

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

BY K.-D. JAHNKE AND G. BAHNWEG

Abteilung für Molekulare Genetik der Gesellschaft für Strahlen- und Umweltforschung, Grisebachstr. 8, 3400 Göttingen, F.R.G.

AND J. J. WORRALL

Department of Botany and Plant Pathology, Nesmith Hall, University of New Hampshire, Durham, New Hampshire 03824-3597, U.S.A.

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; Kozłowski & 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	<i>Armillaria mellea</i> (Vahl: Fr.) Kummer	M 3	England, Norfolk, on <i>Fraxinus excelsior</i>	J. Rishbeth
2	<i>Armillaria mellea</i> (Vahl: Fr.) Kummer	PP	France, Vaucluse, on <i>Prunus persica</i>	Guillaumin & Pierson (1978)
3	<i>Armillaria bulbosa</i> (Barla) Romagn.	B1	England, Norfolk, isolated from rhizomorphs	J. Rishbeth
4	<i>Armillaria bulbosa</i> (Barla) Romagn.	780811.1.1	F. R. G., Ebergötzen near Göttingen, on <i>Pseudotsuga menziesii</i>	K. Korhonen
5	<i>Armillaria obscura</i> (Pers.) Herink = <i>A. ostoyae</i> (Romagn.) Herink	780811.1.2	F.R. G., Ebergötzen near Göttingen, on <i>Picea omorika</i>	K. Korhonen
6	<i>Armillaria obscura</i> (Pers.) Herink = <i>A. ostoyae</i> (Romagn.) Herink	0 3	England, Suffolk, on <i>Pinus sylvestris</i>	J. Rishbeth

No.	Sp.
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2	<i>A. me</i>
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Table 2. Nuclear GC contents and nuclear DNA-DNA homologies of *Armillaria* spp.

No.	Species/Strain	Homology (%)*						mol% G+C†
		1	2	3	4	5	6	
	<i>A. mellea</i> M 3	—	97±4	64±9	55±5	56±2	n.d.	47.3
	<i>A. mellea</i> PP	—	—	45±9	44±2	65±5	62±5	46.6
	<i>A. bulbosa</i> B 1	—	—	—	100±0	57±11	57±6	46.0
	<i>A. bulbosa</i> 780811.1.1	—	—	—	—	59±4	70±11	46.8
	<i>A. obscura</i> 780811.1.2	—	—	—	—	—	90±8	48.1
	<i>A. obscura</i> 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.

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DNA by ultracentrifugation in CsCl-bisbenzimidide
gradients based on the DAPI-CsCl procedure of
Klimczak & Prell (1984). Instead of DAPI,
however, bisbenzimidide (Hoechst Dye 33258) was
used. Approximately 1.5 mg of DNA were dis-
solved 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 bisbenzimidide (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 iso-
propanol and dialysis against 0.1 × SSC buffer,
the DNAs were concentrated by isopropanol pre-
cipitation (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 Kozłowski & 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 measure-
ments, 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 approxi-
mately 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 & Pierson
(1978)

J. Rishbeth

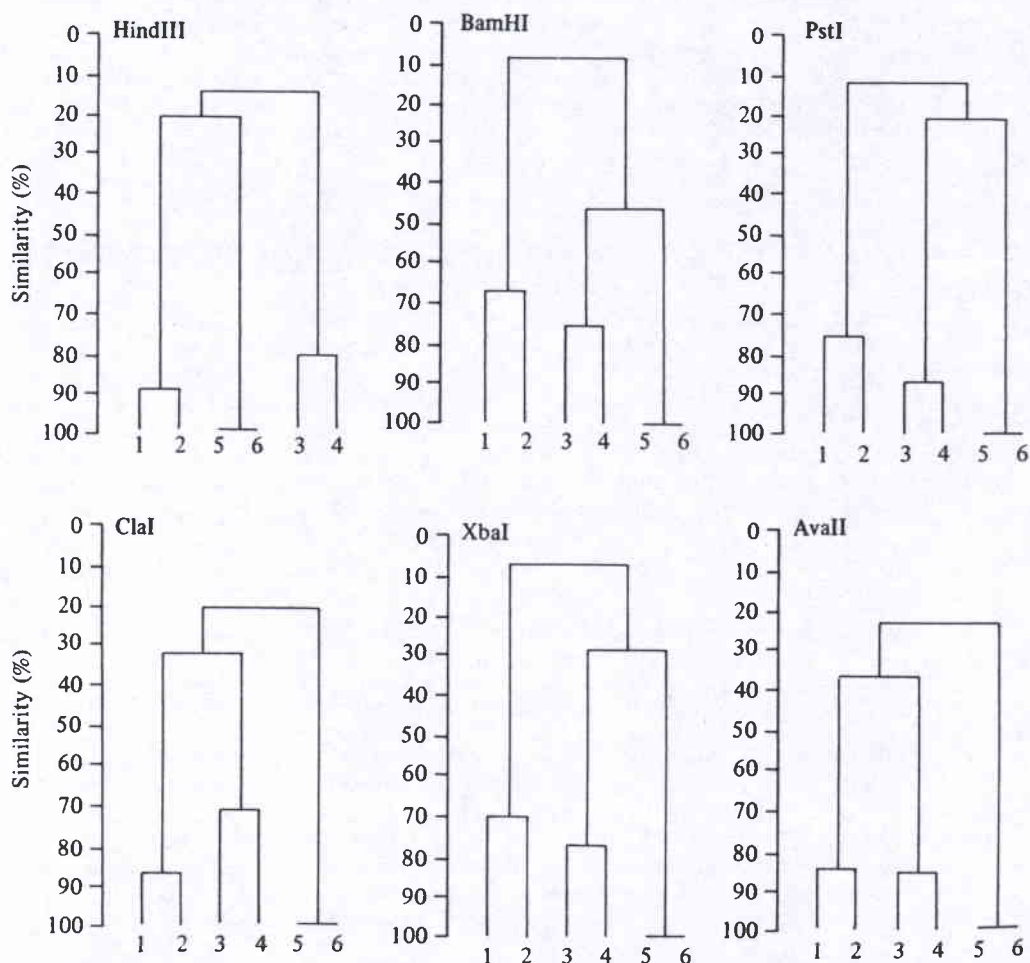
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J. Rishbeth

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	<i>A. mellea</i> M 3	24	11	13	14	16	24	89.45 ± 10.51
2	<i>A. mellea</i> PP	25	10	18	16	16	28	96.60 ± 12.33
3	<i>A. bulbosa</i> B 1	22	7	19	12	19	22	93.07 ± 10.99
4	<i>A. bulbosa</i> 780811.1.1	22	9	22	13	20	22	98.79 ± 7.35
5	<i>A. obscura</i> 780811.1.2	24	7	14	13	18	20	89.32 ± 12.25
6	<i>A. obscura</i> 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* (Kozłowski & Stepien, 1982) should therefore be regarded with caution. Obviously resolution power increases with numbers of generated fragments.

The potential of mtDNA at subspecific level has been shown in different races of *Aspergillus* (1987). *Gerlachia* isolates were clearly separated by their mtDNA patterns.

In the present study there is a clearcut correlation between nuclear DNA-DNA hybridization and mtDNA similarity. The mtDNA of *A. bulbosa* and *A. obscura* obtained are consistent with those proposed by Roll-Haas

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