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ORIGINAL ARTICLE

Isolation and identification of two flavonoid-producing endophytic fungi from *Ginkgo biloba* L.

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Abstract Strain ST22 and SX10 isolated from the twigs of *Ginkgo biloba* L. were found to be able to produce phenolic and flavonoid compounds. They were systematically identified by both morphological and molecular methods. Morphological identification, which employed light microscope and scanning electron microscope, showed that ST22 and SX10 are members of *Aspergillus*. ITS1-5.8S-ITS2 regions of these two strains were cloned in order to carry out a similarity alignment. ST22 was identified as *Aspergillus nidulans* and SX10 as *Aspergillus oryzae*. In addition, the total phenolic and flavonoid contents were measured via UV-spectrophotometry. The total phenolic contents in ST22 and SX10 were 0.1413±0.0098 and 0.1450±0.0154 mg/ml, respectively. The amounts of total flavonoids in ST22 and SX10 were 0.01162±0.0014 and 0.01256±0.00378 mg/ml, respectively. It was concluded that these two strains may have potential as sources of natural medicines or prodrugs.

Keywords Antioxidant · *Aspergillus* · Endophytic fungi · Flavanoids · *Ginkgo biloba* L. · Phenolics

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Introduction

Endophytic fungi, by definition, are the fungi which spend the whole or part of their lifecycle colonizing inter- and/or intracellularly inside the healthy tissues of the host plant, typically causing no apparent symptoms of disease (Carroll 1988; Petrini 1991). They are an important ecological component of forest ecosystems (Sherwood and Carroll 1996). Considering the unique specific symbiotic relationships between host plants and their associated endophytes, these microorganisms may have special biochemical metabolic pathways in comparison with others. It is noteworthy that some plants generating bioactive natural products have associated endophytes that can produce the same compounds (Strobel 2003). The reason might be related to a genetic recombination of the endophytic fungi with their host that occurs in evolutionary time (Tan and Zou 2001). It is the rationale to screen natural medicines from this particular source. In the past decades, plenty of secondary metabolites from endophytic fungi have been tested and found to have applications as medicinal and agrochemical candidates (Strobel 2003; Huang et al. 2008). Pioneeringly, Stierle et al. (1993) obtained a taxol-producing fungus from Pacific yew in 1993.

Ginkgo biloba L. is one of the most ancient plants on earth with fossil records dating back more than 200 million years. It has been widely used in China as an important and traditional medicine for various ailments, and contains flavones and lactones (Chen et al. 2007). As a class of main drug efficacy ingredients in *G. biloba*, flavones have been proved to have beneficial effects against atherosclerosis, osteoporosis, diabetes mellitus, and certain cancers (Cermak 2008).

On the basis of the rationale mentioned above, 51 strains of endophytic fungi isolated from the twigs of *G. biloba* were screened to see whether they can produce flavonoids

like their host plant, As a result, strains ST22 and SX10 were detected and regarded as candidates for further research. It occurred to us that these two strains could be used as potential natural medicines or prodrug producers. In this paper, we identified strains ST22 and SX10 at the species level using morphological and molecular methods. The yields of total phenolic and flavonoids of the two strains were quantified basically, and the hydroxyl radical scavenging activity of the culture broth was considered.

Materials and methods

Plant materials

The healthy twigs of *G. biloba* (1 year old) were collected, in August 2007, from mature, healthy-looking trees (approximately 10 years old) located in Tianmu Mountain Nature Reserve and Xiasha, Hangzhou, Zhejiang Province, P.R. China. The samples were then stored in freezer bags at 4°C.

Isolation and culture of the endophytic fungi

Initially the excised twigs were cleaned under running tap water. The samples were surface-sterilized successively with 70% ethanol for 1 min and then rinsed with sterile water. Mercuric chlorine ($HgCl_2$, 0.1%, w/v) was used to sterilize the tissues for 3 min, and again rinsed with sterile water five times. The surface-sterilized twigs were cut into about 0.5×1×0.5 cm (length × width × thickness) pieces and placed on new Petri dishes with potato dextrose agar (PDA) medium to which 0.1% (w/v) gentamicin was added (Vega et al. 2005). These Petri dishes were incubated at 28°C for 7 days. Pure fungal cultures were obtained by the methods described by Lacap et al. (2003) and Promputtha et al. (2005). The strains were preserved on PDA slants and stored at 4°C.

