronchitis, cough, arthritis, adrenal insufficiency and allergies. The active substances and secondary metabolites from Licorice roots may be associated with a specific microbial agent. It is believed that many endophytic fungi from medically important plants can produce the same or similar secondary metabolites as the plant. Endophytic fungi are also thought to play an important role in plant communities by increasing fitness of the host plant. To clarify whether endophytic fungi are found in the licorice, fungi were isolated from Licorice root and characterized morphologically and using molecular identification. The morphological examination showed that the endophytic fungi from Licorice were *Aspergillus* and *Chaetomium* species. Further identification was achieved by sequence similarity comparison and phylogenetic analysis of the ITS regions. Results showed that strains L10L2 and L4 belong to *Aspergillus* species, L3 identified to *Chaetomium*. A neighbor-joining tree showed the relationships between the isolate's sequence data and the closest identified relatives from GenBank. These fungi could have significance as a source of pharmaceutical natural products.

Key words: *Aspergillus spp*, *Chaetomium spp*, Gan-Cao, *Glycyrrhiza* spp.

Licorice, or Gan-Cao, the roots of *Glycyrrhiza* spp., is one of the most popular and widely consumed herbs in the world. It has been used for more than 4 millennia as a medicine to treat gastric or duodenal ulcers, sore throat, bronchitis, cough, arthritis, adrenal insufficiency, and allergies (Fintelmann 1991; Carbonell-Barrachina *et al*., 2003; Kamei *et al*., 2004; Haggag *et al*., 2003). It is also used as a flavoring agent in foods, beverages, and tobacco due to its sweet taste. Licorice is extensively used in the traditional Chinese medicine (TCM) prescriptions, and is the second most prescribed herb in China following Ginseng (Miller 1998). One of the major active components of licorice is glycyrrhizin (GC). It also has many other components, such as flavonoids, isoflavonoids, chalcones, sugars, starches, sterols, amino acids, gums, essential oil and triterpene saponins (Asl and Hosseinzadeh 2008, Chin 2007). It has been reported to display various pharmaceutical functions, including anti-cancer, anti-inflammation, anti-ulcer, anti-tumor, anti-depressive, antivirus, hepatoprotective, anti-allergy, liver function improvement, anti-oxidative, anti-microbial, superoxide scavenging, anti-spasmodic, antidiabetic, anti-carcinogenic, expectorant and memory enhancing activities (Kim *et al*., 2006; Visavadiya and Narasimhacharya 2006;

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Ahn *et al*., 2006; Shin *et al*., 2007; Mae *et al*., 2003; Jo *et al*., 2005; Leung *et al*., 2003, Dhingra and Sharma 2006, Dhingra *et al*., 2004, Chen *et al*., 2004, Cinatl *et al*., 2003, Ko *et al*., 2007).

In recent years, scholars have isolated endophytic fungi and bacteria from in a variety of medicinal plants (Sun *et al*., 2008; Chen *et al*., 2008; Huang *et al*., 2008). Endophytic fungi are microorganisms that live inside the tissues and organs of healthy plants without causing discernible manifestation of disease (Strobel 2003). They play important roles in the process of host plant growth and systematic evolution (Tan and Zou 2001; Saikkonen *et al*., 2004). Endophytic fungi are often not only involved in the synthesis and transformation of plant secondary metabolites, but also can produce abundant secondary metabolites independently. Those secondary metabolites have antibacterial, antiviral, anti-tumor, anti-oxidation, immune enhancer activity, which is becoming one of the most important sources of natural products and are highly valuable as new types of biological resources (Yao and Wei 2011; Aly *et al*., 2010, Jalgaonwala 2011; Tenguria 2011; Gunatilaka 2006). Isolating endophytic fungi from medicinal plants and screening determine which can synthesizes active products and secondary natural products could help replace the lack of wild medicinal plant resources. It is not known if Licorice contains endophytic fungi and if so whether they can synthesize the active substances and secondary metabolites found in the plant.

