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SPECIES DELIMITATION IN THE ARMILLARIA MELLEA COMPLEX BY ANALYSIS OF NUCLEAR AND MITOCHONDRIAL DNAs

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Nuclear and mitochondrial DNAs were isolated from mycelia of two different isolates of Armillaria mellea, A. bulbosa and A. obscura originating from widely separated localities. Nuclear GC contents ranged from 46.0 to 48.1 mol%. Nuclear DNA–DNA homologies obtained by a fast spectroscopic method were 90–100%, for interspecific hybrids and 44–70% for intergeneric hybrids. Restriction enzyme cleavage patterns of mitochondrial DNAs using six different restriction enzymes yielded interspecific similarities from 0 to 30% and intraspecific similarities from 67 to 100%. Phylogenetic distances of the fungal species could not be deduced unequivocally from the mtDNA restriction data. Average mitochondrial genome sizes were estimated to be between approximately 90 and 100 kb.

Armillaria species are among the most damaging root- and butt-rotting pathogens of trees. Species identification based on slight morphological differences proved to be difficult, and taxonomic confusion surrounding Armillaria has long hampered investigations of this important phytopathogenic genus (Watling, Kile & Gregory, 1982). Introduction of DNA–DNA hybridizations (Jahnke & Bahnweg, 1986) and restriction enzyme analysis of mitochondrial DNAs to elucidate taxonomic problems in fungi at the species level (McArthur & Clark-Walker, 1983; Kozlowski & Stepren, 1982) have facilitated recognition of species boundaries and evolutionary distances between closely related species. The aim of this study was to explore the correlation of such molecular methods with other techniques of taxonomy using three taxonomically, genetically and physiologically well-defined intersterile pairs of Armillaria isolates (Korhonen, 1978; Roll-Hansen, 1985; Worrall, unpubl.).

Fungal cultures used for DNA analysis are listed in Table 1. These isolates were deposited in the German Collection of Micro-organisms (DSM), Grisebachstr. 8, 3400 Göttingen, F.R.G. Mycelia for DNA extraction were grown in submerged culture in large culture vessels containing 2500 ml glucose/asparagine medium (Moody & Weinhold, 1972) on a reciprocal shaker at 100 strokes per min at room temperature. Whole-cell DNA was isolated as described by Jahnke & Bahnweg (1986). Mitochondrial DNA was separated from nuclear DNA by ultracentrifugation gradients based on Kohn & Fuchs (1972), however, bisbenzimide was used. Approxim. 100 000 g were discovered from 1 mg of DNA and 60 ml of bisbenzimide. The DNA was fragmented by centrifugation in tubes (12 000 g) and the DNA was recovered from the reaction mixture using centrifugation. Restriction enzyme digestion was performed in 20 ml of 100 mm Tris–HCl, 100 mm NaCl, 10 mm MgCl₂, and 1 unit of the enzyme. The DNA was digested for 1 h at 37 °C. The DNA fragments were separated by electrophoresis through 0.8% agarose gels using Tris–HCl buffer (pH 7.5) at 100 V, 1 °C/h. The gels were stained with ethidium bromide and exposed to a light source. The restriction patterns were compared with the restriction of the EII as standard.

Table 1. Organisms

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Isolate</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Armillaria mellea (Vahl. Fr.)</td>
<td>M 3</td>
<td>England, Norfolk, on Fraxinus excelsior</td>
<td>J. Rishbeth</td>
</tr>
<tr>
<td>2</td>
<td>Armillaria mellea (Vahl. Fr.)</td>
<td>PP</td>
<td>France, Vaucule, on Prunus persica</td>
<td>Guillaumier &amp; Pierson (1978)</td>
</tr>
<tr>
<td>3</td>
<td>Armillaria bulbosa (Barla) Romagn.</td>
<td>B1</td>
<td>England, Norfolk, isolated from rhizomorphs</td>
<td>J. Rishbeth</td>
</tr>
<tr>
<td>4</td>
<td>Armillaria bulbosa (Barla) Romagn. 780811.1</td>
<td></td>
<td>F. R. G., Eberglötz en near Göttingen, on Pseudotsuga menziesii</td>
<td>K. Korhonen</td>
</tr>
<tr>
<td>5</td>
<td>Armillaria obscura (Pers.) Herink = A. ostoyae (Romagn.)</td>
<td>780811.1.2</td>
<td>F. R. G., Eberglötz en near Göttingen, on Picea omoëka</td>
<td>K. Korhonen</td>
</tr>
<tr>
<td>6</td>
<td>Armillaria obscura (Pers.) Herink = A. ostoyae (Romagn.)</td>
<td>0 3</td>
<td>England, Suffolk, on Pinus sylvestris</td>
<td>J. Rishbeth</td>
</tr>
</tbody>
</table>

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Table 2. Nuclear GC contents and nuclear DNA–DNA homologies of Armillaria spp.

