APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1187–1193 Vol. 61, No. 4
0099-2240/95/$04.00+0

Wide Distribution of Mitochondrial Genome Rearrangements in Wild Strains of the Cultivated Basidiomycete *Agrocybe aegerita*

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We used restriction fragment length polymorphisms to examine mitochondrial genome rearrangements in 36 wild strains of the cultivated basidiomycete *Agrocybe aegerita*, collected from widely distributed locations in Europe. We identified two polymorphic regions within the mitochondrial DNA which varied independently: one carrying the *Cox II* coding sequence and the other carrying the *Cox I*, *ATP6*, and *ATP8* coding sequences. Two types of mutations were responsible for the restriction fragment length polymorphisms that we observed and, accordingly, were involved in the *A. aegerita* mitochondrial genome evolution: (i) point mutations, which resulted in strain-specific mitochondrial markers, and (ii) length mutations due to genome rearrangements, such as deletions, insertions, or duplications. Within each polymorphic region, the length differences defined only two mitochondrial types, suggesting that these length mutations were not randomly generated but resulted from a precise rearrangement mechanism. For each of the two polymorphic regions, the two molecular types were distributed among the 36 strains without obvious correlation with their geographic origin. On the basis of these two polymorphisms, it is possible to define four mitochondrial haplotypes. The four mitochondrial haplotypes could be the result of intermolecular recombination between allelic forms present in the population long enough to reach linkage equilibrium. All of the 36 dikaryotic strains contained only a single mitochondrial type, confirming the previously described mitochondrial sorting out after cytoplasmic mixing in basidiomycetes.

Mitochondrial genomes, because of their rapid rate of sequence divergence, are appealing molecules to use in the study of eukaryotic population biology or evolutionary biology (21). For example, in animals, evolution by nucleotide substitutions proceeds 10 to 100 times faster in the highly compact mitochondrial genome than in the larger nuclear one (3). In this context, comparison of mitochondrial genomes provides interesting information to estimate genetic variability, as well as powerful tools that can be used to molecularly fingerprint individuals. Such tools are particularly important in the higher fungi, in which the clonal individual is difficult to define because the thallus is a network of anastomosing hyphae (18).

In the ascomycete and basidiomycete fungi, a highly polymorphic organization of the mitochondrial genome between classes, genera, and even species has been described (for a review, see reference 2). In these fungi, the mitochondrial genetic information is usually located on circular or, rarely, linear, double-stranded DNA molecules whose sizes range from 19 kb in the yeast *Schizosaccharomyces pombe* (12) to 170 kb in the basidiomycete *Agaricus bitorquis* (10).

In ascomycetes, as well as in other lower eukaryotes and in flowering plants, mitochondrial restriction fragment length polymorphisms (RFLPs) result primarily from length mutations whose origin and characteristics are largely unknown (22). Additionally, in ascomycetous strains subcultured for a long time in laboratories, altered mitochondrial DNAs (mtDNAs) due to well-known molecular rearrangements have been associated with modifications of the mycelial (*Neurospora crassa* [22]) or cellular growth (*Saccharomyces cerevisiae* [4]) or with senescence (*Podospora anserina* [6] or *Neurospora intermedia* [8]). Similarly, changes in mtDNA organization may be related to phenotypic variability or vegetative decline during subcultures, leading to spawn production and, more generally, to variations in growth in commercial strains of *Agaricus bisporus* (11).

Accordingly, mtDNA RFLPs are becoming increasingly important for correlating mitochondrial genes with seemingly unrelated functions and for the study of mtDNA organization, rearrangements, and heredity, factors that are important in breeding research for mushroom improvement.

In basidiomycetes, mtDNA organization has been described for only five species: *A. bisporus* (9), *Coprinus cinereus* and *Coprinus stercorarius* (23), *Schizophyllum commune* (20), and *Agrocybe aegerita* (13). The four molecules differ greatly in size and gene order. Within the same species, a large size and restriction pattern polymorphism were observed in 10 *A. bitorquis* wild isolates (10). In the basidiomycete *Armillaria bulbosa*, Smith et al. (18) showed that mtDNA RFLPs were useful markers for distinguishing clonal individuals in a local population. However, data on the molecular organization and RFLPs of the mtDNA from basidiomycetes are scarce.

The biological model used in our study, *A. aegerita*, is a cultivated basidiomycete which can be used as a model for the application of genetics and molecular biology to the genetic improvement of cultivated mushrooms (15, 17). An mtDNA library from the wild-type strain (WT-3) was constructed in *Escherichia coli* (14). A restriction and genetic map of this mtDNA was constructed, and a recombination-generated rearrangement involving a large inverted repeat was detected (1).

