Molecular Phylogenetics of Exophiala Species Isolated from Korea

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Background: Recently, identification of fungi have been supplemented by molecular tools, such as ribosomal internal transcribed spacer (ITS) sequence analysis. According to these tools, morphological Exophiala species was newly introduced or redefined. Objective: This study was designed to investigate the phylogenetics based on ribosomal ITS sequence analysis from clinical Exophiala species isolated in Korea. Methods: The strains of Exophiala species were 4 clinical isolates of phaeohyphomycosis agents kept in the department of dermatology, Dongguk University Medical Center(DUMC), Gyeongju, Korea. The DNAs of total 5 strains of Exophiala species were extracted by bead-beating method. Polymerase chain reaction of ITS region using the primer pairs ITS1-ITS4, was done and phylogenetic tree contributed from sequences of ITS region from 5 Korean isolates including E. dermatitidis CBS 109154 and comparative related strains deposited in GenBank. Results: The strains of Exophiala species were 3 strains of E. dermatitidis, 1 strain of E. jeanselmei and 1 strain of Exophiala new species. Among the 3 subtypes (type A, B, C) of E. jeanselmei, E. jeanselmei DUMC 9901 belonged to type B. Of the 2 main types of E. dermatitidis (type A, B) and 3 subtypes of E. dermatitidis type A (A0, A1 and A2), two strains (E. dermatitidis CBS 709.95, E. dermatitidis CBS 109154) belonged to A0 subtypes, 1 strain (E. dermatitidis DUMC 9902) A1 subtype, respectively. Conclusion: Phylogenetic analysis of ITS region sequence provided useful information not only for new species identification but for the subtyping and origin of Exophiala species. (Ann Dermatol 24(3) 287∼294, 2012)

Keywords- Exophiala, Phylogeny, Ribosomal internal transcribed spacer

INTRODUCTION

Exophiala is the main genus of black yeasts, characterised by annellidic conidiogenesis and mostly isolated from environmental substrates, including soil, wood, and other plant material. The majority of these infections are cutaneous and subcutaneous, but fatal systemic infections can occur. Genus Exophiala includes E. jeanselmei complex, E. dermatitidis and E. spinifera complex. E. jeanselmei complex has darkened rocket-shaped conidiogenous cells without multicellular conidiophores. E. spinifera, unlike E. jeanselmei, has large multicellular conidiophores and capsular material around budding cells. E. dermatitidis has numerous conidiophores and conidiogenous cells either intercalary or free, and flask shaped. This species grows at up to 42°C, shows no growth with nitrate and nitrite and is sometimes called Wangiella dermatitidis. With recent advances in molecular biological techniques such as internal transcribed spacer (ITS) sequences analysis and phylogenetic analysis, Exophiala species has been further classified and new species have been identified and named. As a result, E. jeanselmei have recently been molecular biologically re-identified as including E. jeanselmei, E. xenobiotica, E.
Table 1. Korean isolates of *Exophiala* species

| Species and strain number | Source                     | Locality |
|---------------------------|----------------------------|----------|
| *E. jeanselmei* DUMC 9901 | Subcutaneous phaeohyphomycosis | Daegu    |
| *E. jeanselmei* DUMC 0501 | Subcutaneous phaeohyphomycosis | Jinju    |
| *E. dermatitidis* DUMC 9902 | Subcutaneous phaeohyphomycosis | Daegu    |
| *E. dermatitidis* CBS 709.95 | Subcutaneous phaeohyphomycosis | Chonnam  |
| *E. dermatitidis* CBS 109154 | Cerebral phaeohyphomycosis    | Busan    |

*E.:* Exophiala, DUMC: Dongguk University Medical Center.
homology were considered missing data, and the branch distances were calculated by using the average pathway method\textsuperscript{19}.

**RESULTS**

**PCR and sequence analysis**

The ITS regions of four Korean isolates of *Exophiala* species preserved in our hospital were amplified to produce approximately 643-bp fragments. After the ITS sequences of *E. dermatitidis* CBS 109154 and relative strains that represent each subgroup of *Exophiala* species were obtained, the length of ITS nucleotides was compared by multiple alignment of ITS sequences. The number of ITS nucleotides was around 552 in *E. jeanselmei* and around 584 in *E. dermatitidis*. There were almost no significant differences in length.

