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Molecular Analysis of *Exophiala* Species Using Molecular Markers

Hee Youn Chee* and Yoon Kyoung Kim

Division of Biological Sciences, Medical School, Konyang University, Nonsan, Chungnam 320-711, Korea

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Genetic relatedness of medically important *Exophiala* species such as *E. dermatitidis*, *E. mansonii*, and three *E. jeanselmei* varieties: *jeanselmei*, *lecanii-corni*, and *heteromorpha* was examined using PCR-RFLP (restriction fragment length polymorphism) of ribosomal DNA, M-13, $(GTG)_5$, and nucleotide sequences of ribosomal ITS (internal transcribed space) II regions. Three *E. jeanselmei* varieties showing distinct band patterns for each DNA markers as well as different nucleotide sequences of ribosomal ITS II regions could be considered as a separate species. *E. dermatitidis* and *E. mansonii* demonstrated the identical band patterns of RFLP of ribosomal DNA, M-13, and $(GTG)_5$ markers. However, nucleotides sequences of ribosomal ITS II region were different between these two species.

KEYWORDS: ***Exophiala*, Genetic relatedness, $(GTG)_5$, M-13, PCR-RFLP, Ribosomal DNA**

Exophiala is an ascomycetous black yeast forming annelide with balls of conidia at their apices. Main characteristics of *Exophiala* species capable of differentiating from other ascomycetous black yeast genera such as *Phialophora* and *Wangiella* is the formation of annelides rather than phialides. The ecological habitats of *Exophiala* species are diverse. They can be found on decaying wood, soil, and many other sources. Some species of *Exophiala* are known to cause a subcutaneous infection disease in human and other vertebrates. Specially, *Exophiala* species such as *E. dermatitidis* and *E. jeanselmei* are the primary pathogen of systemic mycoses in human.

The classification and identification of *Exophiala* species have been considered to be complicated by their morphological variability and the adaptability of morphological traits to environmental changes (Uijthof, 1996). Identification on the basis of morphological characteristics, therefore, may not lead to proper classification of *Exophiala* species. In *E. jeanselmei* four varieties had been described, *jeanselmei*, *lecanii-corni*, *heteromorpha* and *castellani* (de Hoog, 1997; Iwatsu and Udagawa, 1990). However, there are increasing evidences showing that these varieties should be reconsidered as a separate speices (Uijthof, 1996; Kawasaki, 1999). Taxonomic relationship between *E. dermatitidis* and *E. mansonii* is not clearly defined (de Hoog and McGinnis, 1987).

In recent molecular characters based upon the analysis of nucleic acid have been widely used to classify and identify *Exophiala* species. Matsuda *et al.* (1989) used DNA hybridization to differentiate *E. dermatitidis* from *E. jeanselmei*. Kawasaki *et al.* (1990) also differentiated *E. dermatitidis* from *E. jeanselmei* by mitochondrial DNA analysis. Uijthof *et al.* (1994) estimated genetic diversity of *E. dermatitidis* strains from infected patients by using ribotyping of the small subunit rDNA and random amplified polymorphic DNA (RAPD). Uijthof *et al.* (1995) also analyzed genetic relationship between several *Exophiala* species and other synonym members on the basis of internal transcribed space (ITS) I DNA sequences. Kawasaki *et al.* (1999) discriminated *E. jeanselmei var. lecanii-corni* from *E. castellanii* using mt DNA analysis.

Repetitive DNA markers with tandem repeats of a consensus sequence have been used to study intraspecific genetic variation in fungi. PCR amplification with the M-13 forward sequences primer allowed for strain typing in *Lentinula edodes* (Kwan *et al.*, 1992). Genetic variation in *Heterobasidion annosum* was revealed by M-13 markers (Stenlid *et al.*, 1994). Freeman and Rodriguez (1997) used microsatellite such as $(CAG)_5$ and $(GACA)_5$ to estimate genetic variation among strawberry isolates of *Colletotrichum acutatum*.