In this paper, we describe the mtDNA RFLPs from field strains of *A. aegerita*. Our goal was to determine the frequency

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TABLE 1. Geographic origin and mitochondrial haplotype of the wild strains of *A. aegerita* used in this study

Strain	Collection no.[a]	No. in international collection[b]	Geographic origin (zone)[c]	Mitochondrial haplotype
WT-1	SM 51		Pont de la Maye, France (A)	I
WT-2	SM 48	ATCC 96069	Ordonnac, France (A)	II
WT-3	SM 47		Agen, France (A)	I
WT-5	SM 871027	ATCC 96072	Albaladejito, Spain (D)	III
WT-6	SM 871012	ATCC 96070	Tripoteau, France (A)	II
WT-7	SM 871102	ATCC 96077	Montpellier, France (B)	III[d]
WT-8	SM 871103	ATCC 96076	Montpellier, France (B)	III
WT-9	SM 871021	ATCC 96071	Albaladejito, Spain (D)	IV
WT-10	SM 750905	ATCC 96074	Camarsac, France (A)	II
WT-11	SM 751002	ATCC 96073	Pont de la Maye, France (A)	III[d]
WT-12	SM 750904	ATCC 96075	Pont de la Maye, France (A)	III
WT-13	SM 750901		Le Mans, France (C)	III
WT-14	SM 50		Czechoslovakia (E)	III
WT-15	SM 871106		Scotland (F)	III
WT-16	SM 750902		Villefort, France (C)	III
WT-17	SM 750903		Villamblard, France (A)	II
WT-18	SM 760901		Loches, France (C)	IV
WT-19	SM 770601		Bègles, France (A)	III
WT-20	SM 841005		St.-André de Cubzac, France (A)	III[d]
WT-21	SM 871107		Germany (G)	III
WT-22	SM 930202	MUCL 28847	Merelbeke, Belgium (H)	III
WT-23	SM 46		Epannes, France (C)	III
WT-24	SM 160		Toulouse, France (A)	III
WT-25	SM 161		Lyon, France (B)	IV
WT-26	SM 741		Versailles, France (C)	I
WT-27	SM 750801		St.-Morillon, France (A)	III
WT-28	SM 751001		Talence, France (A)	IV
WT-29	SM 900601		Saumur, France (C)	III
WT-30	SM 162		Lyon, France (B)	I
WT-31	SM 771		Ile d'Arcin, France (A)	III
WT-32	SM 750802		Busloup, France (C)	IV
WT-33	SM 49		Italie (I)	IV
WT-34	SM 880201		Langeais, France (C)	III
WT-35	SM 920502		Langeais, France (C)	III
WT-36	SM 930901		Villenave d'Ornon, France (A)	III
WT-37	SM 930902		Villenave d'Ornon, France (A)	III

[a] The collection number corresponds to the collection nomenclature of the Laboratory of Molecular Genetics and Improvement of Cultivated Mushrooms.
[b] ATCC, American Type Culture Collection, Rockville, Md.; MUCL, Mycothèque de l'Université Catholique de Louvain, Louvain-La-Neuve, Belgium.
[c] The geographic zones are defined in the text.
[d] The corresponding mtDNA possessed an additional punctual mutation described in the text.

at which mtDNA rearrangements occurred in nature and the regions of the mtDNA molecule that were affected.

MATERIALS AND METHODS

Strains, media, and culture conditions. The tetrapolar basidiomycete *A. aegerita* belongs to the class *Agaricales*. Thirty-six wild dikaryotic strains of *A. aegerita* were used; the source and geographic origin of each strain are indicated in Table 1. The geographic area in which the strains were collected was divided into nine zones at least 300 km distant from each other. Twenty-nine strains were derived from basidiocarps collected in France: 16 in the southwestern zone (A), 4 in the southeastern zone (B), and 9 in the northwestern zone (C). Two strains were from Spain (zone D), and the others were from Czechoslovakia (zone E), Scotland (zone F), Germany (zone G), Belgium (zone H), and Italy (zone I). A piece of each wild basidiocarp was subcultured to generate a vegetative dikaryotic mycelium.

Mycelia were cultured vegetatively on complete (CYM) solid or liquid medium as previously described (17).

In vitro manipulation of DNAs. Total DNA from each strain was extracted from 5 g of vegetative mycelium grown in Roux flasks by the *N*-cetyl-*NNN*-trimethyl ammonium bromide (CTAB) method, adapted for *A. aegerita* by Noël and Labarère (16).

DNA digestion with *Hin*dIII or *Hae*III was performed according to the enzyme supplier's instructions (Bethesda Research Laboratories, Gaithersburg, Md., or Eurogentec, Seraing, Belgium).