Morphological identification of the endophytic fungi

The fungal cultures were cultured on the PDA plates at 28°C for 5 days. Macroscopic characters of colony such as shape, size, color, odor, and surface texture were observed and described according to the method of Photita et al. (2005). The growth rate of the colony incubated in the same conditions was recorded in detail. The size and shape of hyphae and conidia of strains grown on PDA and incubated for 2–3 days were measured by microscopy. A Nikon Eclipse E200 microscope with Panasonic digital camera WV-CP470/CH using MOTIC program was employed to capture images. Morphological identification was referenced by Wei (1979).

The morphological characteristics of the fungi were also observed through scanning electron microscope (SEM). The methods mainly referred to Ho et al. (1999), but were improved. The specimens were first fixed with 2.5% glutaraldehyde in phosphate buffer (PBS, 0.2M, pH7.0) for more than 4 h and then washed three times. They were postfixed with 1% (w/v) osmium tetroxide (OsO_4) in PBS for 1 h and again washed three times. The specimens were dehydrated by a graded series of ethanol (50, 70, 80, 90, 95, and 100%) for about 15–20 min at each step, transferred to the mixture of alcohol and iso-amyl acetate (1/1, v/v) for about 30 min, then transferred to pure iso-amyl acetate for about 1 h. Finally, they were dehydrated in critical point dryer (HITACHI Model HCP-2) with liquid CO_2 and coated with gold-palladium. Philips Model XL30 ESEM was employed to scan the surface of the specimen.

DNA extraction, PCR amplification, cloning and DNA sequencing of fungal ITS1-5.8S-ITS2 region

The endophytic fungi were grown in 500-ml Erlenmeyer flasks containing 150 ml of potato dextrose liquid medium by shaking continuously for 7 days at 28°C. The mycelia were harvested from the medium by filtration and thoroughly ground with liquid nitrogen. The genomic DNA was extracted according to the methods of Guo et al. (2000). ITS region of rDNA was amplified and sequenced with universal primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′). PCR amplification was performed according to the method of Guo et al. (2000). These PCR products were purified using the PCR Purification Kit (AXYGEN) according to the manufacturer's protocol. The purified PCR products were cloned into pMD18 T-easy vector (TaKaRa) in accordance with the manual, and transformed into *Escherichia coli* TG1 competent cells (Sambrook and Russell 2001). The recombinant plasmid DNA was purified and sequenced in the ABI PRISM 377 DNA sequencer. The results of sequencing were then submitted to GenBank using SEQUIN program. The accession numbers of ITS sequences of ST22 and SX10 are FJ378070 and FJ645738, respectively.

Phylogenetic analysis

The two sequences were used as query sequences to search for similar sequences from GenBank using the BLAST program. The most similar reference sequences with query sequences were obtained and used for subsequent phylogenetic analyses. These sequences were aligned using the CLUSTALX program (Thompson et al. 1997). To construct the relevant phylogenetic tree, MEGA software was used (Tamura et al. 2007). Ambiguous positions which might not be homologous

were eliminated, and gap positions were completely deleted manually. The alignment data were subsequently analyzed by the neighbor-joining method (Kimura two-parameter distance calculation). For each search, 1,000 replicates of random stepwise sequence addition were performed. Statistical support for the internal branches was estimated by bootstrap analysis with 1,000 replications.

Preparation of culture broth

The two strains were inoculated in PDA plates and cultured in 28°C for 3 days. Then the conidia were washed out with sterile water and collected. The concentrations of the conidia were adjusted to 104–105 CFU/ml. The 1% (v/v) conidia suspension would be inoculated into 300 ml PDA liquid media at 28°C at 100*g* for 20 days. The broth was obtained by filtration and conserved in −20°C.