Recently, endophytic fungi of medicinal plants have aroused great interest from industry. These fungi not only can produce the same or similar active substances as the host plants, but also some endophytic fungi of medicinal plants are entirely responsible for the active products produced (Lin *et al*., 2007; Ganley *et al*., 2004; Deng *et al*., 2004; Zhang *et al*., 2009; Liu *et al*., 2009; Karen *et al*., 2003). Other medicinal plants can-not synthesize the medicinal ingredients without the endophytic fungi. Yet other medicinal plants in arid and semi-arid regions, contain endophytic fungi that use fermentation to produce medicinally materials. Helping solve the lack of medicinal plants is a new research direction and current drug research hot spot.

This work was done to determine if endophytic fungi exist in the root of licorice and their distribution. A licorice planting in (Kaifeng, Henan Province, China) was used for isolation and culture of endophytic fungi. The isolated endophytic fungi were identified based on morphological observation, mycelial morphology, spore morphology and structure, ITS sequence comparison and phylogenetic analysis.

MATERIALS AND METHODS

Plant material

Healthy Licorice root were collected from Jinming (Kaifeng field, Henan province, China) and stored in sterile polythene bags in the laboratory at 4°C for isolation of endophytic fungi.

Isolation of endophytic fungi

Fungal isolation was conducted within 12 h of sample collection. Isolation of the endophytic fungi was performed based on the procedures described previously by (Braun *et al*., 2003).The collected samples were washed under running water thoroughly and then air-dried. The cleaned roots were surface-sterilized as follows: 75% ethanol (v/v) for 30 s, 20% bleach for 3 min (v/v), sterilized water for 30 s and dry on a sterilized paper towel. The sterilized samples were cut into pieces of about 1.0 cm2 and placed onto petri dishes containing water agar at 28 °C, 35% humidity for 2 wks to attain conidia production. Conidia were collected and transferred to Potato dextrose agar (PDA) medium (200 g potato, 20 g D-glucose, 15 g agar, 1000 ml deionized water) supplemented with penicillin (100 units/ml) and streptomycin (0.08 mg/ml) to inhibit bacterial contamination and was used for the isolation and purification of endophytic fungi.

Morphology characteristic of endophytic fungi

All fungal species that isolated from Licorice were examined periodically and identified based on the morphological characteristics of their colony culture spores. Sporulating isolates were identified morphologically to genus or species level. To examine the spore characteristics, the culture was transferred into a drop of 0.01% cotton blue in 60% lactic acid on a microscope slide using a sterile needle, covered with a cover slip, and observed using a light microscope.

DNA extraction

Genomic DNA was extracted from mycelium collected from 7 to 10 d fresh cultures

growing on PDA media plates, using the PlantGen DNA Kit (CWBIO, China). A total of 0.1 g fungal mycelium was ground to a fine powder in a mortar and pestle in liquid nitrogen. Powdered sample was transferred to a 2.0 ml microcentrifuge tube and processed according to the manufacturer's protocol. Isolated DNA from isolates was stored at -20 °C for further use. DNA samples were examined on 1.8 % agarose gel containing ethidium bromide. Total DNA was stored at -20 °C for further use.

PCR amplification and Sequencing

The ITS regions of the selected fungi were amplified by PCR. The PCR primers were universal primers ITS1 (52 -TCCGTAGGTGAACCTGCGG-32) and ITS4 (52 -TCCTCCGCTTATTGATATGC-32). PCR amplifications were performed in a reaction mixture containing 2×Taq Master Mix (SinoBio, China), DNA extracts, and the primer pair. PCR amplification was performed with the following cycling parameters: an initial 95 °C denaturation for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, 1min of annealing at 55 °C, 30 s of extension at 72 °C, followed by a final extension at 72 °C for 10 min. The PCR product was analyzed by electrophoresis in a 1.8% (w/v) agarose gel. The amplified products were sequenced using the same primers. The PCR products were purified and sequenced using a 3730 Genetic Analyzer (Applied Biosystems) after the sequence reaction with a BigDye Terminator version 3.1 Cycle Sequencing kit.