**Phylogenetic analysis**

A phylogenetic tree was constructed by NJ analysis, and the evolutionary disturbances between individual strains were described as horizontal branches. The ITS sequences of twenty five *Exophiala* strains including the five Korean isolates and twenty representative strains were compared. The five Korean isolates did not show morphological diversity and only three species, including one strain of *E. jeanselmei*, three strains of *E. dermatitidis* and one strain of other *Exophiala* species were identified. *E. jeanselmei* is taken as an outgroup. ITS: internal transcribed spacer, *E.: Exophiala*, *R.: Rhinocladiella*, sp.: species, DUMC: Dongguk University Medical Center.

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Fig. 1. Neighbor-joining tree based on sequences of the ITS region from the 25 members of *Exophiala* species and relatives; neighbor-joining algorithm with 1,000 bootstrap replicates. *R. aquaspersa* CBS 313.73 is taken as an outgroup. ITS: internal transcribed spacer, *E.: Exophiala*, *R.: Rhinocladiella*, sp.: species, DUMC: Dongguk University Medical Center.
DUMC 0501 did not show any identical ITS sequences to *Exophiala* species, which was regarded as a new species (Fig. 1).

*E. jeanselmei* was classified into three subtypes: type A, which showed identical ITS sequences to the type strain, *E. jeanselmei* CBS 507.90, and type B and C which were not. Kawasaki et al. have reported intraspecies variation of the genotypes of *E. jeanselmei* isolated from patients. To compare with subtypes of this study, we treated with suppositive restriction enzyme. It is presumed that types A and B identified by this study would be identical with E5 identified by Kawasaki et al. and that type C identified by this study would be identical with E2 or E3. *E. jeanselmei* DUMC 9901 belonged to the type B reported in Japan and United States, and showed identical ITS sequences with the Japanese species *E. jeanselmei* CBS 116.86. Korean strains and Japanese strains caused only skin infections, although there is a lack of information on human infections (Table 2, Fig. 2).

Matos et al. subclassified *E. dermatitidis* as group A (clinical strain), group B (environmental strain), group C or group D. In this study, we also subclassified the isolates as groups A, B and D. Since the ITS sequences of group C was not available, group C was excluded. Group A was further divided into 3 subgroups: group A0 which was identical with *E. dermatitidis* CBS 207.35, and groups A1 and A2 which were similar to *E. dermatitidis* CBS 207.35. All three Korean isolates were in group A. *E. dermatitidis* CBS 709.95 and *E. dermatitidis* CBS 10915 belonged to group A0, and *E. dermatitidis* DUMC 9902 belonged to

**Table 2.** Strains of *Exophiala jeanselmei* grouped by similarities in ITS region sequences

| Strain     | GenBank     | Nation   | Source                      | Genotype | Other name                  |
|------------|-------------|----------|-----------------------------|----------|-----------------------------|
| CBS 507.90 T | AY156963    | Uruguay  | Man, mycetoma               | A        | dH15933; ATCC 34123; CBS 664.76; IP 71.52 |
| CBS 528.76  | AY857530    | -        | Man, skin                   | A        | dH15968; dH3058; IP 1792.88  |
| IP 70.52   | DQ836793    | -        | -                           | A        |                            |
| BMU 00014  | AF549447    | -        | Man, arm                    | A        | UTHSCB672; UTMB2674         |
| CBS 116.86  | AY163556    | Japan    | Man, chromomycosis          | B        | dH15309                     |
| DUMC 9901  | AY163556    | Korea, Daegu | Man, subcutaneous infection | B        |                              |
| UTHSC-3338 | EF025411    | USA      | -                           | B        |                              |
| UTHSC94-28 | EF025410    | USA      | -                           | B        |                              |
| CBS 677.76  | AY163553    | UK, England | Man, skin, abscess          | C        | dH16163; ATCC 34123; IHM 1586 |
| UTHSC93-2459 | EF025412 | USA      | -                           | C        |                              |

ITS: internal transcribed spacer, DUMC: Dongguk University Medical Center.