Analysis of total phenolic compound

The total phenolic contents of the samples were determined with the Folin-Ciocalteau reagent. This employed method was mentioned by Lister and Wilson (2001), but modified. Different concentrations of gallic acid equivalent were prepared in 95% ethanol, and the absorbance was recorded at 273 nm. The standard curve was plotted. Samples (1 ml), diluted to 1 in 20 with water were dissolved in 0.5 ml of the Folin-Ciocalteau reagent and 1.5 ml of ddH_2O. Then, 1 ml of 20% sodium carbonate (Na2CO3) solution was added. The mixtures were shaken and incubated at room temper-

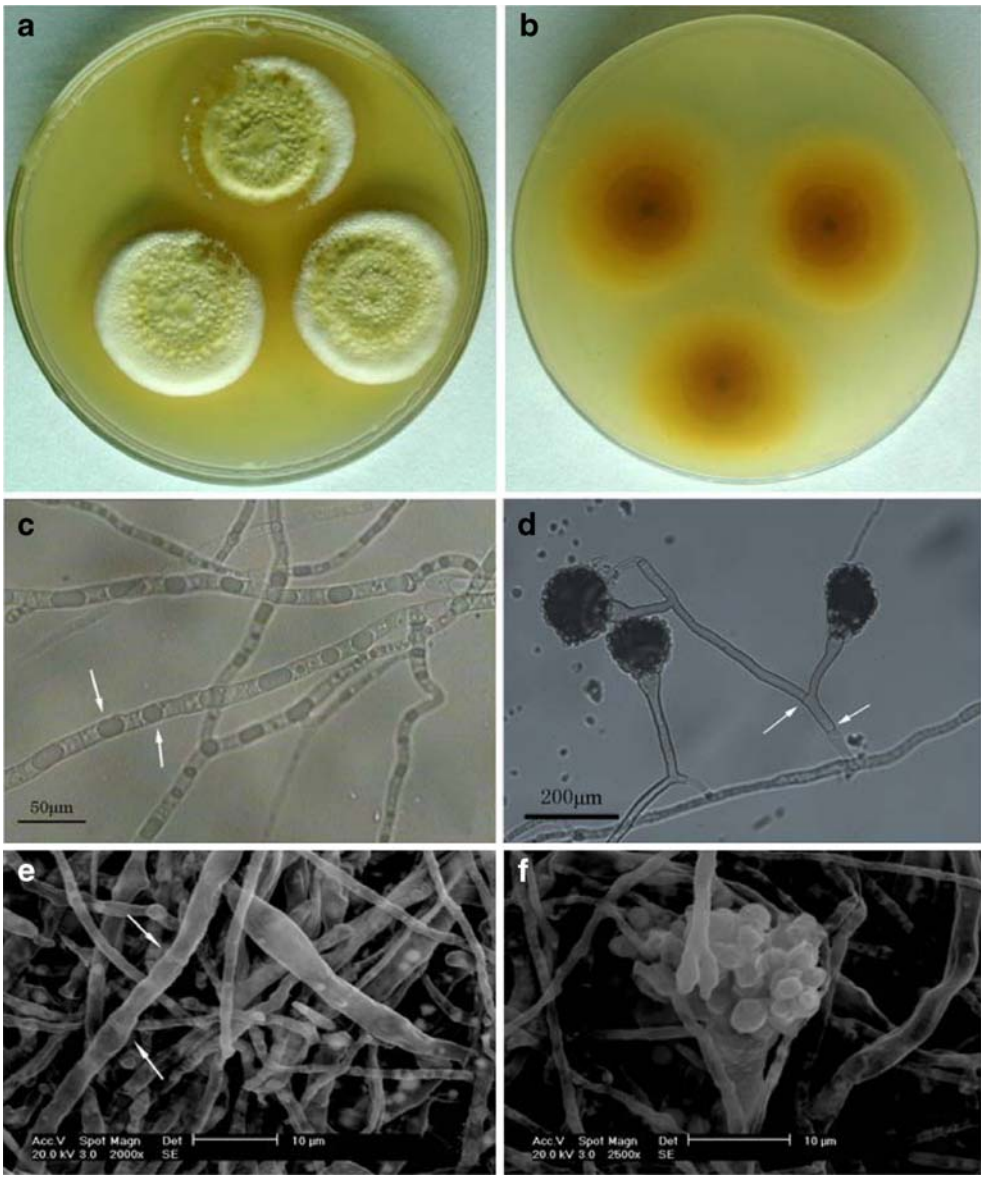

Fig. 1 Morphological examination of strain ST22. **a**,**b** The obverse (**a**) and reverse (**b**) of colonies of ST22 growing on a PDA plate for 5 days. **c**,**d** Light micrographs of the branching hyphae (**c**) and conidiophores (**d**). The vacuoles in (**c**) could be observed vividly (*white arrows*). **d** The conidiophores, which originated from the basal foot cell located on the supporting hyphae and terminate in a vesicle at the apex. **e**,**f** Scanning electron micrographs of branching hyphae (**e**) and conidiophores (**f**) of strain ST22. The septums in (**e**) indicated by *white arrows*

ature for 2 h in the dark. Water was set as the control. The absorbance was measured at 760 nm. The results were expressed in milligram of gallic acid equivalent per milliliter of culture broths.

Estimation of flavonoids content

The total flavonoids contents in extracts were determined spectrophotometrically according to the method of Christel et al. (2000). Rutin equivalent was employed to make the standard curve. Samples (1 ml) were mixed with 1 ml of 2% aluminium chloride ($AlCl_3$) methanolic solution. The mixtures were incubated at room temperature for 15 min, and then the absorbance was determined at 430 nm. The total flavonoids content was expressed in milligram per milliliter of rutin equivalent.