Sequencing and phylogenetic analysis

The sequencing of the eluted fragment was done at Chromous, Bangalore. The BLAST analysis was performed with full length ITS sequences compared with the sequences available in GenBank using BLAST searches to obtain its taxonomy. The highest homology and total score were noted for further analysis. The full length ITS sequences were aligned by Clustal W, a phylogenetic neighbor-joining (NJ) tree was constructed following MEGA ver 5.0.

RESULTS

Fungal identification

The endophytic fungi isolated from Licorice were white color in the beginning (Fig. 1 A), then change to brown, densely spores, brown matrix. Examination of pure cultures and spores (FIG. 1 C, D, E, F, G, H, I, J, K and L) revealed that the fungal isolates were from the genus *Aspergillus* and *Chaetomium*. Condiophore swelling into a spherical in the top without branch, colorless, spherical or oval conidia arranged in chain. The morphologies of the colonies, conidiophore, conidiospores and hyphae of *Aspergillus* and *Chaetomium* were shown in Figure 1.

Sequence and phylogenetic analysis

Genomic DNA and molecular analysis from the pure cultures confirmed the morphological identifications. All samples amplified the rDNA ITS region and yielded a single 500-600 bp DNA fragment, which was sequenced and homology analysis. Homology and BLAST research of sequences retrieved from fungal cultures showed a higher sequence similarity to a known sequence in the Database NCBI. Sequencing of pure cultures isolated from Licorice revealed high similarities to *Aspergillus calidoustus* and *Chaetomium globosum* at 100% and 100%, respectively. This supports our morphological examinations as *Aspergillus* and *Chaetomium*. The majority of sequences had highest similarity to members from *Aspergillus calidoustus*, followed by *Chaetomium globosum* (Figure 2).

Taking together morphological and genetic characteristics, the endophytic fungi isolated from root of licorice belonged to the *Aspergillus* except for L3, L3 belong to the *Chaetomium*.

DISCUSSION

A total of four strains of endophytic fungi were isolated from the medicinal plant licorice root, L1, L2, L3 and L4, respectively. According to the morphological characteristics and spore production, L1, L2, L4 was identified as *Aspergillus*, L3 belong to *Chaetomium*. In order to further prove the validity of the results, the genomic DNA sequence identification and ITS phylogenetic tree was analyzed. It has been demonstrated that the endophytic fungi isolated from licorice root belong to *Aspergillus* and *Chaetomium*.

All the four isolates were produce spores, L1, L2, L4 produce the same spores but L3 spores was different from L1, L2, L4 (Figure. 1. I, J, K, L). These isolates were characterized based on their