In this study, we examined genetic relatedness of *Exophiala* species including *E. jeanselmei* varieties: *jeanselmei*, *lecanii-corni*, and *heteromorpha* using RFLP of ribosomal DNA, M-13, $(GTG)_5$, and nucleotide sequences of ribosomal ITS regions and provided molecular datas to evaluate the classification of *Exophiala* species.

Materials and Methods

Isolates. Isolates were obtained from Korean Collection of Type Culture (KCTC). Isolates used in this study are as follows: *Exophiala moniliae* (KCTC 6801), *E. dermatitidis* (KCTC 6794), *E. jeanselmei* var. *jeanselmei* (KCTC 6795), *E. jeanselmei* var. *heteromorpha* (KCTC 6796), *E. jeanselmei* var. *lecanii-corni* (KCTC 6797) and *E. mansonii* (KCTC 6798). Isolates were grown in malt extract broth in 150 m*l* flask on an orbital shaker at 25°C. After

*Corresponding author

12 day-incubation, mycelia were harvested by vaccum filtration, frozen overnight. The mycelia were stored at -20°C.

DNA preparation. Total DNA was extracted by miniprep procedures (Uijthof *et al.*, 1994). Mycelia were dissolved in 1 m*l* TES buffer (0.1 mM Tris-HCl, pH 8.0; 10 mM EDTA; 2% SDS). Glass beads (diam. 0.45~0.50 mm) were added to mycelial suspension. The mexture was vortexed for 5 min. 20% sodium dodecyl sulfate (SDS) was added, mixed, and the tubes were incubated at RT with gently shaking for 1 h. 5 M NaCl and 10% CTAB/NaCl were added, and incubated at 65°C for 30 min. An equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) was added and then centrifuged at 12,000 rpm for 10 min. The upper phase was precipitated by adding 0.1 volumn of 3 M sodium acetate and 0.6 volume isopropanol. The tubes were centrifuged for 5 min. The pellet was rinsed with 70% ethanol and dried. The pellet was resuspended in distilled water.

RFLP of ribosomal DNA. PCR was performed in a total of 100 μl containing 100 ng template DNA, 10 μl of 10× reaction buffer, 5 mM $MgCl_2$, 200 uM dNTP, one unit of *Taq* DNA polymerase and 0.5 μl of primer ITS 1 and 4. DNA was amplified in the thermocycler (Perkin-Elmer) for 40 cycles of 1 min at 94°C, 2 min at 48°C and 2 min at 72°C. Final extension was carried out for 7 min at 72°C. PCR products were digested with 6 restriction endonucleases (*Alu*I, *Rsa*I, *Hae*III, *Dde*I, *Tru9*I, *Cfo*I) according to the manufacturer's instruction. The digested DNA was run through a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

DNA sequencing. PCR was performed in a total of 50 μl containing 50 ng template DNA, 5 μl of 10× reaction buffer, 5 mM $MgCl_2$, 200 uM dNTP, 4 unit of *Taq* DNA polymerase and 50 pmol of each primer. Primer ITS 3 and ITS 4 were used for amplification. DNA was amplified in the thermocycler for 4 cycles of 1 min at 94°C, 2 min at 48°C and 2 min at 72°C. The remaining primers and dNTP were removed by isolating the products with Gene Clean kit according to the instruction of the menufacturer. The amplicons were sequenced with a dye terminating protocol on an ABI automatic sequencer with primer ITS 3 and ITS 4.