DNA labelling and DNA-DNA hybridizations. Total digested DNA (1 μg) was transferred, after agarose (0.8% [wt/vol]) gel electrophoresis in Tris-borate-EDTA buffer, to Hybond N^+ (Amersham, Amersham, United Kingdom) membranes by the method of Southern (19). Membranes were treated for covalent DNA fixation according to the manufacturer's recommendations. For analytical electrophoresis and Southern hybridization, the Appligene (Illkirch, France) Raoul molecular weight marker set (22 DNA fragments ranging from 234 to 48,000 bp) was used. *Hin*dIII restriction fragments from WT-3 mtDNA were previously inserted into the *Hin*dIII site in pBR322. Probes were made by digesting 20 ng of plasmid DNA with *Hin*dIII and then were labeled with 25 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol, [Amersham]) with the Bethesda Research Laboratories random primers DNA labelling kit. All probes had a specific radioactivity higher than 10^8 cpm/μg of DNA.

Prehybridizations and hybridizations were performed at 65°C for 4 and 16 h, respectively, in a Techne hybridizer (Cambridge, United Kingdom), model HB-1, in 50 ml of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin, 0.5% sodium dodecyl sulfate (SDS), and denatured salmon sperm DNA at a final concentration of 100 μg/ml. Filters were washed two times in 6× SSC–0.5% SDS at 30°C (5 min each), three times (10 min each) in 2× SSC–0.5% SDS at 65°C, and then three times (15 min each) in 0.2× SSC–0.5% SDS at the hybridization temperature. The filters were wrapped in plastic bags, and hybrids were identified by exposure of the blots for 24 h at −80°C to Kodak XAR-5 film (Rochester, N.Y.) with intensifying screens. All hybridization experiments were run in duplicate with total DNA extracted from two independently grown vegetative cultures used for each strain. Chemicals were obtained from Sigma (St.-Quentin Fallavier, France).

RESULTS

Determination of the polymorphic regions of the *A. aegerita* mtDNA molecule. *Hin*dIII-digested total DNA from 12 wild-

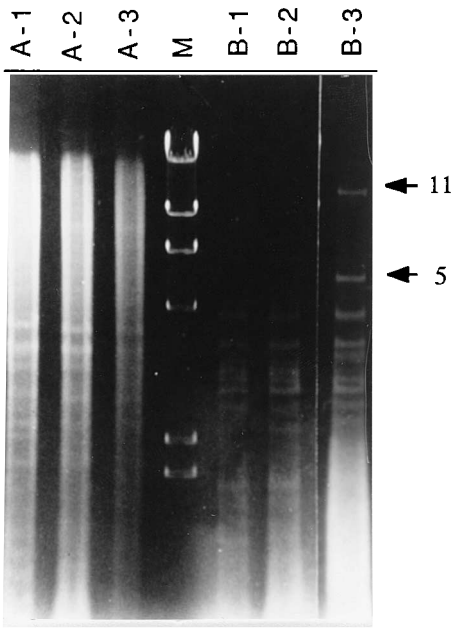

FIG. 1. Agarose (0.8% [wt/vol]) gel electrophoresis pattern of the *Hin*dIII (lanes A)- or *Hae*III (lanes B)-digested DNA from three *A. aegerita* wild strains (lanes A1 and B1, WT-11; lanes A2 and B2, WT-13; lanes A3 and B3, WT-3). The polymorphic *Hae*III fragments of the WT-3 strain are indicated by arrows, and their sizes are expressed in kilobases. The *Hin*dIII restriction fragments of the bacteriophage λ DNA (23.13, 9.416, 6.557, 4.361, 2.322, 2.027, 0.564, and 0.125 kb) were used as molecular size markers (lane M).

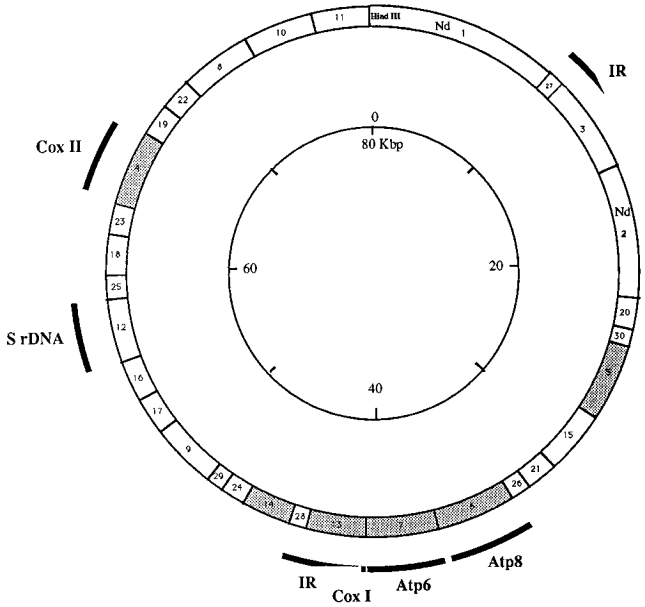

FIG. 2. Location of RFLP probes (shaded) on the *Hin*dIII restriction map of *A. aegerita* WT-3 mtDNA. The *Hin*dIII map, the location, and orientation of the inverted repeat (IR) sequences, and the location of the 5′ coding end of the mitochondrial genes (*Cox I*, *Cox II*, *Atp6*, *Atp8*, and the gene coding for the small rRNA (S rDNA) were previously described (13). Nd, not determined (i.e., these two probes were not used in this study).