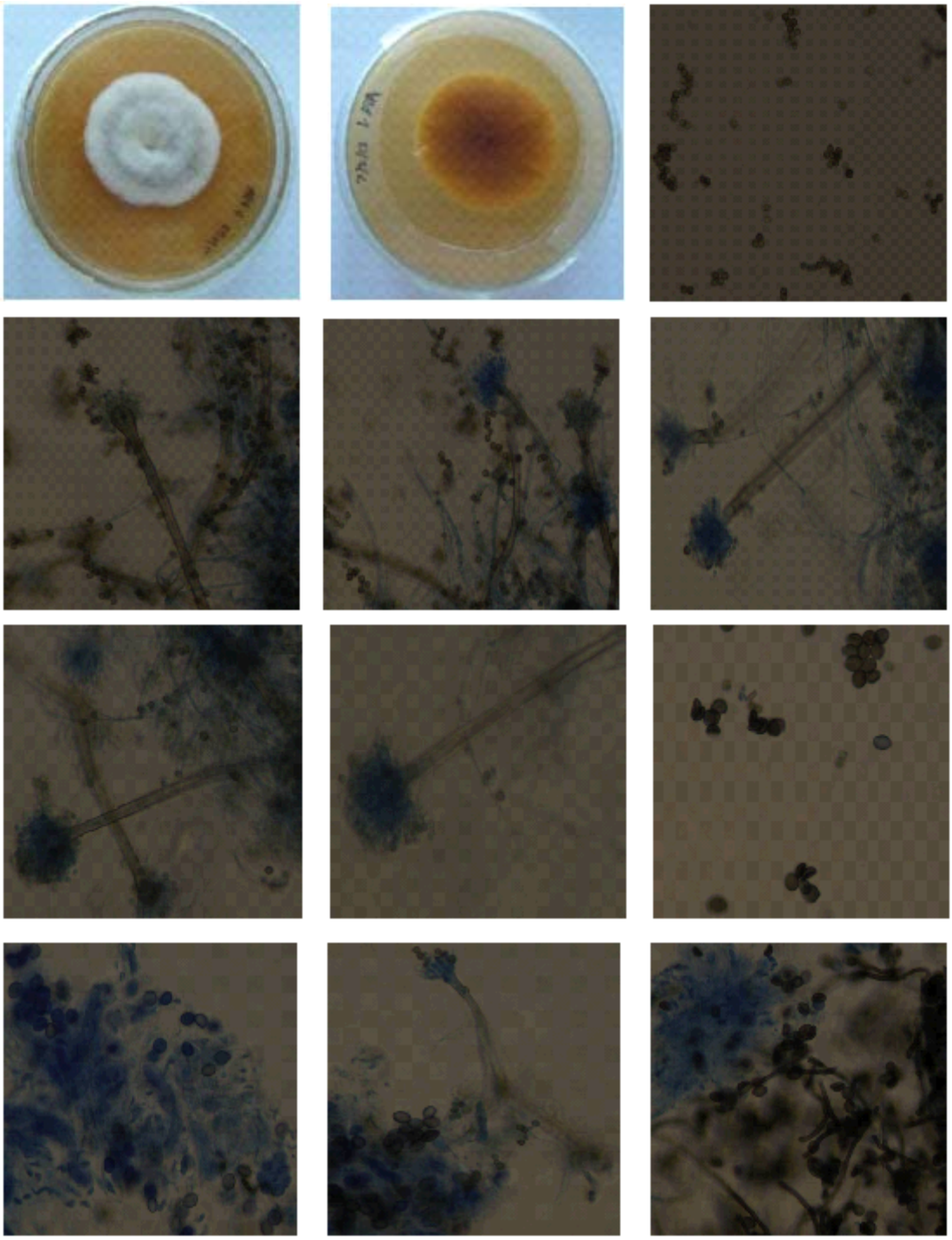

Fig. 1. Morphologies colonies, conidiophore, conidiospores and hyphae of *Aspergillus* and *Chaetomium*: (A) Endophytic fungi front colonies cultivated for 7 d in PDA culture plate; (B) The back of colonies; (C) Microscopic observation of *Aspergillus* spores morphology; (D, E, F, G, H) Microscopic morphologies; Hyphae and conidia Microscopic morphologies of *Aspergillus*. (I, J) Microscopic observation of *Chaetomium* spores morphology; (K, L) Hyphae and conidia Microscopic morphologies; Morphologies colonies, conidiophore, conidiospores and hyphae of *Chaetomium*

morphological and molecular characteristic. The ITS rDNA sequence data of L1, L2, L4 gave a closest match (100% similarity) in the NCBI GenBank database to *Aspergillus* (Figure 2). L3 isolate was identified as *Chaetomium* by its morphological characteristics. The identity of the fungus was further confirmed by ITS rDNA analysis. The closest match (100% similarity) was to *Chaetomium* (Figure 2).

