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Full Length Research Paper

Biodiversity of cultivable fungi in hair samples from tree shrews

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Diverse fungal species live on the surfaces of plant tissues, some of which presumably occur in a mutualistic association. Some fungal species are widespread and can be found in many different animal species, whereas others are highly specific to single hosts. In this study, we investigated the taxonomic identities and phylogenetic relationships of fungal species isolated from tree shrew hair samples, using a combination of morphological and molecular approaches. Morphological differences among the seventy-one fungal isolates indicate that diverse distinct morphotypes might be present on the hosts. Seven representative isolate taxa were selected for further molecular phylogenetic analysis using nuclear ribosomal internal transcribed spacer (ITS1 and ITS2) DNA sequencing. The 71 endophytes were identified to the species level based on fungal sequences with known identities in GenBank. Our results suggest that 7 fungal genera are the dominant fungal parasites on the tree hairs.

Key words: Tree shrew, cultivable fungi, phylogeny, taxonomy.

INTRODUCTION

The tree shrews are small animals belonging to the family Tupaiidae, mainly found in Southern China, India, and Southeast Asia. The 17 known species of tree shrews are classified in genus *Tupaia*, family Tupaiidae. Among them, northern tree shrew (*Tupaia belangeri*) is typical species. Tree shrews are now being tried as possible models for medical and biological research. Because of the susceptibility of the tree shrews (*T. belangeri*) and their hepatocytes to infection with human hepatitis B virus (HBV) *in vivo* and *in vitro,* these animals have been used to establish human hepatitis virus-induced hepatitis models. As these animals are phylogenetically close to primates in evolution and have a well-developed visual system and color vision in some species, they have been utilized to establish myopia models. In brief, the tree shrews holds significant promise as research models and great use could be made of these animals in biomedical research (Novacek et al., 1992; Cao, 2003).

In natural or domesticated state, tree shrew (*T. belangeri)* is proved to be infected with several bacteria, viruses, and parasites (Bahr and Darai, 2001; Tidona et al., 1999; Saitou and Nei, 1987). Based on these facts, when tree shrews are used as experimental model, microbiological quality control over tree shrews is compulsory. In this report, we describe the isolation and identification of seven fungal species from the hair samples of adult *T. belangeri* tree shrews from wild situations.

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MATERIALS AND METHODS

Animal and fungus isolation

Forty wild adult tree shrews were captured from urban mountains of

Great Kunming region, hair samples were taken from three sites (head, back, and abdomen) of every tree shrew by using sterile scissors and were cut to powder-like pieces. The samples was spotted by inoculation loop on the surfaces of Potato dextrose agar (PDA), Czapek medium and Sabouraud's Medium dish for fungi isolation, and incubated in Petri dishes in the dark at 27°C. Fungal colonies growing from the plant tissues, usually after 5 to 10 days, were picked with hyphal tips and transferred to PDA plates to determine for culture purity. Pure fungi were sub-cultured on PDA slopes after incubation for up to 7 days. Individual fungi were grouped according to the gross morphology of colonies. Appropriate controls were also set up to ensure nice medium quality and no contamination from experimental operation. Fungal Isolates were placed in 20% (vol/vol) glycerol and stored at -70°C or further study.

Morphological identification of fungal isolates

Morphological identification of the 71 fungal isolates from 40 tree shrews was first carried out according to colony or hyphal morphology of the fungal cultures, characteristics of the spores, and reproductive structures if discernible (Barnett and Hunter, 1998; Carlile et al., 2001; Watanabe, 2002; Gadd et al., 2007) . Based on these features, the 72 isolates could be classified into 7 different morphological taxa. Using the traditional morphological techniques, only a few of the fungal isolates could be identified to the genus level.

Molecular identification of fungal isolates

DNA extraction and amplification fungi

A total of 7 representative isolates of various morphological fungi isolated from tree shrew hair samples were selected for molecular identification. Fungal genomic DNA was prepared using Biospin Fungus Genomic DNA Extraction Kit (Bioer Technology Co., Ltd., Hangzhou, P.R. China). The DNAs were transferred to the new tubes and stored at -20°C respectively.

DNA detection concentration was performed by electrophoresis on a 2% (wt/vol) agarose gel stained with ethidium bromide. A volume of 10 µl of DNA and 2 µl of Ficoll dye was loaded in each lane. Electrophoresis condition was 110 V for 50 min in 1× TE buffer.