type dikaryotic mycelia (Table 1, strains WT-1, -2, -3, -5, -6, -7, -9, -10, -11, -13, -14, and -15) that ranged in size from 25 kb to 0.5 kb, with major bands corresponding to multicopy fragments (Fig. 1, lanes A). No polymorphism was detected with this enzyme.

When the DNA was digested with *Hae*III, polymorphic restriction fragments could be detected (Fig. 1, lanes B). As expected, *Hae*III digestion resulted in more fragments than did *Hin*dIII digestion and, consequently, the size continuum was displaced to the bottom of the gel, from 2,500 bp to less than 500 bp. The fragments which migrated slower than this smear might be AT-rich sequences, such as the mitochondrial DNA, and revealed clear differences between strains. For example, an 11-kb *Hae*III restriction fragment was observed in only five strains (WT-1, -2, -3, -6, and -10). Similarly, a specific 5-kb *Hae*III fragment was observed only in strains WT-1, -3, and -9.

Electrophoretic analysis of the digested total DNA allowed us to identify AT-rich RFLPs in *A. aegerita*. To confirm the mitochondrial origin of these polymorphic fragments, *Hin*dIII and *Hae*III digests from the same 12 wild strains were hybridized with the mitochondrial probes previously cloned from *A. aegerita* WT-3 (13).

Among the 30 previously characterized mitochondrial fragments, the two largest ones, H1 (9.5 kb) and H2 (6.5 kb), were eliminated because of their weak hybridization signals, imputed to their high molecular weight (13). Of the 28 remaining probes, five (H4, H5, H7, H13, and H14) revealed polymorphic *Hin*dIII and *Hae*III fragments, and one (H6) revealed polymorphism only with the *Hae*III fragments. The other 22 probes did not detect polymorphic *Hin*dIII or *Hae*III fragments. The locations of the 28 probes are indicated on the *Hin*dIII restriction map of strain WT-3 (Fig. 2). The monomorphic regions could account for 43.5 kb (54%) of the mitochondrial genome. The polymorphic regions were located in regions containing several mitochondrial genes (*Cox I*, *Cox II*, *ATP6*, and *ATP8*).

Evidence for a large and widely distributed rearrangement in the *A. aegerita* mitochondrial genome. To study polymorphism in a larger population and, consequently, to strengthen the accuracy of the analysis of mutations in the natural population of *A. aegerita*, the probes revealing polymorphisms were hybridized with total DNA from 24 additional strains.

The hybridization of the H4 probe with *Hin*dIII or *Hae*III-digested DNAs detected polymorphic fragments in both *Hin*dIII and *Hae*III digests (Fig. 3A and Table 2). This polymorphism allowed the characterization of two mitochondrial types. (i) Mitochondrial type H4-1 (strains WT-5, WT-7 to WT-9, WT-11 to WT-16, WT-18 to WT-25, WT-27 to WT-29, and WT-31 to WT-37) was characterized by a 3.5-kb *Hin*dIII fragment and two *Hae*III fragments (4 and 5.3 kb) homologous to H4. (ii) Mitochondrial type H4-2 (the other eight strains) was characterized by two *Hin*dIII fragments (2.4 and 4.2 kb) and three *Hae*III fragments (4, 5.3, and 11 kb) homologous to H4.

It will be noticed that the polymorphism of the *Hae*III and *Hin*dIII fragments detected by the H4 probe divided the strains into the same two groups. Thus, this result suggested that the *Hin*dIII and *Hae*III polymorphisms were correlated. More precisely, the DNA rearrangement in which the 3.5-kb *Hin*dIII restriction fragment of the H4-1 mitochondrial type was replaced by two *Hin*dIII fragments in the H4-2 mitochondrial type modified the *Hae*III pattern by the addition of a high-molecular-weight fragment (11 kb).