Arbitrarily primed PCR (AP-PCR). PCR reactions were performed in a total of 25 μl containing 10 ng template DNA, 2.5 μl of 10× reaction buffer, 5 mM $MgCl_2$, 200 uM dNTP, 4 unit of *Taq* polymerase and 2 uM primer. DNA was amplified in the thermocycler for 30 cycles of 30 sec at 94°C, 30 sec at either 52°C for M-13 primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') or 48°C for (GTG)5 primer and 1 min at 72°C. Final extension was carried out for 7 min at 72°C. The amplification products were run through a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

Result and Discussion

Amplification of the ITS1-ITS4 region resulted in an approximately 600 bp fragment for all the *Exophiala* species studied. In order to differentiate isolates at species level, PCR products were subject to digestion with 6 different restriction endonucleases. Fig. 1 shows the banding pattern of the fragments obtained with the digested riboso-

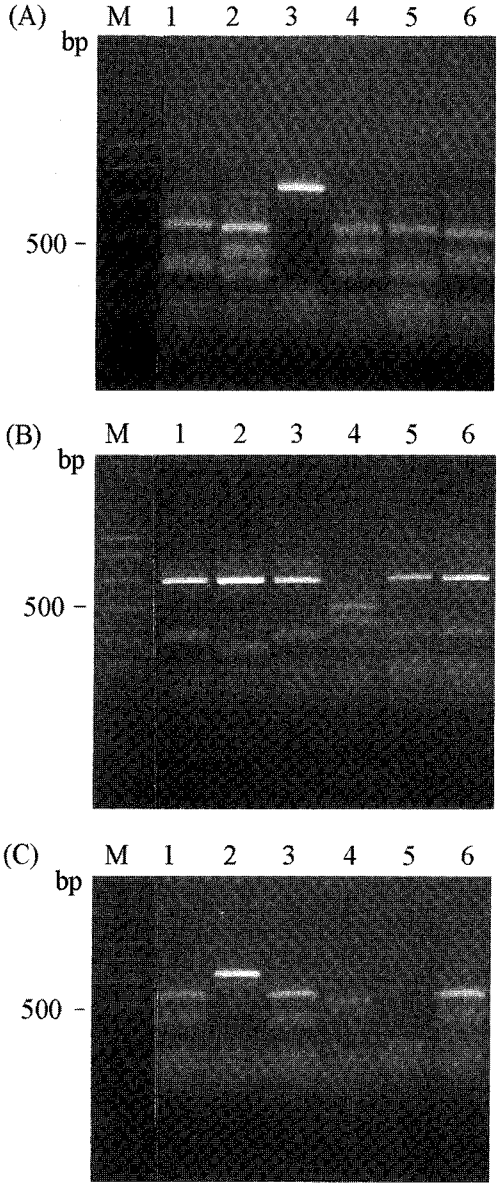

Fig. 1. Restriction band pattern of ITS of rDNA after digestion with restriction endonuclease. (A) *Dde*I, (B) *Rsa*I, (C) *Hae*III, M. Molecular weight marker, 1. *Exophiala dermatitidis*, 2. *E. jeanselmei* var. *jeanselmei*, 3. *E. j.* var. *heteromorpha*, 4. *E. j.* var. *lecanii-corni*, 5. *E. mansonii*, 6. *E. moniliae.*

