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Short Communication

Molecular Characterization of *Aspergillus tubingensis* and *Eurotium amstelodami* Associated with Black Brick Tea

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Abstract

Two kinds of fungi, named strain B and strain Y were isolated and purified from the Black Brick Tea. Under the standardized culture condition for fungus, these two strains were identified by the traditional morphology and the method of phylogenetic tree construction after cloning the 18S rDNA-ITS segment, respectively. Further analysis of these results demonstrated that strain B was *Aspergillus tubingensis*, while strain Y was *Eurotium amstelodami*. Our results constitute the first valuable resource to further investigate application of the new Black Brick Tea strains. © 2016 Friends Science Publishers

Keywords: Black brick tea; Identification; Phylogenetic tree; *Aspergillus tubingensis*; *Eurotium amstelodami*

Introduction

The quality of the brick tea is influenced by microorganism in its manufacturing process. For example, the quality of FuZhuan brick tea is directly influenced by *Eurotium cristatum* (Wang *et al.*, 1991; Zhao *et al.*, 1991; Kai *et al.*, 1992). From the last 50s to now, various fungus have been isolated from several kinds of brick tea by researchers. For example, *Aspergillus niger*, *A. glaucus*, *A. oryzae* and *A. tubingensis* had been isolated from PuEr brick tea (Jian *et al.*, 1979; Hu and Hu, 1957).

With the development of molecular biology, this technology which offers a more comprehensive and scientific basis is gradually used to identify Fungi (Su *et al.*, 2006; Deepak and Alok, 2007). The ITS region is the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genic regions of rDNA, variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. In addition to the standard ITS1+ITS4 primers used by most labs, several taxon-specific primers have been described that allow selective amplification of fungal sequences (Gardes and Bruns, 1993). ITS region is nowadays being used to know the genetic diversity among different strains of fungi by sequencing the ITS gene.

Materials and Methods

Isolation of Fungi

The black brick tea is provided by the Experiment Center of Hunan Agricultural University. The improved soluble starch culture medium (Yun and Han, 2006), contained soluble starch, 15 g; sucrose, 150 g; agar, 15 g; yeast agar, 5 g; KH_2PO_4, 1 g and $MgSO_4$, 0.5 g per litre distilled water (pH 6.5). The black brick tea was cut into pieces, washed with sterilised water and placed on soluble starch culture medium plates. The plates were incubated inversely at 30˚C and spread with different colonies after 5 days. Take a portion of the colonies by means of a wire and stab inoculate to the plates, incubated inversely at 30˚C for 5 days. Individual colonies were sub-cultured into nutrient agar plates containing until pure culture was isolated.

Morphological Characteristics

The isolated strain was inoculated on an improved soluble starch culture medium plate and incubated at 30˚C. The diameter of its colony was measured every 3 days and the color and surface characteristics of the colony was observed. The characteristic of mycelium and spore were observed and imaged by using Microscopic Imaging System-MVC2000 and Transmission Electron Microscope- JSM6360LV.

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Molecular Identification

Genome DNA was extracted from individual spargana by SDS/proteinase K treatment, column-purified (Wizard SV Genomic DNA Purification System, Promega) and eluted into 60 μL H_2O according to the manufacturer recommendations (Zhao *et al.*, 2009a, b).

A portion of the ITS gene was amplified with universal primers ITS1 and ITS4 (White *et al.*, 1990). PCR reactions (25 μL) were performed in 4 mM of $MgCl_2$, 5 μM of each primer, 2.5 μL 10×rTaq buffer, 0.2 mM of each dNTPs, 1.25U of rTaqDNA polymerase (TAKARA) and 1 μL of DNA sample in thermocycler (Biometra) under the following conditions: an initial denaturation at 94˚C, for 5min, then 35 cycles of 94˚C for 30s (denaturation), 55˚C for 30s(annealing), 72˚C for 30s (extension); followed by a final extension at 72˚C for 10 min.Samples without genomic DNA (no-DNA controls) and host genomic DNA (host-DNA controls) were included in each amplification run, and in no case were amplicons detected in these controls (not shown). Each amplification (5 μL) was examined by agarose gel electrophoresis.

Concentration of DNA was determined by nanodrop spectrophotometer (Nanodrop™ 1000). The amplified PCR products were purified with a Wizard PCR purification kit (Qiagen Ltd., Crawley, United Kingdom). The sequencing was done at Sangon Biotect (Shanghai) Co., Ltd. The obtained sequences were submitted to BLAST search in NCBI database for phylogenetic relationship.