**Fig. 2.** Neighbor-joining tree based on sequences of the ITS region from the 11 members of *E. jeanselmei* and relatives; neighbor-joining algorithm with 1,000 bootstrap replicates. *E. spinifera* CBS 899.68T is taken as an outgroup. ITS: internal transcribed spacer, *E.: Exophiala.*
A1. Since groups A0 and A1 have been isolated in many countries, including Japan, China, United States and Germany, there is no significant regional difference in isolated strains between countries. Human infections ranged in virulence from skin infections to fatal systemic infections (Table 3, Fig. 3).

A new *Exophiala* species DUMC 0501 was similar to *Pseudocladosporium* species and *E. salmonis* CBS 157.67, but no identical ITS sequences were found. This new species was presumed to originate from soil by the ITS sequences analysis with GenBank (Table 4, Fig. 1, 4).

**DISCUSSION**

*Exophiala* species invades the human body, mainly causing phaeohyphomycosis which shows brown hyphae or

![Fig. 3](image-url)
Table 4. Strains of *Exophiala* sp. DUMC 0501 (new species) and relatives according to similarities in the sequences of the ITS region

| Strain                          | GenBank     | Nation   | Source                      |
|---------------------------------|-------------|----------|-----------------------------|
| *Exophiala* sp. DUMC 0501       | DQ420723    | Korea, Jinju | Man, subcutaneous infection |
| Uncultured soil fungus clone 167-1 | DQ008140    | USA, Minesota | Soil, 553/557                |
| Uncultured ascomycete           | AM901745    | Finland  | House dust, 552/555         |
| *Pseudocladosporium* sp. CBS 115143 | DQ420723    | Australia | Bottled spring water, 541/545 |
| Fungal sp. GMG_C6                | FJ439580    | UK; Drigg | Coast soil, 549/556         |
| Uncultured soil fungus clone T1-A12 FL | GU083143    | USA; Alaska | Raw soil, 550/555           |
| *Exophiala* sp. WW-2009a MDL-15-44h | FJ65274     | Canada    | Roots of aspen, 550/555     |
| Uncultured *Herpotrichiellaceae* clone LTSP_EUKA_P6P13 | FJ554453     | Canada    | Forest soil, 550/555        |
| *Exophiala salmonis* CBS 157.67 | AF050274    | Canada    | Man, mycetoma, 538/561      |

DUMC: Dongguk University Medical Center, ITS: internal transcribed spacer, sp: species.

**Fig. 4.** Neighbor-joining tree based on sequences of the ITS region from the 9 members of *Exophiala* species DUMC 0501 (new species) and relatives; neighbor-joining algorithm with 1,000 bootstrap replicates. ITS: internal transcribed spacer, DUMC: Dongguk University Medical Center, E.: Exophiala, u.: uncultured, f.: fungal.

yeast-like spores in involved tissue. The species rarely causes either chromoblastomycosis in which round-shaped sclerotic cells or muriform cells with a thick wall are observed or eumycotic mycetoma which is characterized by the development of abscesses, draining sinuses, and the formation of fistulae discharging granules. Phaeohyphomycosis usually occurs in the skin or subcutaneous tissue. The skin lesion appears as pustules or verrucous plaques. It is rarely disseminated to the internal organs.

In Korea, three strains of *E. jeanselmei* species and three strains of *E. dermatitidis* were isolated from patients with phaeohyphomycosis. Most of these strains caused subcutaneous infections, but only one strain of *E. dermatitidis* species involved the brain. More strains of *Exophiala* species were isolated in many countries: 188 strains in the United States, 76 strains in Japan and 20 strains in China. It is expected that more strains will appear in Korea. In this study, we used two strains of *E. jeanselmei* and two strains of *E. dermatitidis* which were isolated and preserved in our hospital as well as one strain of *E. dermatitidis* CBS 109154 obtained from GenBank. One strain of *E. jeanselmei* isolated by Kim et al. was excluded from the study because no molecular biological information was available. Of these five strains, one was identified as *E. jeanselmei* by Suh et al. through morphological analysis. However, this strain was regarded as a new strain because it had no identical ITS sequences with *Exophiala* species. Therefore, molecular biological analysis is recommended as a supplementary method if morphological analysis is inadequate.

