Original Article

Isolation and Genotype Analyses of Ascospores Produced between Genetically Different *Arthroderma benhamiae* Strains

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Abstract

Thirty-one single ascospore cultures were obtained from one ascoma produced in mating of RV 26678 (+) (Institut de Medicine Tropicale, Antwerp, Belgium) and KMU 4169 (−) (Dept. Dermatology, Kanazawa Medical University, Uchinada, Japan), which are genetically different strains of *Arthroderma benhamiae*. The isolation was performed with the aid of an inverted microscope and a binocular microscope. The internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene of all the ascospore cultures were analyzed by restriction fragment length polymorphism (RFLP) with the restriction enzyme Hinfl. The mating types of all the single-ascospore cultures were also checked. Eight cultures had mating type (+) and RV 26678 genotype, 10 had mating type (−) and RV 26678 genotype, 6 had mating type (+) and KMU 4169 genotype and 7 had mating type (−) and KMU 4169 genotype. There was no linkage between the mating types and the genotypes, implying that the genes control the mating behavior and the genes of ribosomal RNA are on different chromosomes from each other. The hybrids comprised half of the isolates and so they were actually from the ascospores and not from the microconidia or the peridial hyphae.

Key words: *Arthroderma benhamiae*, single ascospore culture, mating type, genotype, hybrid

Introduction

*Arthroderma benhamiae* is comprised of two genetically different races: an African race and an Americano-European race[1-2]. Recently, isolates of both races have been found in Japan[3,4]. We successfully mated a clinical isolate, KMU 4169, from Gifu prefecture, Japan with RV 26678 (Institut de Medicine Tropicale, Antwerp, Belgium) which is *A. benhamiae* Americano-European race mating type (+). However, the restriction fragment length polymorphism (RFLP) pattern of the mitochondrial DNA (mtDNA) of the clinical isolate KMU 4169 was unexpectedly identical with that of *T. verrucosum*. The isolate was therefore presumed to be genetically different from the African and Americano-European races, even though it was identified as *A. benhamiae* from the mating tests. To deepen our knowledge of the sexual reproduction in dermatophytes, we studied the genotypes of ascospores from an ascoma produced from KMU 4169 and RV 26678. In this paper, we report our technique of making single-spore cultures and our studies on the ascospores, which involved RFLP analysis of the ribosomal RNA gene and mating tests.

Materials and Methods

Strains used in this study:
The *Arthroderma benhamiae* Americano-European race mating type (+) RV 26678, and the clinical isolate KMU 4169, which originated from a skin lesion on the face of a pet shop employee in Gifu, Japan were used in this study. KMU 4169 was identified as *T. mentagrophytes* based on the morphological features, as *A. benhamiae* based on the mating tests and as *T. verrucosum* based on the mtDNA-RFLP pattern. We studied one of many ascomata produced between the two isolates (Fig. 1).

Single-ascospore culture:
One ascoma randomly chosen and picked up
from the mating plate with a sterilized sewing needle, was put on a new agar plate under a binocular microscope (Nikon SMZ-10, Japan) and rolled around gently on the agar plate to remove any microconidia and hyphae. The ascoma was cracked open and a clump of yellow asci and ascospores was scratched out and put on a new agar plate with a new needle. The cells were confirmed to be ascospores under an inverted microscope (Nikon DIAPHOT, Japan) at 400x magnification (Fig. 2). Under a binocular microscope, ascospores were drawn into a 5 ml syringe with a needle and suspended in 1 ml of Sabouraud’s broth medium. The suspension was dropped onto lined squares of a new agar plate (Fig. 3), then the remaining suspension was diluted with an equal volume of the Sabouraud’s broth medium and dropped again. This was repeated until all the squares had been used. The plate was then kept at room temperature for 36
hours. Germination is noticeable by the surface of the spores becoming shiny, making them easier to see (Fig. 4). A germinating spore distant from other spores was chosen and confirmed under 400x magnification to be a single entity. The magnification was again reduced to 40x. The area around an ascospore to be isolated was marked by making pits with a needle (Fig. 5) while viewing under the inverted microscope. The plate was then put under the binocular microscope at 40x magnification. The agar was cut into a tiny block with the ascospore on top of it and, using the tip of the needle, transferred onto one of the lined squares with numbers of a new agar plate (Fig. 6). The agar plate from which the ascospore was picked up and the plate onto which it was inoculated were both observed at 400x magnification to confirm that no other cells were transferred. The procedure was repeated for each of the 75 ascospores. After incubation at 25°C for 3 days, the tiny colony in each square was transferred again under the binocular microscope to a separate slant agar medium, given the square number as a reference number and kept at 25°C until used.

Mating tests:
All the isolated single-ascospore cultures were crossed with tester strains of *A. benhamiae* Americano-European race, RV 26678 (+) and RV 26680 (−). They were confronted on 1/10 Sabouraud dextrose agar medium. The mating types of 31 single-ascospore cultures were determined based on the production of gymnothecia or pseudo-gymnothecia using *A. benhamiae* Americano-European race tester strains. Fourteen of 31 cultures were mating type (+) and 17 were mating type (−). Results of the mating tests are shown in Table 1.