Some early research indicated that *Aspergillus calidoustus* produced natural product metabolites, such as neoechinulin A, neoechinulin B, echinulin, preechinulin, neoechinulin E epiheveadride, questin, sesquiterpene drimane, flavoglaucin, auroglaucin, isotetrahydroauroglaucin and methyl isoquinoline alkaloids (Gregory et al., 2009). Antifungal metabolites (chaetoglobosin A and chaetoglobosin C) from *Chaetomium globosum* suppressed and inhibited mycelial growth and conidial germination of numerous phytopathogenic fungi especially *Setosphaeria turcica* on potato dextrose agar medium (Zhang *et al*., 2013). The use of *C. globosum* as a biocontrol agent against the late blight pathogen *Phytophthora* infestans was evaluated in potato plants, and found that *C. globosum* can be a potential biocontrol agent in the management of late disease in potato plants (Shanthiyaa *et al*., 2013; Xu *et al*., 2013). Three novel azaphilone alkaloids, namely chaetomugilides, Cytotoxic azaphilone alkaloids from *C. globosum* TY1, an endophytic fungus isolated from *Ginkgo biloba*. The isolated compounds exhibited highly cytotoxic activities against human cancer cell line HePG2 (Li *et al*., 2013; McMullin *et al*., 2013).

Recently, endophytic fungi have attracted many researches due to its importance in contribute to their host plant and its secondary metabolites (Mayer and Hamann 2004; Bourguet-Kondracki and Kornprobst 2005). Many endophytic fungi have been isolated from medicinal plants and proven to produce bioactive compounds, such as *Coelomycetous viz*., *Chaetomell raphigera*, *Colletotrichum falcatum*, *Fusicoccum* sp. and *Pestalotiopsis neglecta* (Zhu *et al*., 2008; Venkatachalam *et al*., 2008). Filamentous fungus *Aspergillus niger* isolated from *Taxus cuspidate* was shown to produce Taxol (Zhao *et al*., 2009). In our present study, three

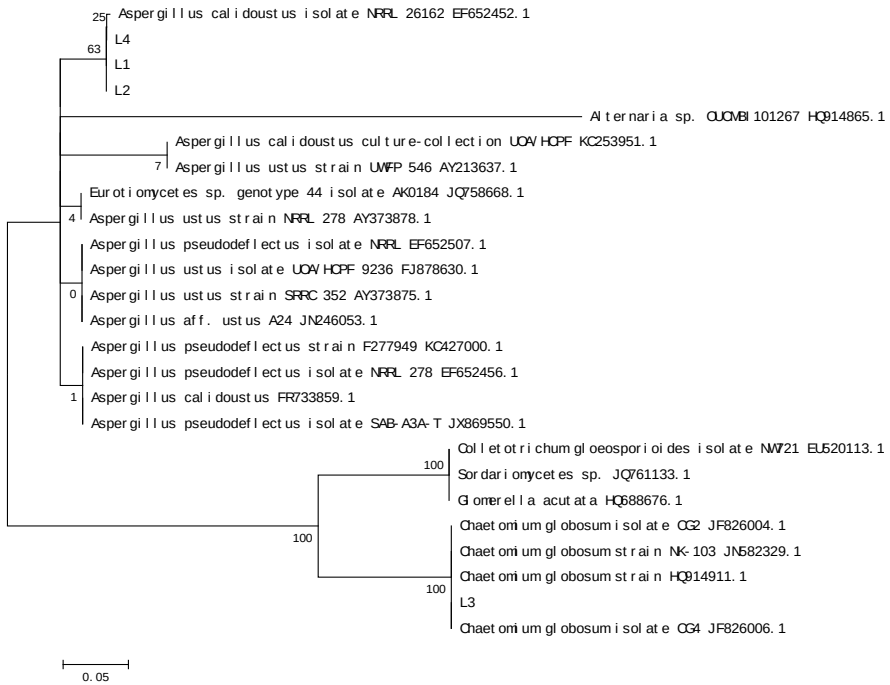

Fig. 2. Neighbor-joining (NJ) phylogenetic tree based on ITS rDNA sequence of endophytic fungi isolated from Licorice and its closest ITS rDNA matches in the GenBank

isolated endophytic fungi were isolated from Licorice belong to *Aspergillus*, one isolated belong to *Chaetomium*. In further studies we will determine if endophytic fungi isolated from Licorice produce the secondary metabolites. It would be produce precious resource for the pharmaceutical natural products that are originally from the medicinal plant.

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