Analysis of hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured via the method of Smirnoff and Cumbes (1989). Ferrous sulfate ($FeSO_4$) solution (0.18 M, 1 ml), sodium salicylate (C7H5O3Na) solution (2.4 mM, 1 ml) and 0.1 ml samples were mixed together. Then, 0.9 ml hydrogen peroxide (H2O2) solution (7.2 mM) was added into the mixtures and kept at room temperature for 1 h. The absorbance was measured at 520 nm with water as control. Inhibitory rate was calculated as:

$$(\mathrm{IR}) = [T - (\mathrm{T}_1 - \mathrm{T}_2)]/\mathrm{T} \times 100\%$$

where T is the absorbance of the control system, T_1 is the absorbance of sample system, and T_2 is the absorbance of the sample system, but with no color developing agent.

Results and discussion

Screening the flavonoids producer

Fifty-one fungal strains were isolated from the twig tissues of *G. biloba*. Among these, 20 strains were from the tissues collected from Tianmu Mountain Nature Reserve, and others from the twigs picked from Xiasha.

Using hydrochloric acid-magnesium powder (HCl-Mg) reaction (this reaction is a universal method for identifying flavonoids in solution), the culture broths of ST22 from Tianmu Mountain Nature Reserve and SX10 from Xiasha were detected to be positive. These two strains were selected as the objects that need to be examined.

Identification of strain ST22

Colonies of strain ST22 could reach 3–4 cm diameter on a PDA plate after 5 days, and displayed as slightly protuberant, lanose, and sometimes with exudates on the surface. The color of the colony surface was white to light yellow, and the reserve was yellow owing to the secretion of the pigment. The margin was smooth. Interestingly, the odor of the culture was sometimes refreshing (Fig. 1a, b).

Figure 1c–f shows the results of light and scanning electron microscopic studies of strain ST22. The hyphae were always branching and contained vacuoles. Conidiophores were smooth to finely rough walled. They were 200–300 μm long, up to 10 μm in diameter, enlarging gradually into vesicles of approximately 20 μm diameter. Long metulae was absent, and phialides were ampulliform, 8–10 μm long, with a short neck. The conidia were mostly subglobose-globose,