The *Hin*dIII-digested DNA from strain WT-3 contained two fragments homologous to the H4 probe (4.2 and 2.4 kb), suggesting that a portion of the H4 fragment has been duplicated. In the *Hae*III patterns, three H4-hybridizing fragments (4, 5.3, and 11 kb) were identified. This result contrasts with the restriction map of the cloned H4 fragment (Fig. 3B). Indeed, according to the map, in WT-3, the H4 probe should hybridize

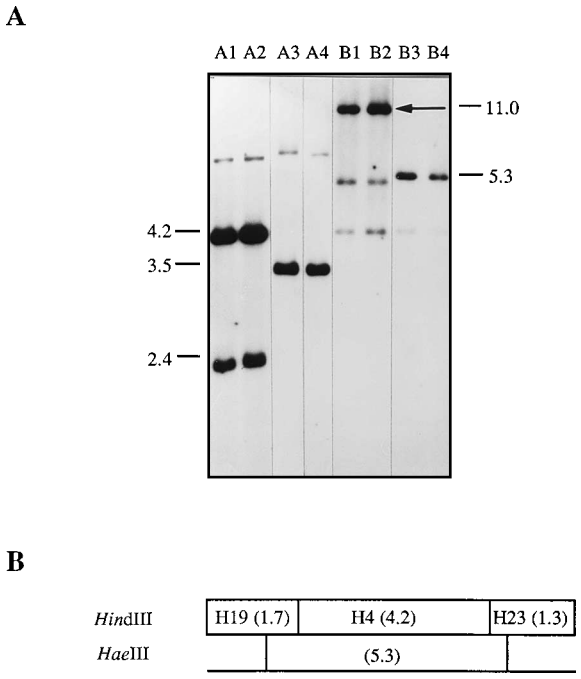

FIG. 3. Restriction polymorphism of the H4 mitochondrial region. (A) Hybridization pattern of the *Hin*dIII (lanes A)- or *Hae*III (lanes B)-digested total DNA of four *A. aegerita* strains with the H4 mitochondrial probe (lanes A1 and B1, WT-3; lanes A2 and B2, WT-1; lanes A3 and B3, WT-9; lanes A4 and B4, WT-13). The arrow shows the polymorphic 11-kb *Hae*III restriction fragment. The sizes of the fragments are indicated in kilobases. (B) Schematic representation of the *Hin*dIII and *Hae*III restriction maps of the WT-3 mtDNA in the H4 region (13). The sizes of the fragments (in kilobases) are indicated in parentheses.

with a unique 5.3-kb *Hae*III fragment, because H4 does not contain a *Hae*III recognition site, and two *Hae*III sites are present in the two adjacent *Hin*dIII fragments (H19 and H23). The 2.4-kb *Hin*dIII fragment and the 4- and 11-kb *Hae*III fragments, which are additional to the fragments deduced from the WT-3 map (Fig. 3B), could result from a duplication of a portion of the H4 sequence or, more likely, from a large insertion into the H4 fragment. Such insertions have been widely described in the mitochondrial genomes of various eukaryotic species, including fungi (7). For example, optional introns have been described in the mitochondrial gene coding for the cytochrome oxidase subunit I of *N. crassa* (5).

In the restricted DNA from 36 strains, the H14 probe distinguished two mitochondrial types (Table 2), each characterized by specific *Hin*dIII and *Hae*III restriction fragments. (i) Mitochondrial type H14-1 (strains WT-5, WT-7 to WT-9, WT-11 to WT-16, WT-18 to WT-25, WT-27 to WT-29, and WT-31 to WT-37) was characterized by a specific 4.2-kb *Hin*dIII fragment and a specific 4.5-kb *Hae*III fragment. (ii) Mitochondrial type H14-2 (the other eight strains) was characterized by a specific 11-kb *Hae*III fragment.

The H14-1 and the H4-1 mtDNA types were both detected in the same 28 strains, while the H14-2 and H4-2 mtDNA types were both recovered in the other 8 strains. Moreover, the H4-2 and H14-2 mitochondrial types were defined by a specific large *Hae*III restriction fragment of the same 11-kb size. This large *Hae*III fragment was clearly evident as a unique fragment (i.e., it appeared in equimolar quantity with the other *Hae*III fragments) on the *Hae*III restriction pattern of the mtDNA from strain WT-3, suggesting that the H4 and H14 probes hybridized to the same large *Hae*III fragment in the H14-2 (and H4-2) defined strains.

Evidence for a short length mutation in the *A. aegerita* mitochondrial genome. Hybridizations of the *Hae*III-digested total DNAs with probes H6, H7, and H13 were analyzed together because they had numerous common fragments, as expected from their contiguous location on the mtDNA map (14). The sizes of the *Hae*III fragments allowed us to define two additional mitochondrial types (Table 2) which divide the strains into two groups (Fig. 4A). (i) Mitochondrial type H7-1 (strains WT-1, WT-3, WT-9, WT-18, WT-25, WT-26, WT-28, WT-30, WT-32, and WT-33) was characterized by a 5 $\pm$ 0.2-kb *Hae*III fragment that hybridized to all three probes. (ii) Mitochondrial type H7-2 (the other 26 strains) was characterized by two *Hae*III fragments (1.7 and 3.1 kb) that hybridized to the H7 probe, a unique 1.7-kb fragment that hybridized to the H6 probe, and a 3.1-kb fragment that hybridized to the H13 probe.