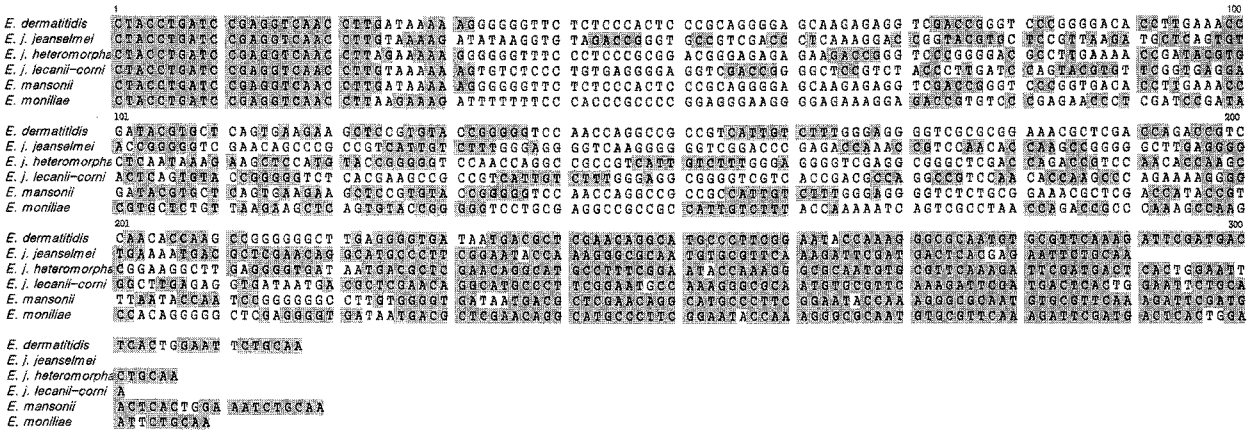

Fig. 2. Partial ribosomal ITS II sequence of *Exophiala* species.

mal DNA of isolates. All the restriction endonuclease produced polymorphic bands among isolates. Digestion of ITS1-4 amplicon resulted in the species-specific pattern (Fig. 1). Each species can be differentiated by combined results of ribotyping from different restriction endonucleases.

In *E. jeanselmei*, on the basis of morphological characters, 4 varieties had been discribed as *jeanselmei*, *lecanii-corni*, *heteromorpha* and *castellani* (de Hoog, 1977). However, recent studies demonstrated that varieties of *E. jeanselmei* could be considered as a separate species. On the basis of ribosomal ITS I DNA sequences, Uijthof (1996) observed that *E. jeanselmei* varieties are found in 4 different clusters and claimed the status of independent species. In this study three *E. jeanselmei* varieties could be differentiated from each other by digestion with *Hae*III, *Rsa*I, and *Dde*I (Fig. 1). Each restriction endonuclease produced distinct band patterns for all the *E. jeanselmei* varieties. For *E. jeanselmei* var. *jeanselmei*, restriction site of *Hae*III was absent in amplified products whereas *E. jeanselmei heteromorph* and *E. jeanselmei lecanii-corni* has different restriction sites for *Hae*III. Thus, digested products of *Hae*III, *Rsa*I and *Dde*I could be useful RFLP markers to discriminate *E. jeanselmei* varieties in ribotyping.

Ribosomal ITS II region sequences was determined by sequencing ITS3-ITS4 PCR products. DNA sequences of ITS II region showed that different species contain different length of amplicon. The length of the amplification products ITS3-ITS4 among *Exophiala* species was variable. Many variable domains were interspersed with conserved region (shaded area) of the ITS II region (Fig. 2). Nucleotides sequences of all the species could not be aligned properly since each species contained several variable domains at many sites in which alignment was ambiguous. Thus, different nucleotide length of ITS II region for each *Exophiala* species could be explained by variable size and the number of insert variable domains (Fig. 2). From our results, we concluded that ITS II region of each species contain variable domains interspersed between conserved domains. Especially, although ITS II sequences could not provide proper analysis of phylogenetic relationship between isolates, DNA sequences highly varied between *E. jeanselmei* varieties.

In addition, M-13 and $(GTG)_5$ revealed distinct band patterns among these varieties (Fig. 3 and 4). All these results are consistent with Uijthof's data analysis of ITS I sequences, forming independent cluster of each variety (Uijthof's, 1996). Thus our results based on ribotyping, M-13, $(GTG)_5$, and ITS II DNA sequences strongly supported that these varieties could be treated as a separated species. *E. dermatitidis* and *E. jeanselmei* varieties could be clearly differentiated by M-13 and $(GTG)_5$ markers as well as RFLP of ribosomal DNA. Clear distinction between *E. dermatitidis* and *E. jeanselmei* is consistent with the result of Matsuda *et al.* (1989).