TaKaRa fungi Identification PCR Kit (Code No. D317) was used in amplifying the internal transcribed spacer (ITS) region of 18 to 26S nuclear ribosomal DNA with primers ITS4 (5'-TCCTCCGCTTATTG ATATGS- 3') and ITS5 (5' -GGAAGTAAAA GTCGT AACAAGG-3'). An initial denaturating step (94°C for 5 min) was followed by 30 cycles (with each cycle consisting of DNA denaturating at 94°C for 30 s, primer annealing at 55°C for 30 s, and elongation at 72°C for 1 min) and a final extension step at 72°C for 5 min. A no-template negative control was included in each PCR run. The PCR products were purified with a TaKaRa Agarose Gel Purification Kit Ver.2.0 (Code No. DV805A, TaKaRa Biotechnology (Dalian) Co., Ltd.).

Sequencing and bioinformational analysis of fungal ITS

Direct DNA sequencing and sequence analysis was performed using primers ITS3 (5 -GCATCGATGA AGAACGCAGC-3) and ITS5 (White et al., 1990) with an automated sequence analyzer (ABIPRISM^{TM} 3730XL DNA Sequencer; Applied Biosystem) following the manufacturer's instructions (Applied Biosystems, Inc.). Sequence similarity searches were performed for each of the 7 representative fungal sequences against the non-redundant database maintained by the National Center for Biotechnology Information using the BLAST algorithm (http://www.ncbi.nlm.nih.gov). Alignment search tool (BLAST) at the National Center for Biotechnology Information, and percent homology scores were generated to identify fungi. Fungi with amplified ITS sequences >98% similar were considered to be the same phylotype. Each ITS DNA sequence was compared by using the BLAST alignment program with data available from GenBank at the National Institutes of Health. The computer alignment provides a list of matching organisms, ranked in order of similarity between the unknown sequence and the sequence of the corresponding organism from the database. The percentage and absolute number of matched base pairs from each BLAST match were reported.

RESULTS AND DISCUSSION

Seventy one pure fungal isolates from hair samples of tree shrew (*T. belangeri*), which were tentatively identified, based on phenotypic analysis by colonial character, pigment, stain and fungal morphology, and then these isolates are classified into 7 morphological taxa of fungi: F5 , F12-14, F14-122, F16-9, F24-1, 24-121, and F25-7 (summarized in Table 1).

Genomic DNAs of Seven morphological taxa of fungi were extracted and its ITS regions were amplified by PCR and sequenced separately Figure 1. Sequence similarity searches for ITS region DNA were performed for each of the 7 representative fungal sequences against the non-redundant database maintained by the National Center for Biotechnology Information using the BLAST algorithm. Each ITS DNA sequence was compared by using the BLAST alignment program with data available from GenBank at the National Institutes of Health. The computer alignment provides a list of matching organisms, ranked in order of similarity between the unknown sequence and the sequence of the corresponding organism from the database, Fungi with amplified ITS sequences >98% similar were considered to be the same phylotype (Table 2).

Seventy-one pure fungal isolates from hair samples of tree shrew (T. belangeri), which were tentatively identified, based on phenotypic analysis by colonial character, pigment, stain and fungal morphology, and then these isolates are classified into 7 morphological taxa of fungi: F5 , F12-14, F14-122, F16-9, F24-1, 24-121, and F25-7.

Genomic DNAs of seven morphological taxa of fungi were extracted and its ITS regions were amplified by PCR and sequenced separately. Sequence similarity searches for ITS region DNA were performed for each of the 7 representative fungal sequences against the non-redundant database maintained by the National Center for Biotechnology Information using the BLAST algorithm. Each ITS DNA sequence was compared by using the BLAST alignment program with data available from GenBank at the National Institutes of Health. The computer alignment provides a list of matching organisms, ranked in order of similarity between the unknown sequence and the sequence of the corresponding

Table 1. Morphological taxa of fungi from tree shrew hair.