The classification and identification of *Exophiala* species have been performed by morphological examination, including colony morphology and light microscopy, as well as a physiological examination, including sugar assimilation tests and heat tolerance. Molecular biological analysis has recently been used as a supplementary method. *E. jeanselmei* species is morphologically and molecular biologically heterogeneous, whereas *E. dermatitidis* species is homogeneous.

In the past, *E. jeanselmei* species were re-identified as *E.
jeanselmei var. jeanselmei, E. jeanselmei var. heteromorpha and E. jeanselmei var. lecanii-corni by morphological and cultural features. After then, this species were further classified as E. jeanselmei var. jeanselmei, E. jeanselmei var. heteromorpha and E. jeanselmei var. lecanii-corni and E. lecanii-corni by mitochondrial DNA analysis. Furthermore, E. jeanselmei was newly subclassified as E. jeanselmei as E. jeanselmei, E. xenobiotica, E. oligosperma, E. lecanii-corni and E. heteromorpha by sequence analysis of the ITS regions, elongation factor 1-α (EF1-α) and β-tubulin (β-TUB). Currently, E. jeanselmei is much more subdivided into seven species, including E. jeanselmei, E. xenobiotica, E. oligosperma, E. exophialae, E. nishimurae, E. bergeri and E. nigra by sequence analysis of the ITS regions. The above-mentioned species was confirmed as fifteen subtypes by ITS-RFLP analysis. In this study, we classified E. jeanselmei as types A, B and C by ITS-RFLP analysis, and matched with fifteen subtypes suggested by Kawasaki et al.; types A and B are considered to be subtype E5, whereas type C is considered to be subtype E2 or E3. In addition, since E. jeanselmei DUMC 9901 corresponds to type B, and subtype E5 is the most commonly isolated subtype in Japan, there is a possibility that more type B strains will be identified in Korea. Although the DUMC O501 strain isolated in Korea was identified as E. jeanselmei by morphological and physiological analyses, it had a 84% (432/512) homology to E. jeanselmei by ITS sequences analysis. While this strain was close to Pseudocladosporium species and E. salmonis, this is regarded as a new strain because no identical ITS sequences were detected from GenBank. Since most of the strains with similar ITS sequences originate from soil, this strain is also thought to originate from soil. Because morphological and physiological analyses have some limitations in the identification of strains, molecular biological analysis is recommended as supplementary method. Since one of the five Korean isolates is a new species, further new species will be identified in Korea.

Since E. dermatitidis species is very homogeneous, it had not been subclassified by morphological or molecular biological analysis before the studies by Uijthof et al., which reported that of the five subgroups, group I is most common and the number of nucleotides in groups II to V is different by 1 to 4 from that in group I. In 2003, Matos et al. further classified E. dermatitidis species as groups A, B, C and D by ITS sequence analysis and M-13 fingerprinting. They also stated that most strains belong to groups A and B, and that group A strains are clinical isolates, whereas group B strains are environmental isolates. In this study, it was found that the 3 strains isolated in Korea were classified as A0, A1 and A2, although there were no significant differences in locations and clinical features between A0, A1 and A2.

In conclusion, the five Korean isolates did not show more diversity than western isolates and only three species, including one strain of E. jeanselmei, three strains of E. dermatitidis and one strain of other Exophiala species were identified. All these strains were isolated from patients with phaeohyphomycosis. Despite the low number of the strains included in this study, E. dermatitidis is the most commonly isolated strains from phaeohyphomycosis patients in Korea. In the Western world, however, E. jeanselmei is the main causative agent of phaeohyphomycosis.

Taken together, ITS sequence analysis and phylogenetic analysis can supplement traditional morphological and physiological analyses in the identification of Exophiala species as well as the evaluation of its distribution, determination of subtypes and detection of new species.

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