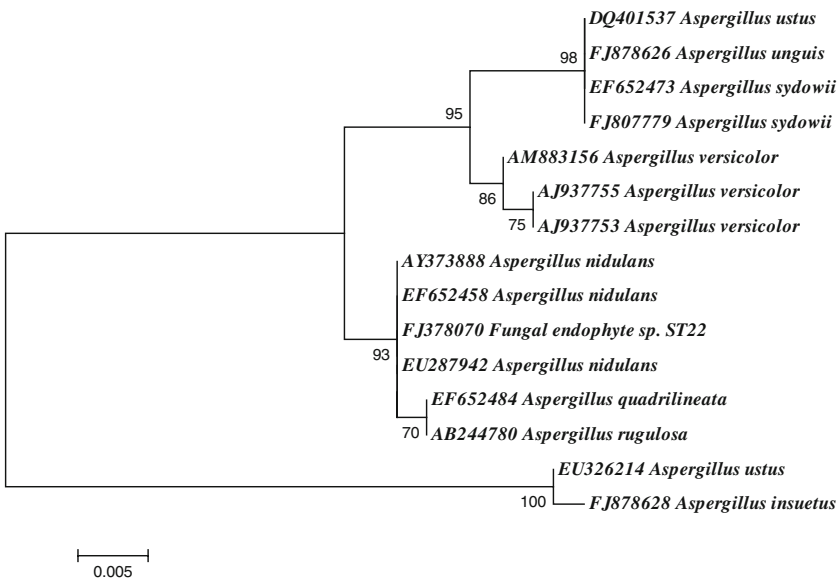

Fig. 2 Neighbor-joining tree of *Fungal* endophyte sp. ST22 based on ITS1-5.8S-ITS2 rDNA sequences. Confidence values above 50% obtained from a 1,000-replicate bootstrap analysis are shown at the branch nodes. Bootstrap values from neighbor-joining method were determined. *Aspergillus insuetus* (FJ878628) and *Aspergillus ustus* (EU326214) were used as the outgroup

2–3×1.5–2 μm in size. Vesicle was the typical formation for the genus *Aspergillus*. According to these characteristics described above, strain ST22 was identified as a definite species of *Aspergillus*. It was hard to locate this strain into a named species thanks to the lack of the morphological information. Thus, the molecular identification was employed to identify it.

A search for similar ITS region sequences from GenBank showed that strain ST22 had higher sequence similarities with the species *Aspergillus nidulans* than any other reference taxa. All 14 sequences of reference taxa were obtained for constructing phylogeny. In the neighbor-joining (NJ) tree (Fig. 2), strain ST22 and other three reference taxa, *Aspergillus nidulans* (AY373888), *Aspergillus nidulans* (EF652458,) and *Aspergillus nidulans* (EU287942) formed a clade with 93% bootstrap support. In this clade, two reference taxa, *Aspergillus quadrilineata* (EF652484) and *Aspergillus rugulosa* (AB244780), also cluster together with them. The results of similarity comparisons of the ITS1-5.8S-ITS2 region sequence revealed that strain ST22 had the highest nucleotide similarities with *Aspergillus nidulans*. Referring to the results of the morphological and molecular