<table>
<thead>
<tr>
<th>Species/Strain</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>mol % G+C†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. mellea M 3</td>
<td>—</td>
<td>97±4</td>
<td>64±9</td>
<td>55±5</td>
<td>56±2</td>
<td>n.d.</td>
<td>47±3</td>
</tr>
<tr>
<td>A. mellea PP</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>46±6</td>
</tr>
<tr>
<td>A. bulbosa B 1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>45±9</td>
<td>44±2</td>
<td>—</td>
<td>46±6</td>
</tr>
<tr>
<td>A. bulbosa 780811.1.1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>100±0</td>
<td>—</td>
<td>—</td>
<td>46±6</td>
</tr>
<tr>
<td>A. obscura 780811.2.3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>46±6</td>
</tr>
<tr>
<td>A. obscura O 3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>47±1</td>
</tr>
</tbody>
</table>

* Measurements in 2 × SSC (0.03 M trisodium citrate 0.03 M-NaCl, pH 7.0).
† GC content obtained from two measurements in 0.1 × SSC (0.0015 M trisodium citrate, 0.0015 M-NaCl, pH 7.0).

DNA by ultracentrifugation in CsCl–bisbenzimide gradients based on the DAPI–CsCl procedure of Ghimczak & Prell (1984). Instead of DAPI, however, bisbenzimide (Hoechst Dye 33258) was used. Approximately 1.5 mg of DNA were dissolved in 7 ml 0.1 × SSC buffer (0.001 M-NaCl + 0.0015 M trisodium citrate, pH 7.0), and 77 g CsCl and 60 μl of bisbenzimide (1 mg/ml) were added. The DNA was centrifuged in a Beckmann L8-70M ultracentrifuge in a 75 Ti rotor at 42 000 rev. min⁻¹ (120 000 g) for 38 h at 20 °C. The DNA bands were visualized by u.v. irradiation and recovered from the gradient using a gradient fractionator developed by Jahnke & Prell (1986). After removal of the fluorescing dye from the DNA by extraction with buffered-CsCl-saturated isopropanol and dialysis against 0.1 × SSC buffer, the DNAs were concentrated by isopropanol precipitation (Maniatis, Fritsch & Sambrook, 1982).

Spectroscopic measurement of GC contents and nuclear DNA–DNA homologies were performed previously described (Jahnke & Bahnwe, 1986; DeLey, Cattoir & Reynaerts, 1970). Second-order rate plots were calculated from renaturation curves by the method of Wetmur & Davidson (1968) as described by Jahnke (1984).

Fungal mtDNAs were analysed by restriction analysis following basic protocols described by Maniatis et al. (1982). Ten units of restriction enzyme were incubated per μg of fungal mtDNA for 4 h at 37 °C. Restriction enzymes HindIII, PstI, XbaI, AvaII, ClaI and BamHI were purchased from Boehringer, Mannheim, F.R.G. For direct comparison the six different mtDNAs cleaved with the same particular restriction enzyme were always run on the same agarose gel. Molecular weights of mtDNA fragments were determined in 1.2% agarose gels using A-phage fragments generated by restriction with HindIII, PvuI, BamHI and XbaI as standards. DNA–DNA homologies were calculated from restriction patterns for each pair of the isolates as described by Kozlowski & Stepień (1982) using the formula

$$S = \left(\frac{2e_{xy}}{u_x + u_y + e_{xy}}\right)100,$$

where $e_{xy}$ represents the number of bands common to isolates x and y, and $u_x$ and $u_y$ represent numbers of unique bands. Hypothetical family trees were constructed by average linkage cluster analysis from these homology data as described by Bahnweg & Jäckle (1986).

The GC range of the nuclear DNAs of the six *Armillaria* strains investigated was narrow, ranging from 46.0 to 48.1 mol% G+C (Table 2). Second-order rate plots calculated from the renaturation curves for all isolates were markedly straight, indicating that second-order conditions prevailed almost instantly after the start of the reaction. This may be due to the absence of mitochondrial DNA in the sample. Since large amounts of mtDNA may significantly influence hybridization measurements, the use of purified nuclear DNA instead of whole-cell DNA is recommended.

Isolates belonging to the same species had very high spectroscopic DNA–DNA homologies of close to 100%. Interspecific hybridizations gave values between 44 and 70% (Table 2). This is in good agreement with results obtained previously on other basidiomycetes (Jahnke & Bahnweg, 1986). DNA–DNA homology values of 30–40% obtained with the spectroscopic method of DeLey et al. (1970) do not indicate close relatedness (Huss, V. A. R., Festl, H. & Schleifer, K. H., 1983; Koops & Harms, 1985; Jahnke & Bahnweg, 1986).

