# 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 intraspecific hybridizations and 44–70% for interspecific hybridizations. Restriction enzyme cleavage patterns of mitochondrial DNAs using six different restriction enzymes yielded interspecific similarities from 0 to 50% 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 & Stepien, 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

Table 1. *Organisms*

| No. | Species | Isolate | Origin | Reference |
|---|---|---|---|---|
| 1 | *Armillaria mellea* (Vahl: Fr.) Kummer | M 3 | England, Norfolk, on *Fraxinus excelsior* | J. Rishbeth |
| 2 | *Armillaria mellea* (Vahl: Fr.) Kummer | PP | France, Vaucluse, on *Prunus persica* | Guillaumin & Pierson (1978) |
| 3 | *Armillaria bulbosa* (Barla) Romagn. | B1 | England, Norfolk, isolated from rhizomorphs | J. Rishbeth |
| 4 | *Armillaria bulbosa* (Barla) Romagn. | 780811.1.1 | F. R. G., Ebergötzen near Göttingen, on *Pseudotsuga menziesii* | K. Korhonen |
| 5 | *Armilaria obscura* (Pers.) Herink = *A. ostoyae* (Romagn.) Herink | 780811.1.2 | F.R. G., Ebergötzen near Göttingen, on *Picea omorika* | K. Korhonen |
| 6 | *Armillaria obscura* (Pers.) Herink = *A. ostoyae* (Romagn.) Herink | 0 3 | England, Suffolk, on *Pinus sylvestris* | J. Rishbeth |

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

| No. | Species/Strain | Homology (%)* 1 | 2 | 3 | 4 | 5 | 6 | mol% G+C† |
|---|---|---|---|---|---|---|---|---|
| | *A. mellea* M 3 | — | 97±4 | 64±9 | 55±5 | 56±2 | n.d. | 47·3 |
| | *A. mellea* PP | — | — | 45±9 | 44±2 | 65±5 | 62±5 | 46·6 |
| | *A. bulbosa* B 1 | — | — | — | 100±0 | 57±11 | 57±6 | 46·0 |
| | *A. bulbosa* 780811.1.1 | — | — | — | — | 59±4 | 70±11 | 46·8 |
| | *A. obscura* 780811.1.2 | — | — | — | — | — | 90±8 | 48·1 |
| | *A. obscura* O 3 | — | — | — | — | — | — | 47·1 |

* Measurements in 2 × SSC (0·03 M trisodium citrate 0·3 M-NaCl, pH 7·0).
† GC content obtained from two measurements in 0·1 × SSC (0·0015 M trisodium citrate, 0·015 M-NaCl, pH 7·0). Average S.D. expected from other measurements ±0·4 mol% G+C.

DNA by ultracentrifugation in CsCl–bisbenzimide gradients based on the DAPI–CsCl procedure of Klimczak & 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 (0·015 M-NaCl + 0·0015 M trisodium citrate, pH 7·0), and 7·7 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 42000 rev. $min^{-1}$ (120000 *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 as previously described (Jahnke & Bahnweg, 1986; De Ley, 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°. 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 one particular restriction enzyme were always run on the same agarose gel. Molecular weights of mtDNA fragments were determined in 1·2% (w/v), 1·0% (w/v), 0·6% (w/v) and 0·4% (w/v) agarose gels using λ-phage fragments generated by restriction with HindIII, PvuI, BamHI and BstEII as standards. DNA–DNA homologies were calculated from restriction patterns for each pair of the isolates as described by Kozlowski & Stepien (1982) using the formula

$$S = (2c_{xy}/(u_x + u_y + c_{xy}))\,100,$$

where $c_{xy}$ 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 De Ley *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

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

| No. | Species/Strain | Numbers of fragments generated by | | | | | | mtDNA genome size (kbp) |
|---|---|---|---|---|---|---|---|---|
| | | HindIII | BamHI | XbaI | ClaI | PstI | AvaII | |
| 1 | *A. mellea* M 3 | 24 | 11 | 13 | 14 | 16 | 24 | 89·45 ± 10·51 |
| 2 | *A. mellea* PP | 25 | 10 | 18 | 16 | 16 | 28 | 96·60 ± 12·33 |
| 3 | *A. bulbosa* B 1 | 22 | 7 | 19 | 12 | 19 | 22 | 93·07 ± 10·99 |
| 4 | *A. bulbosa* 780811.1.1 | 22 | 9 | 22 | 13 | 20 | 22 | 98·79 ± 7·35 |
| 5 | *A. obscura* 780811.1.2 | 24 | 7 | 14 | 13 | 18 | 20 | 89·32 ± 12·25 |
| 6 | *A. obscura* O 3 | 24 | 7 | 14 | 13 | 18 | 20 | 89·32 ± 12·25 |