These two mitochondrial types can be explained by the appearance of a *Hae*III site in the H7 region of the H7-1 mitochondrial type (Fig. 4B). In the H7-2 mitochondrial type, two H7-homologous *Hae*III restriction fragments (3.1 and 1.7 kb) replaced the 5-kb *Hae*III fragment of the H7-1 mitochondrial type. The sum of the sizes of the two *Hae*III fragments specific to the H7-2 type mtDNA was slightly smaller than the 5-kb fragment specific to the H7-1 type. From these data, we cannot determine whether a single-base-pair change or a small deletion is responsible for this polymorphism. Indeed, the size difference between the polymorphic fragments (5 kb and 1.7 + 3.1 kb) might be due to the imprecision of the fragment size determination. Because mitochondrial types H7-1 and H7-2 were found in several geographically distant strains, this RFLP could be attributed to a molecular event that is widely distributed among geographically distant strains. However, this wide distribution may be the result of an event occurring repetitively at different locations or the result of a single event that has since been widely dispersed.

Evidence for a point mutation in the *A. aegerita* mitochondrial genome. In 34 strains, the H7 probe hybridized to a 3.7-kb *Hin*dIII restriction fragment, but in two strains (WT-11 and WT-20) the probe detected two *Hin*dIII fragments with sizes of 2.3 and 1.4 kb (Fig. 4A). This polymorphism might be due to the creation of an additional *Hin*dIII recognition site by a point mutation in the 3.7-kb *Hin*dIII fragment.

The WT-11 and WT-20/H7 polymorphism also could be detected with the H13 probe. These probes probably share a common sequence, because H13 hybridizes to a 3.7-kb fragment in the 34 monomorphic strains but hybridizes only to the 2.3-kb fragment in strains WT-11 and WT-20. Moreover, the cloned H13 fragment was previously shown to be a part of an inverted repeated sequence (1) and to cross-hybridize with the H3 fragment (4.7 kb). As indicated above (Fig. 2), this H3-homologous *Hin*dIII fragment was monomorphic in the first 12 strains studied.

Thus, the same probe could be used to identify two types of RFLP-generating mutations: a point mutation and a large insertion.

Similar results were obtained with the H5 probe (3.9 kb). The H5-homologous *Hin*dIII fragment (3.9 kb) was isomorphic in 35 strains; the WT-7 strain had a slightly smaller *Hin*dIII fragment (3.5 kb). This fragment might be due to (i) the appearance of an additional *Hin*dIII recognition site in the 3.9-kb H5 fragment, leading to two *Hin*dIII fragments (3.5 and 0.4 kb), the smallest one not detectable by hybridization in our experimental procedure; or (ii) a small deletion.

In *Hae*III digests, the H5 probe hybridized to five fragments: two monomorphic (1.2 and 1.0 kb) and three polymorphic (Table 2). The 1.9-kb *Hae*III fragment in strains WT-1, WT-3, WT-9, WT-18, WT-25, WT-26, WT-28, WT-30, WT-32, and

TABLE 2. Sizes of mitochondrial *Hin*dIII or *Hae*III restriction fragments revealed by the polymorphic probes

Mitochondrial haplotype[a]	Size (kb) of mitochondrial restriction fragment revealed by probe (endonuclease)[b]							
	H4 (*Hin*dIII)	H4 (*Hae*III)	H14 (*Hin*dIII)	H14 (*Hae*III)	H5 (*Hae*III)	H6 (*Hae*III)	H7 (*Hae*III)	H13 (*Hae*III)
I	2.4[c]	4	2.4	5.6	1	0.5	5[c]	0.7
	4.2[c]	5.3		11[c]	1.2	1.4		1.3
		11[c]			1.9[c]	5[c]		5[c]
	[_____]		[_____]		[____]	[_____]		
	H4-2		H14-2		H5-1	H7-1		
II	2.4[c]	4	2.4	5.6	0.8[c]	0.5	1.7[c]	0.7
	4.2[c]	5.3		11[c]	0.9[c]	1.4	3.1[c]	1.3
		11[c]			1	1.7[c]		3.1[c]
					1.2			4
	[_____]		[_____]		[____]	[_____]		
	H4-2		H14-2		H5-2	H7-2		
III	3.5[c]	4	2.4	4.5[c]	0.8[c]	0.5	1.7[c]	0.7[c]
		5.3	4.2[c]	5.6	0.9[c]	1.4	3.1[c]	1.3
					1	1.7[c]		3.1[c]
					1.2			4
	[_____]		[_____]		[____]	[_____]		
	H4-1		H14-1		H5-2	H7-2		
IV	3.5[c]	4	2.4	4.5[c]	1	0.5	5[c]	0.7
		5.3	4.2[c]	5.6	1.2	1.4		1.3
					1.9[c]	5[c]		4
			[_____]		[____]	[_____]		5[c]
	H4-1		H14-1		H5-1	H7-1		

[a] Mitochondrial haplotypes are defined by the combination of the mitochondrial types (in brackets) defined by the polymorphic restriction fragments. The haplotypes are cross-listed with the strains in Table 1.
[b] Endonuclease used for total DNA digestion.
[c] Polymorphic restriction fragment.