Genotype analysis:
The genotype was analyzed using restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS) regions (ITS-RFLP) of the ribosomal DNA (rDNA). First, the total DNA of the KMU 4169 was prepared and then the nucleotide sequence of the ITS region was determined by the method of Makimura *et al.* In brief, a small amount of mycelium grown on a Sabouraud dextrose agar slant was treated with 70% ethanol for a day and crushed with a conical grinder in lysis buffer, then incubated at 100°C for 5 min. After adding a half volume of 3 M sodium acetate it was kept at-20°C for 10 min and then centrifuged at 12,000 Xg for 5 min. The supernatant was extracted with phenol-chloroform. The total DNA was precipitated with an equal volume of isopropanol, dried and dissolved in 30 μl of distilled water. Using 2 μl of total DNA as a template, the ITS regions of the rDNA were amplified with primers of ITS1 and ITS4 by the polymerase chain reaction (PCR). PCR was performed under the following conditions: 94°C for 60 sec, 58°C for 120 sec, and 72°C for 90 sec for 35 cycles in the 20 μl of mixture using Taq DNA Polymerase (QIAGEN GmbH, Germany, www.qiagen.com). The sequencing reaction was also performed using the primer set of ITS1 and ITS4, ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits (PE Biosystems, USA), and the automated sequence readings were performed with a model ABI PRISM 310 Genetic Analyzer (PE Biosystems). Then one restriction endonuclease was selected to discriminate the two parental rDNAs on the basis of their ITS sequences.

The ITS-RFLP analysis of each ascospore culture was performed as follows. The total DNA of each culture prepared by the method of Makimura *et al.* was dissolved in 30 μl of distilled water. Using 2 μl of total DNA as a template, the ITS regions of the rDNA were amplified with primers of ITS1 and ITS4 by PCR under the above-mentioned conditions. The resultant ITS fragments were digested with the restriction endonuclease Hinfl and electrophoresed on a 5% acrylamide gel and stained with ethidium bromide. The RFLP patterns appearing on a gel were observed under ultraviolet light.

Results
Of 75 isolated ascospores, 31 formed colonies. Mating types of 31 single-ascospore cultures were determined based on the production of gymnothecia or pseudo-gymnothecia using *A. benhamiae* Americano-European race tester strains. Fourteen of 31 cultures were mating type (+) and 17 were mating type (−). Results of the mating tests are shown in Table 1.

The nucleotide sequence of an ITS fragment from KMU 4169 was identical to that of KMU 4136 (GenBank Accession No. AB048192)
previously reported\(^1\). On the basis of the ITS sequences, the restriction enzyme *Hinfl* was selected as the most suitable for discriminating the ITS fragments of RV 26678 from those of KMU 4169. The ITS-RFLP pattern of KMU 4169 with *Hinfl* was similar to that of the African race strain RV30000. The genotype of each single-ascospore culture based on the ITS-RFLP pattern with *Hinfl* is also summarized in Table 1. Eighteen cultures had the genotype of RV 26678 and the other 13 had the genotype of KMU 4169 (Table 2).

### Discussion

**The isolation technique and its usefulness**

To make single-ascospore cultures, a manipulator or a dilution method has usually been employed\(^2\), but both have many drawbacks. The manipulator method requires a skilled technique and much time, and the dilution method sometimes lacks confirmation of a single ascospore.

In this study, we employed a modified dilution method. Since ascospores were isolated not after the formation of colonies but just after the germination, we were able to confirm them to be single cells, even after isolation on an inverted microscope.

The spores were made easy to find by marking the outline of each drop of spore suspension and placing the undiluted suspension beside the diluted suspension. This technique does not require any special tool or apparatus and is not time-consuming. The only difficult step, which requires some experience and training, is to locate the spore in the area demarcated by the pits under a binocular microscope at 40x magnification.

**Mating type and genotype**

Of 31 single-ascospore cultures, 8 cultures had mating type (+) and RV 26678 genotype, 10 had mating type (−) and RV 26678 genotype, 6 had mating type (+) and KMU 4169 genotype and 7 had mating type (−) and KMU 4169 genotype (Table 2). From the result, no linkage between the mating types and the genotypes was elucidated, implying that the genes control the

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### Table 1. Genotype and mating type of each single ascospore culture

<table>
<thead>
<tr>
<th>Reference No.</th>
<th>ITS-RFLP type</th>
<th>Mating type</th>
<th>Hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-3</td>
<td>+</td>
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<td></td>
</tr>
<tr>
<td>4-6</td>
<td>–</td>
<td>+</td>
<td></td>
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<tr>
<td>4-7</td>
<td>+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>4-9</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>4-10</td>
<td>+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>4-12</td>
<td>–</td>
<td>+</td>
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</tr>
<tr>
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<td>+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>4-16</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>4-17</td>
<td>+</td>
<td>–</td>
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</tr>
<tr>
<td>4-18</td>
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<td>+</td>
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</tr>
<tr>
<td>4-29</td>
<td>+</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

\(^{31}\) strains, \(^{16}\) strains
mating behavior and the genes of ribosomal RNA are on different chromosomes from each other.

The frequency ratio is close to the theoretical ratio, 1:1:1:1, which is expected for 8 ascospores produced by meiosis of an ascogenous cell. Consequently, these 31 arbitrarily selected cultures could be germinated from ascospores and not from microconidia or peridial hyphae.

The single-ascospore cultures established from one ascoma produced between genetically different parents will be very helpful in genetic investigations of sexual reproduction.

**Acknowledgement**

We thank Prof. Atsuhiko Hasegawa, Nihon University, Japan, for important suggestions on starting this study.

**References**


5) Takashio M: Sexual reproduction of some *Arthroderma* and *Nannizzia* on diluted Sabouraud agar with or without salts. Mykosen **15**: 11-17, 1972.


<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mating type (+)</th>
<th>Mating type (-)</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
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<td>10</td>
<td>18</td>
</tr>
<tr>
<td>KMU 4169-type</td>
<td>6</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>14</strong></td>
<td><strong>17</strong></td>
<td><strong>31</strong></td>
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</table>