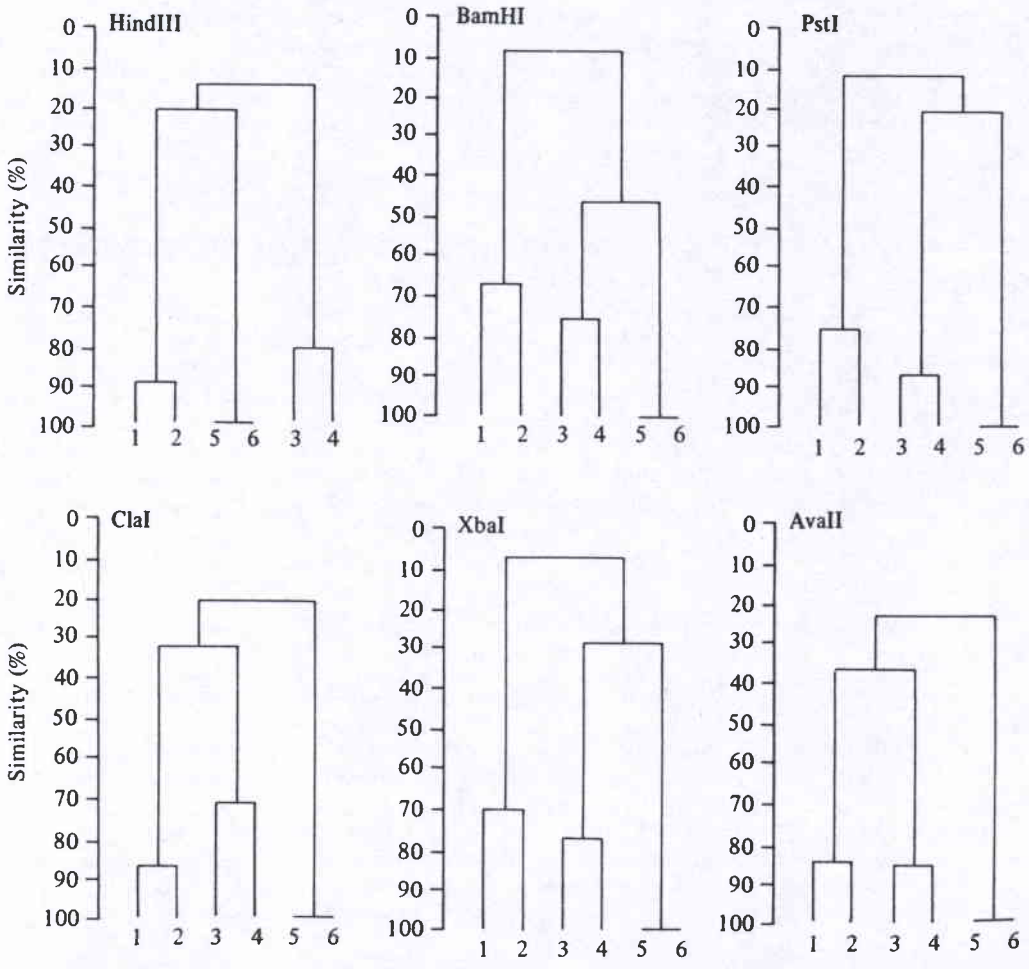
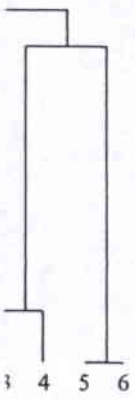


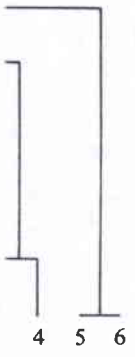

Fig. 1. Hypothetical average linkage dendrograms based on mtDNA restriction data of six different isolates of *Armillaria* using six different restriction enzymes. For species identification see Table 1.

belonging to the same species differed to varying degrees or were identical, resulting in similarity values of 67–100%. Restriction patterns of different species were markedly dissimilar, giving similarity values from 0 to 50%. Hypothetical family trees constructed from mtDNA restriction data depended very much on the restriction enzyme used. We obtained three qualitatively different dendrograms (Fig. 1). Family trees based on restriction-enzyme fragment similarities, such as those proposed for seven species of *Aspergillus* (Kozlowski & Stepien, 1982) should therefore be regarded with caution. Obviously resolution power increases with numbers of generated fragments.

d average mtDNA

| | mtDNA genome size (kbp) |
|---|---|
| | 89·45 ± 10·51 |
| | 96·60 ± 12·33 |
| | 93·07 ± 10·99 |
| | 98·79 ± 7·35 |
| | 89·32 ± 12·25 |
| | 89·32 ± 12·25 |

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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 the present 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 obtained are consistent with the species concept proposed by Roll-Hansen (1985) and others.

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

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A new disease of *Pinus kesiya* caused by *Pseudocercospora montantiana* 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