The ITS sequences of 20 fungus, which relatively show a high similarity to the ITS sequence of the isolated strain were selected from GenBank™ database. A method, namely neighbor-joining (NJ) was used to construct a phylogenetic tree by using PAUP 4.0 Beta 10 program (Swofford, 2003).

Results

The pieces of black brick tea were inoculated inversely on improved soluble starch culture medium plate (Yun and Han, 2006) at 30˚C for 5 days. When the whole plate was spread with colonies, two kinds of colonies, the black-brown one and the yellow one, were observed by naked eye (Fig. 1).

After isolation and identification those two colonies, each of them with the same growth situation and morphological characteristics was separated. The black one was marked as Colony B (Fig. 2). Colony B can grow to the diameter of 27 mm after being incubated at 30˚C on the improved soluble starch culture medium plate for 3 days and the diameter extends to 33 mm for 6 days. The surface of colony was loose with intensive radial lines; the aerial mycelium in the middle of colony has projections; the circle of colony was hair like. The head of spore was spherical while the stem of it had smooth wall (Fig. 3). The structure of the whole spore liked a pie with smooth circle and the diameter of it was about 4.5 μm (Fig. 4). The preliminary results of morphology demonstrate that it belongs to *A. tubingensis*.

The yellow-green colony was marked as colony Y (Fig. 5). Colony Y can grow to the diameter of 16 mm after being incubated at 30˚C on the improved soluble starch culture medium plate for 3 days and the diameter extends to 34 mm for 6 days. The whole colony was dense with clear radial lines, the back of colony is brown, and no exudate was found. The propagation style of it was a combination of asexual reproduction and sexual reproduction. The mycelium was white while the head of the spore was yellow and spherical; the nutrient mycelium had a short and thick structure without separation and the perithecium was strong with a yellow-green color (Fig. 6). The ascospore with clear projections of 5 μm was observed (Fig. 7). Thus the preliminary results of morphology demonstrated that it belongs to *Eurotium amstelodami*.

Molecular Identification

The amplified PCR products (Fig. 8) can be found by agarose gel electrophoresis. By analysing the phylogenetic tree (Fig. 9; Fig. 10), the results demonstrated that Strain B was identified as *A. tubingensis*, while Strain Y was identified as *Eurotium chevalieri.*

Identification of Initial Fermentation Broth Product

We have been determinate the initial fermentation broth product in different samples. Those products include polyphenols, tea polysaccharide, EGCG and free amino acid (Table 1).

Identification of the Influence on Amylase, Pepsin, Trypsin and Lipase

By imitating the human indigestion system, we characterized the influence of amylase, pepsin, trypsin and lipase in the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) that caused by the single or the mixed fermentation liquor (Table 2).

Discussion

In this research, two fungi were isolated and purified in the black brick tea. Strain B was identified as *A. tubingensis* through morphological identification. At the same time, the homology between strain B and *A. tubingensis* was high to 99% after identifying by the method of molecular biology. Strain Y was identified as *Eurotium amstelodami* by means of the morphological identification, however the results of biological identification displayed that strain Y had the highest homology value (98%) with *Eurotium chevalieri*. The results from molecular biology identification and morphological identification were different. *Eurotium*

Fig. 1: Colony (observe)

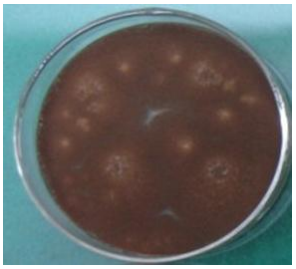

Fig. 2: Colony (Strain B)

Fig. 3: Mycelia and Sporangium (Strain B)

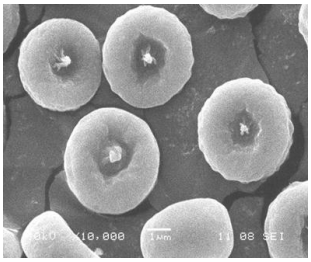

Fig. 4: Spore (Strain B)

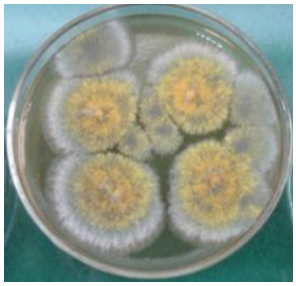

Fig. 5: Colony (Strain Y)