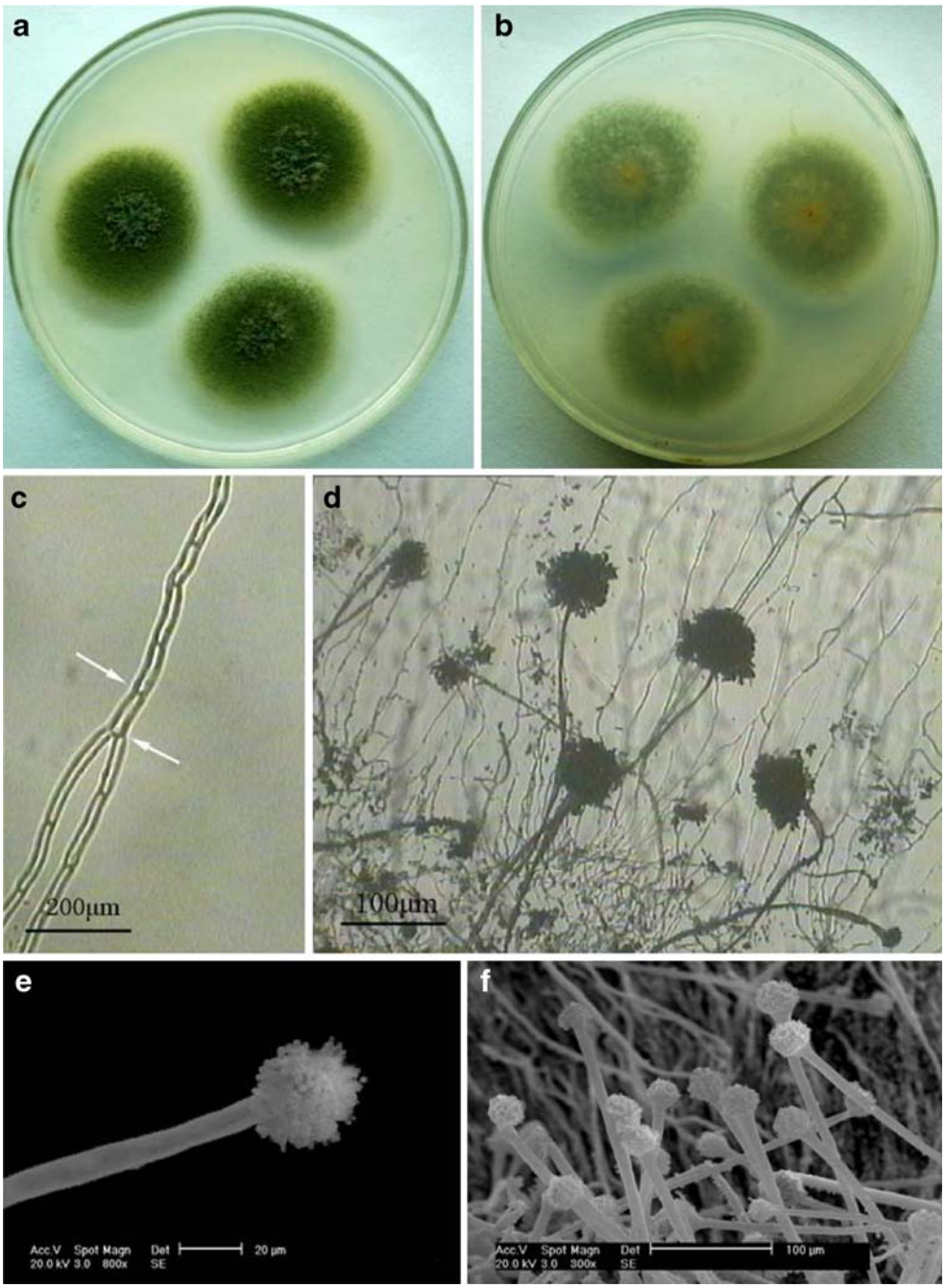

Fig. 3 Morphological examination of strain SX10. **a**,**b** The obverse (**a**) and reverse (**b**) of colonies of SX10 growing on a PDA plate for 5 days. **c**,**d** Light micrograph of branching hyphae (**c**) and conidiophores (**d**). The septums in (**c**) indicated by *white arrows*. **e**,**f** Scanning electron micrographs of conidiophores of strain SX10. The conidia were located on the terminate vesicles

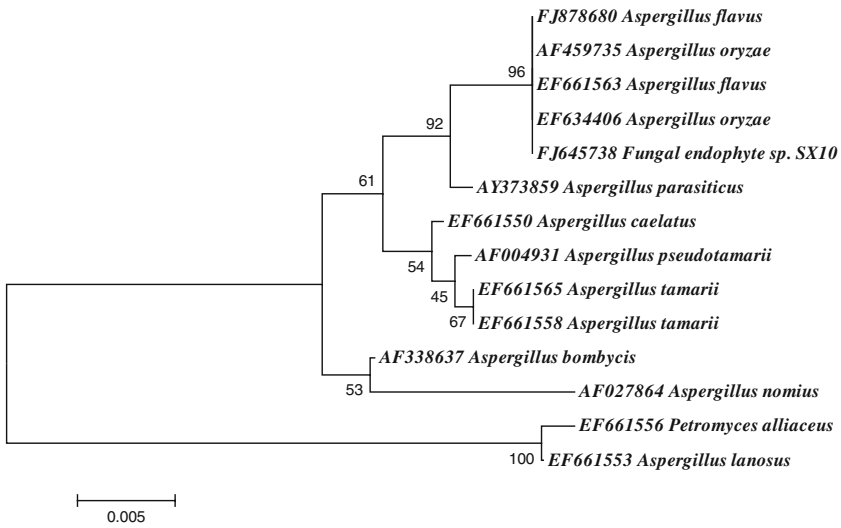

Fig. 4 Neighbor-joining tree of *Fungal* endophyte sp. SX10 based on ITS1-5.8S-ITS2 rDNA sequences. Confidence values above 50% obtained from a 1,000-replicate bootstrap analysis are shown at the branch nodes. Bootstrap values from the neighbor-joining method were determined. *Petromyces alliaceus* (EF661556) and *Aspergillus lanosus* (EF661553) were used as the outgroup

identification, it could be confirmed that ST22 is *Aspergillus nidulans*.