We examined the hypothesized taxonomic relationship

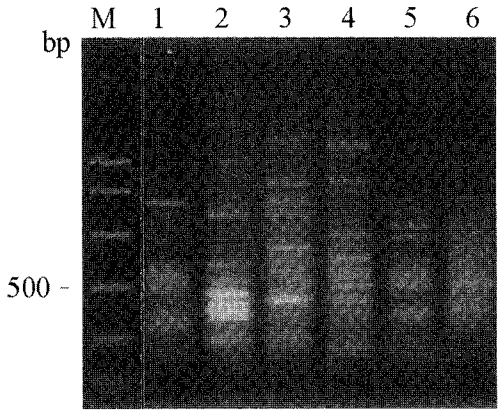

Fig. 3. Agarose gel electrophoresis of PCR products with M-13 primer. M. Molecular marker, 1. *Exophiala dermatitidis*, 2. E. *jeanselmei* var. *jeanselmei*, 3. *E. j.* var. *heteromorpha*, 4. *E. j.* var. *lecanii-corni*, 5. *E. mansonii*, 6. *E. moniliae.*

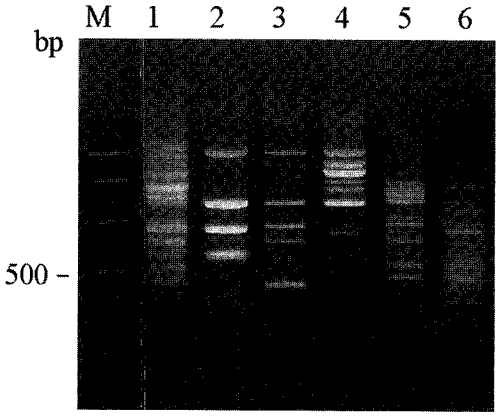

Fig. 4. Agarose gel electrophoresis of PCR products with $(GTG)_5$-primer. M. Molecular marker, 1. *Exophiala dermatitidis*, 2. *E. jeanselmei* var. *jeanselmei*, 3. *E. j.* var. *heteromorpha*, 4. *E. j.* var. *lecanii-corni*, 5. *E. mansonii*, 6. *E. moniliae.*

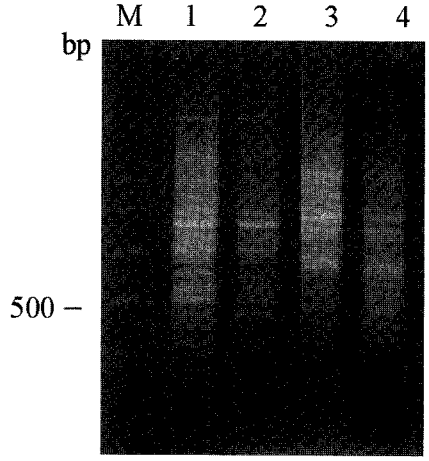

Fig. 5. Agarose gel electrophoresis of PCR products with M-13 and $(GTG)_5$ primer. M. Molecular marker, (M-13): 1. *Exophiala dermatitidis*, 2. *E. mansonii* $(GTG)_5$: 3. *E. dermatitidis*, 4. *E. mansonii.*

between *E. dermatitidis* and *E. mansonii.* These species have been considered as a separate species. However, de Hoog and McGinnis (1987) claimed that these species could belong to the same species as a synonymy. In our result, *E. mansonii* and *E. dermatitidis* represented identical RFLP pattern of ribosomal DNA (Fig. 1). In addition, M-13 and $(GTG)_5$ amplification resulted in the identical band patterns (Fig. 5). This gives surprising consideration that M-13 and $(GTG)_5$ markers are usually distinct between different species. Thus, on the basis of RFLP of ribosomal DNA as well as M-13 and $(GTG)_5$ markers, *E. dermatitidis* and *E. mansonii* showed genetically same species. However, DNA sequences variations of ITS II region were different between these two species (Fig. 2). Ten out of 310 nucleotides was different between *E. dermatitidis* and *E. mansonii.* However, minor genetic variation could occur among isolates belong to the same species. Although the taxonomic relationship between *E. dermatitidis* and *E. mansonii* could not be confirmed by the present study, our results demonstrated that these two species is genetically very similar. Further studies of molecular identifications between these two species will be needed to confirm taxonomic relationship.

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