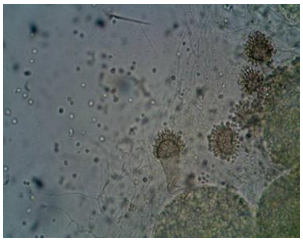

Fig. 6: Mycelia and Sporangium (Strain Y)

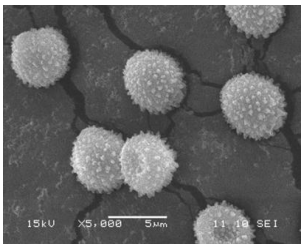

Fig. 7: Spore (Strain Y)

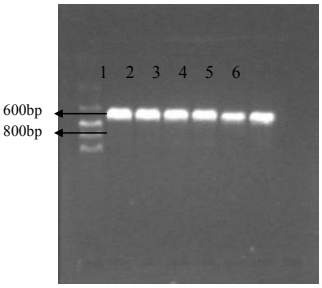

Fig. 8: Result of PCR (1, 2, 3-strain B; 4, 5, 6-strain Y)

chevalieri has the golden dense colony and the back of colony is yellow-green (described in Web of Portal of Chinese Science and Technology Resource). For strainY, morphological identification demonstrated that it varied from the characteristic of *Eurotium chevalieri,* but was very similar to *Eurotium amstelodami*. The features of *Eurotium amstelodami* are as following: the color of colony is yellow-green; the middle part of this colony has projections and partition; the aerial mycelium is white; substrate mycelium is yellow; the spore is yellow granulated and the inner wall of it is smooth without any partition (described in Web of Portal of Chinese Science and Technology Resource). At the same time, strain Y also shared a high homology value with *Eurotium amstelodami,* which were selected from 7 strains of phylogenetic tree. This was a strong molecular biology evidence for the strain that it belonged to *Eurotium amstelodami*.

The same strain *Trichoderma sp.* from several different microbial culture collection centers was studies by Kuhls and Bomber and differences were found in the results of their DNA fingerprints (Kuhls *et al*., 1995). *Fusarium axysporium f sp ciceris* kept for 12 years was contrasted with other strains in this specie by Kelly, and the results demonstrated that this strain has no classic characteristic of molecular fingerprint, and so it may be degenerated (Kelly *et al*., 1994). Those results arrived at a conclusion that different situation of collection may lead to different degree of variation which were reflected at the level of molecular structure and morphological characteristics. Li *et al*. (2007) found one of the *Amanitas* had long-distance relative relationship with other six *Amanitas* when seven of them were investigated and identified initially based on morphological characteristics after cloning and sequencing the segment of rDNA-ITS and contrasting the sequence characteristics (Li *et al.*, 2007). Artiukova found ITS sequences had variations at multiple levels. The variations not only exist in different species but also in the different geographic species under the same species (Artiukova *et al.*, 2005).

Using ITS sequence to identify different species is an effective way to differentiate. The distance of relative relationship has close connection with the degree of differentiation. It seems to have impact on the identification results, but actually it still correctly reflects the closeness of relative relationship and the high homology value in the process of evolution. So it remains a strong molecular biology index for the traditional identification results.

To further study the functions of fungi isolated from Dark Brick Tea on human indigestion system, the five identified fungi were fermented singly and mixed. The content of tea polyphenols, tea polysaccharide, catechin, and free amino acid in the fermentation liquor were measured. At last, by imitating the human indigestion system, the influence of amylase, pepsin, trypsin and lipase that caused by the single or the mixed fermentation liquor was researched. The result showed that it was the metabolites produced by the isolated fungi by using the nutrient component in Dark Brick Tea to improve human indigestion.

Table 1: Determination of functional components

Sample	Polyphenols mg/mL	FAA mg/ 100 mL	Tea polysaccharide mg/mL	EGCG mg/100 mL
Strain A	0.136	2.393	0.133	28.883
Strain B	0.187	2.403	0.124	27.511
Strain C	0.181	2.280	0.120	29.897
Strain D	0.180	2.196	0.178	28.664
Strain E	0.172	2.003	0.153	30.586
Strain A+B	0.173	1.996	0.105	26.440
Strain B+C	0.168	2.344	0.119	31.231
Strain A+E	0.176	1.963	0.159	25.697
Strain C+D	0.179	1.985	0.192	27.332
Strain A+C	0.172	2.013	0.158	26.351
Strain A+B+C+D+E	0.147	1.775	0.160	27.385
Black Brick Tea soup	0.180	4.252	0.200	46.254