WT-33 was replaced in the other 26 strains by a 0.9-kb fragment and a 0.8-kb fragment. As with the H7 polymorphism, this difference could be due to a single-base-pair change or a small deletion. Indeed, the H5 and H7 polymorphisms divided the strains into the same sets. This result suggests that the mtDNA rearrangement events leading to the H7-2 and H5-2 mitochondrial types might be related.

DISCUSSION

Mitochondrial RFLPs in *A. aegerita* were studied through hybridizations between digested total DNA and cloned mtDNA probes from strain WT-3. Two polymorphic regions were defined: one containing the *Cox II* coding sequence and the other containing the *Cox I*, *ATP6* and *ATP8* coding sequences. The two regions varied independently. The frequencies of the RFLPs were determined in a sample of 36 strains from dispersed European locations.

The RFLPs that we observed may be due to point or length mutations. The H7 probe can be used to identify a point mutation in strains WT-11 and WT-20. These strains differ from the other 34 strains because they have two *Hin*dIII fragments (2.3 and 1.4 kb) that hybridize to the H7 probe, while the other 34 strains have a single 3.7-kb fragment. This type of RFLP-generating mutation is characterized by its high degree of strain specificity (i.e., the polymorphic pattern is detected in one or a few strains) and by the equal sums of the homologous fragments in the two different patterns. In this context, it will be noticed that the two strains were collected in the same geographical zone, 30 km apart, and consequently might be derived from a common ancestor. This type of RFLP can be used to study mitochondrial heredity in industrially cultivated mushrooms. Indeed, such molecular markers are not related to mtDNA size variations and consequently limit the risk of being related to a replicative advantage of the mtDNA genome considered.

Another possible point mutation can be found in the H5-hybridizing 3.5-kb *Hin*dIII fragment, which is recovered in the WT-7 strain only. This fragment could result from a point mutation leading to the appearance of an additional *Hin*dIII recognition site in the 3.9-kb H5 fragment or from a small deletion (400 bp).

RFLPs also may result from length mutations, attributable to rearrangements of the mitochondrial genome, such as deletion, insertion, or duplication.

As indicated above, the H4 and H14 probes revealed polymorphic *Hae*III and *Hin*dIII fragments. In this region, length mutations defined two molecular types, suggesting that the length mutation was not randomly generated but instead involved a precise rearrangement. The two molecular types were widely distributed and divided the 36 wild strains into two groups. The first group (mitochondrial type H4-1 or H14-1) contained 28 strains; the second group (mitochondrial types H4-2 and H14-2) contained eight strains (Fig. 5). Both forms were never detected simultaneously in any strain.

In a similar manner, a second polymorphic region (determined on the basis of hybridization with the H5, H6, H7, and H13 fragments) divided the 36 wild strains in two groups. The H7-1 and H7-2 groups contained 10 and 26 strains, respectively (Fig. 5).