Identification of strain SX10

Figure 3a, b shows the obverse and reverse of the colonies of strain SX10. The colonies were flat, just a little convex in the center. The texture was loose. The color varied with state of sporulation from white to viridian. The margin of the colonies was scalloped.

Figure 3c–f displays the branching hyphae and conidiophores of strain SX10. Conidiophores arising from basal hyphae, smooth 200–300×10–20 μm. Metulae could be observed, and phialides are ampulliform, 8–10 μm long. The conidia were mostly globose, 1.5–2.5×1.5–2 μm in size.

For identifying strain SX10, a similarity search was done bythe Blast program. Based on ITS1-5.8S-ITS2 rDNA sequence of strain SX10 and another 13 representative reference taxa, the neighbor-joining tree revealed distinctly that endophytic fungus SX10 was a member of the *Aspergillus* clade (Fig. 4). In this NJ tree, fungal endophyte sp. SX10 formed a clade with *Aspergillus flavus* (FJ878680), *Aspergillus flavus* (EF661563), *Aspergillus oryzae* (AF459735), and *Aspergillus oryzae* (EF634406), with 96% bootstrap support. Also, this big clade including strain SX10 formed a new clade with *Aspergillus parasiticus* (AY373859) with 92% bootstrap value. We can consider that SX10 was a member of *Aspergillus flavus-oryzae.*

From Fig. 4, it was concluded that strain SX10 was a species of *Aspergillus*. However, the ITS of SX10 was most similar not only *with Aspergillus flavus* but also with *Aspergillus oryzae*.

Nowadays, the techniques of molecular biology are widely used for the identification of microorganisms (Arnold et al. 2000; Okane et al. 2001; Baayen et al.

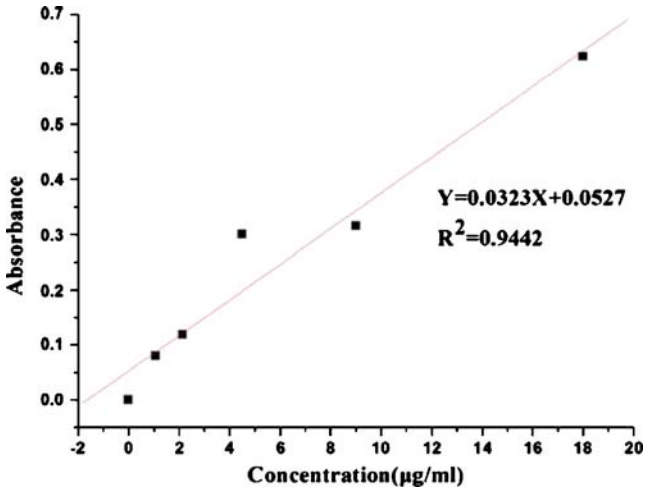

Fig. 5 The standard curve to measure total phenolic content in samples. Absorbance=0.0326 gallic acid (μg/ml)+0.0607 (R^2=0.9422)

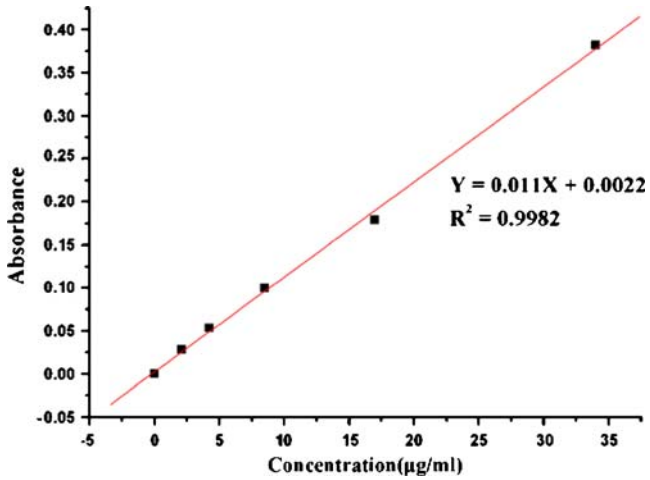

Fig. 6 The standard curve to measure total flavonoids content in samples. Absorbance=0.011 Rutin (μg/ml)+0.0607 (R^2=0.9982)