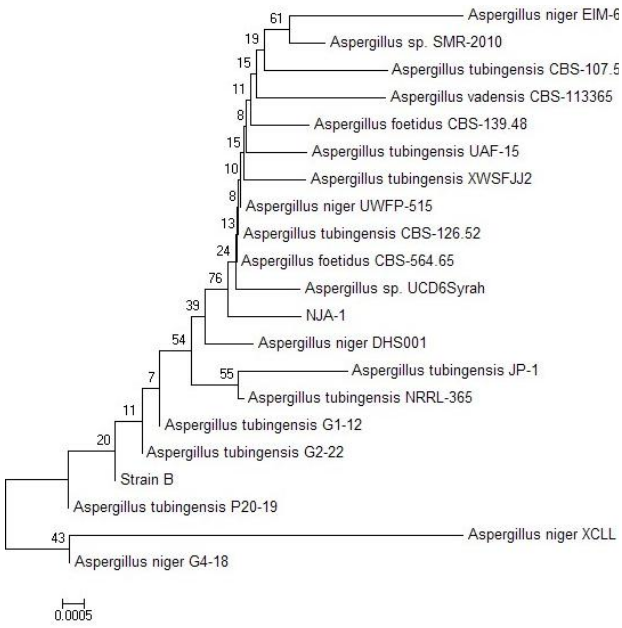

Fig. 9: Phylogenetic tree (Strain B)

Conclusion

The strains B and Y isolated from the Black Brick Tea and analyzed through morphological characteristics and phylogenetic status, demonstrated that strain B was *A. tubingensis*, while strain Y was *Eurotium amstelodami*.

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Table 2: Affects to different enzymatic activity of functional components

Sample	α-amylase U/mL	Pepsin U/mL	Trypsin U/mL	lipase U/mL
SGF		23.62		5.05
SIF	3.13		13.11	25.15
SGF+Black brick tea soup		66.73		4.02
SIF+Black brick tea soup	3.61		25.90	16.56
SGF+Strain A		23.08		5.08
SGF+Strain B		33.27		4.28
SGF+Strain C		50.43		3.03
SGF+Strain D		72.04		2.03
SGF+Strain E		23.62		4.72
SGF+Strain AB		23.62		4.28
SGF+Strain AC		23.62		4.86
SGF+Strain AE		22.19		5.05
SGF+Strain CD		105.11		1.83
SGF+Strain BC		42.13		2.35
SGF+Strain ABCDE		36.55		3.37
SIF+Strain A	3.01		13.03	25.20
SIF+Strain B	4.33		37.75	24.40
SIF+Strain C	4.67		36.62	20.70
SIF+Strain D	5.12		44.31	16.50
SIF+Strain E	3.31		26.34	25.16
SIF+Strain AB	3.02		22.15	25.13
SIF+Strain AC	3.10		13.10	25.16
SIF+Strain AE	2.97		12.97	26.33
SIF+Strain CD	5.23		46.72	15.35
SIF+Strain BC	4.85		40.93	16.73
SIF+Strain ABCDE	3.97		33.71	24.42

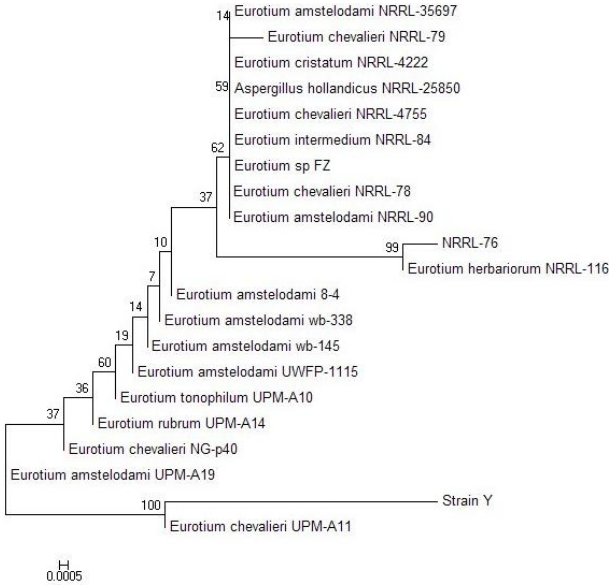

Fig. 10: Phylogenetic tree (Strain Y)

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