Taxa name	Number of isolates	Detection rate (%)
F5	11	17.74
F12-14	19	12.40
F14-122	8	12.90
F16-9	12	19.35
F24-1	5	16.13
24-121	9	14.52
F25-7	7	11.29

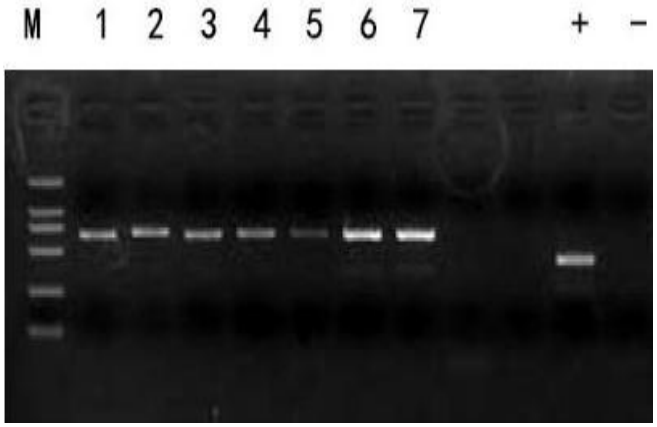

Figure 1. Agarose gel electrophoresis of PCR-amplified ITS regions of 26s rDNA. All products were electrophoresed in 3% agarose gels. M: DL 2,000 DNA marker; (1) Taxa F5 PCR product; (2) F12-14 PCR product; (3) F14-122 PCR product; (4) F16-9 PCR product; (5) F24-1 PCR product; (6) F24-121 PCR product; (7) F25-7 PCR product; + = Positive control; - = Negative control.

Table 2. Molecular identification of fungi from tree shrew hair.

Taxa	Genus	Species
F5-1	*Chaetomium*	*Chaetomium globosum*
F12-14	*Trichosporon*	*Trichosporon mucoides*
F14-122	*Corynascus*	*Corynascus kuwaitiensis*
F16-9	*Ascochyta*	*Ascochyta rabiei*
F24-1	*Alternaria*	*Alternaria alternata*
24-121	*Retroconis*	*Retroconis fusiformis*
F25-7	Debaryomyces	Debaryomyces hansenii

organism from the database, Fungi with amplified ITS sequences >98% similar were considered to be the same phylotype. ITS (internal transcribed spacer) refers to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. Read from 5' to 3', this polycistronic rRNA precursor transcript contains the 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S rRNA and finally the 3'-ETS. The ITS region is separated into ITS-1 and ITS- 2, both imme-diately flanking the 5.8s gene sequence, with the former upstream and the latter downstream of that sequence. The entire ITS region (ITS-1, 5.8s, ITS-2) ranges bet-ween 565 and 700 base pairs in angiosperms, sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because it is easy to amplify even from small quantities of DNA due to the high copy number of rRNA genes and it has a high degree of variation even between closely related species. This can be explained by the relatively low evolutionary pressure acting on such non-functional sequences. For example, ITS has proven especially useful for elucidating relation-ships among congeneric species and closely related genera in Asteraceae (Baldwin, 1992) as well as clinically important yeast species(Chen et al., 2001). The ITS region is now perhaps 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 (for example, small- and large-subunit rRNA). In addition to the standard ITS1+ITS4 primers used by most laboratories, several taxon-specific primers have been described that allow selective amplification of fungal sequences (Gardes and Bruns, 1993). ITS region is now-adays being used to know the genetic diversity among different strains of fungi by sequencing the ITS regions. Rapid advances in DNA sequencing technology led to a major change in the way prokaryotes were classified.

Sequence analysis of highly conserved regions of the bacterial genome, such as the small subunit rRNA gene, now provide us with a universal method of estimating the evolutionary relationships among all organisms. Such gene-based phylogenetic classifications led to many new discoveries about prokaryotes that were not reflected in the classical classification. Phylogenetic classification is now broadly accepted as the preferred method of representing taxonomic relationships among prokaryotes. Many taxa based on shared phenoltypic features may be quite distinct from one another based on phylogenetic evidence (Petti, 2007; Clarridge, 2004).

Conclusively, we investigated the taxonomic identities of fungal species isolated from tree shrew samples by using a combination of morphological and molecular approaches. Seven representative isolate taxa were selected for further molecular phylogenetic analysis using nuclear ribosomal internal transcribed spacer (ITS1 and ITS2) DNA sequencing. The 71 endophytes were identified to the species level based on fungal sequences with known identities in GenBank. Our results suggest that 7 fungal genera are the dominant fungal parasites on the tree hairs.

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