2002), but there are some problems for sequencing the specific DNA fragments or genes. The most important point is what DNA region should be available for identifying the organisms. The ITS region is now perhaps the most widely sequenced DNA region in fungi (Powers et al. 1997). It has typically been most useful for molecular systematics at the species level, and even within species. Because of its higher degree of variation than other genic regions of rDNA, sequencing the ITS region can sometimes identify the fungi to species and subspecies level. However, the ITS sequences are so conserved between *Aspergillus flavus* and *Aspergillus oryzae* that it is difficult to solve SX10 placement exactly in the evolutionary lineage. In some cases, molecular and morphological methods should be integrated. So, we consider that traditional morphological observation is also significant for identification and classification. Based on the characteristics of the colonies and microstructure of hyphae and conidiophores, this fungus resembled *Aspergillus oryzae* (Wei 1979).

Total phenolic and flavonoids content

The standard curves to measure total phenolic and flavonoids content in samples are shownin Figs. 5 and 6. On the basis of the absorbance of the samples, the amounts of total phenolic and flavonoids were calculated from the relevant standard curves as annotated in in the Figures. After ratio conversion, the concentrations of total phenolic and flavonoids in strain ST22 culture broth were 0.1413± 0.0098 and 0.01162±0.0014 mg/ml, respectively. The amounts of total phenolic and flavonoids in strain SX10 samples were 0.1450±0.0154 and 0.01256±0.00378 mg/ml, respectively.

Some endophytic fungi have been determined as the phenolic or flavonoids producers. Huang et al. (2007) isolated 42 endophytic fungal strains from *Nerium oleander* and found that the major bioactive constituents of some fungal cultures were found to contain phenolics and their derivatives, flavonoids. They also clarified that some phenolic compounds were found to more likely coexist with certain endophytic fungi in the same plants. Liu et al. (2007) obtained an endophytic *Xylaria* sp. from *G. biloba*, and this strain has the ability to produce phenolic, especially flavonoids. The antioxidant activity has also been determined.

Certain species of *Aspergillus* are important as agricultural pathogens, especially *Aspergillus flavus-oryzae.* However, there are fewer reports that species of *Aspergillus* as endophytic fungi can produce phenolic and flavonoid compounds. These results suggest that strains ST22 and SX10 can be the potential source of natural products. However, the safety of these two strains and the culture extracts are unknown. More research should be carried out to ensure that the strains as potential natural source are nontoxigenic and nonpathogenic for human. Alternatively, some suitable techniques can be used to isolate the phenolic and flavonoids. These further experiments are in progress.

Hydroxyl radical scavenging activity

For the presence of phenolics and flavonoids in the culture media, the broths were concluded to have antioxidant activities. The antioxidant activity of phenolics is mainly due to their redox properties which make them act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may also have a metallic chelating potential (Rice-Evans et al. 1997). Flavonoids are sometimes polyphenolics and also display the ability to scavenge the hydroxyl radical (Husain et al. 1987). On the basis of the formula listed above, some absorbances should be determined accurately. T value, which represent the absorbance of the control system (no samples), was 0.436±0.035. T_1 values were 0.351±0.025 and 0.244± 0.006, which denoted the absorbance of sample system: ST22 and SX10 respectively. T_2 was the absorbance of the sample system, butwith no color developing agent. The value was 0.064±0.012. The inhibitory rate of ST22 sample was 34.17%. Similarly, SX10 sample showed 58.71% of antioxidant capacity. Reactive oxygen species such as superoxide anion, hydroxyl ('OH), peroxyl, and alkoxyl radicals may attack biological macromolecules giving rise to oxidative stress-originated diseases. The results showed that ST22 and SX10 have the potential to be sources of antioxidant.

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