The similarities of mitochondrial genomes were calculated from restriction patterns using six different restriction enzymes. The genome sizes of all strains were very similar, averaging approximately 90–95 kb. Depending on the restriction enzyme used, 7–28 fragments were generated (Table 3). Restriction patterns of different isolates

Reference
J. Rishbeth
Guillaumin & Piers (1978)
J. Rishbeth
K. Korhonen
K. Korhonen
J. Rishbeth

Table 3. Numbers of mtDNA fragments generated by restriction enzyme digestion and average mtDNA genome sizes

<table>
<thead>
<tr>
<th>No.</th>
<th>Species/Strain</th>
<th>HindIII</th>
<th>BamHI</th>
<th>XbaI</th>
<th>ClaI</th>
<th>PstI</th>
<th>AvaI</th>
<th>mtDNA genome size (kbp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>A. mellea</em> M. 3</td>
<td>24</td>
<td>11</td>
<td>13</td>
<td>14</td>
<td>16</td>
<td>24</td>
<td>89.45 ± 10.51</td>
</tr>
<tr>
<td>2</td>
<td><em>A. mellea</em> PP</td>
<td>25</td>
<td>10</td>
<td>18</td>
<td>16</td>
<td>16</td>
<td>28</td>
<td>96.60 ± 12.33</td>
</tr>
<tr>
<td>3</td>
<td><em>A. bulbosa</em> B 1</td>
<td>22</td>
<td>7</td>
<td>19</td>
<td>12</td>
<td>19</td>
<td>22</td>
<td>93.07 ± 10.99</td>
</tr>
<tr>
<td>4</td>
<td><em>A. bulbosa</em> 780811.1.1</td>
<td>22</td>
<td>9</td>
<td>22</td>
<td>13</td>
<td>20</td>
<td>22</td>
<td>98.79 ± 7.35</td>
</tr>
<tr>
<td>5</td>
<td><em>A. obscura</em> 780811.1.2</td>
<td>24</td>
<td>7</td>
<td>14</td>
<td>13</td>
<td>18</td>
<td>20</td>
<td>89.32 ± 12.25</td>
</tr>
<tr>
<td>6</td>
<td><em>A. obscura</em> O 3</td>
<td>24</td>
<td>7</td>
<td>14</td>
<td>13</td>
<td>18</td>
<td>20</td>
<td>89.32 ± 12.25</td>
</tr>
</tbody>
</table>

Fig. 1. Hypothetical average linkage dendrograms based on mtDNA restriction data of six different isolates of *Armillaria* using six different restriction enzymes. For species identification see Table 1.

The potential of mtDNA-ribosomal DNA hybridization for the identification of both nuclear and mtDNA-DNA hybridization patterns. We thank J. Rishal, J. Korhonen for the figures and the Gerris-like organisms for making their available to us.

REF:


State Forts

A new disease of *P.* The pathogen causes *ces tesiya* Royle et al. (1987) has been described as a khasi pine, an important tree in khasi, Jaintia and Khasi hill areas. *B. mycol. Soc. 88* (4), 1987.
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The potential of mtDNA restriction analysis at the subspecific level has recently been demonstrated in different races of Gerlachia nivalis (Leipoldt, 1987). Gerlachia isolates from rye and wheat were clearly separated by their mtDNA restriction patterns.

In this study we have demonstrated that there is a clearcut correlation between intersterility, nuclear DNA-DNA homologies and mitochondrial DNA similarities among isolates of A. mellea, A. bulbosa and A. obscura. The molecular data are consistent with the species concept proposed by Roll-Hansen (1985) and others.

We thank J. Rishbeth, J. J. Guillaumin and G. Korhonen for the fungal cultures used in this study and the German Collection of Microorganisms for making their spectrophotometer available to us.

REFERENCES


PSEUDOCERCOSPORA NEEDLE BLIGHT, A NEW DISEASE OF PINUS KESIYA FROM INDIA

By M. D. Mehotra

State Forest Service College-cum-Research Centre, Burnihat, Assam, India

A new disease of Pinus kesiya caused by Pseudocercospora montuntiana sp. nov. is described. The pathogen caused needle blight in a 4-year-old experimental plot at Burnihat, India.

Pinus kesiya Royle ex Gord, commonly known as khasi pine, is an important conifer species occurring in khasi, Jaintia and Naga Hills and Manipur in north-eastern India. Its timber is used for building and fuel and it yields high-quality resin.

A new needle blight disease was observed in 4-year-old plants in an experimental plot at...