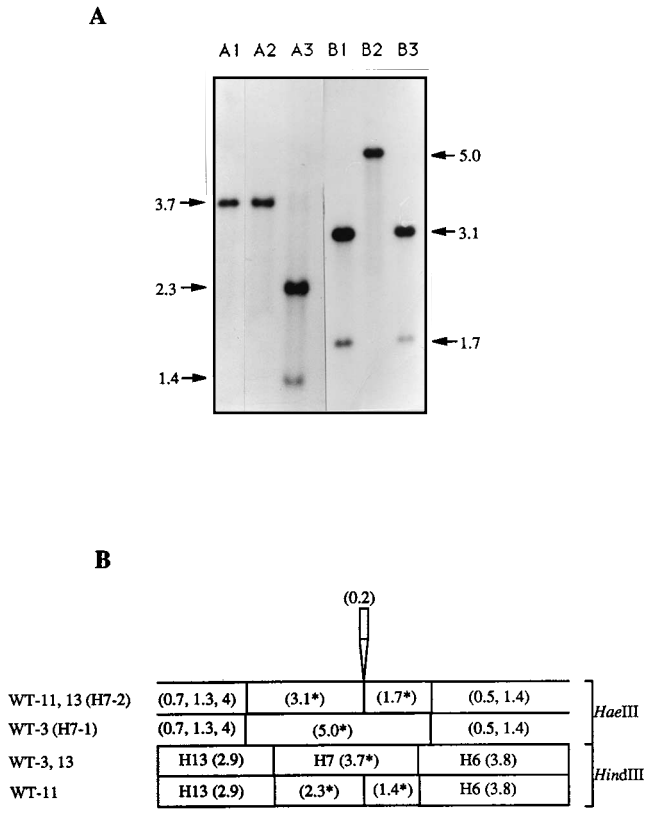

FIG. 4. Restriction polymorphism of the H7 mitochondrial region. (A) Hybridization pattern of the *Hin*dIII (lanes A)- or *Hae*III (lanes B)-digested total DNA of three *A. aegerita* strains to the H7 mitochondrial probe (lanes A1 and B1, WT-13; lanes A2 and B2, WT-3; lanes A3 and B3, WT-11). The sizes of the fragments are indicated in kilobases. (B) Schematic representation of the *Hin*dIII and *Hae*III restriction maps of the H7 mitochondrial region from the WT-3, WT-11, and WT-13 strains. The sizes of the fragments (in kilobases) are indicated in parentheses. Asterisks denote polymorphic restriction fragments.

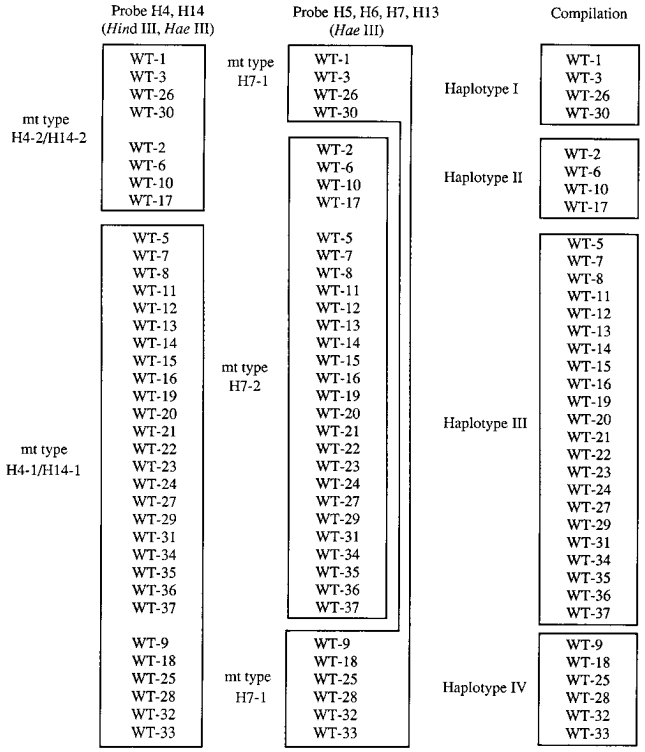

FIG. 5. Distribution of the *A. aegerita* strains in groups sharing the same hybridization pattern according to the probe and restriction endonuclease used (for details see text).

The results show that there is no absolute association between alternate forms of the two regions. By using the H4 and H7 probes, four distinct haplotypes resulting from the different possible combinations of the mitochondrial types involving the two polymorphic regions (H4-H14 and H5-H6-H7-H13) can be identified.

The four mitochondrial haplotypes in *A. aegerita* could be the result of intermolecular recombination between strains carrying different alleles at these two regions. Even though the two independently varying regions were physically adjacent on the map, the relative frequencies of the four haplotypes indicate that these alleles have been present in the population long enough to reach linkage equilibrium; i.e., haplotype frequency is dependent solely upon the allele frequencies. Interestingly, the two regions are separated by a copy of the inverted repeat. This inverted repeat has been shown to be involved in an intramolecular recombination leading to two orientational isomers, one of which was largely preponderant (1). The inverted repeat could also be a hot spot for intermolecular recombination.

Additionally, there was no obvious correlation between the mitochondrial haplotypes and the geographic origin of the strains. For example, all four haplotypes were observed among the 16 strains from zone A in Southwestern France.

Each of the 36 dikaryotic strains contained only a single mitochondrial type. This observation has two possible explanations: there could be a complete lack of mitochondrial mixing during the sexual life cycle, or, after cytoplasmic mixing, the mtDNA types could sort out. Our results, which suggest that the allelic forms have reached linkage equilibrium in the population, are in favor of the second hypothesis. Indeed, the first hypothesis prevents the postulated intermolecular recombination.

Within a polymorphic region, the frequencies of the two allelic forms are different. This difference has several possible explanations. The most probable hypothesis is that no allelic form confers a selective advantage and that nuclear and mitochondrial relationships are critical for the expression of a mitochondrial genome competition. Accordingly, the mitochondrial type would depend on the nuclear haplotypes of the strain.

The second hypothesis is that the major form confers a selective advantage and that the second form is maintained in the mycelium by a mitochondrial incompatibility mechanism, preventing the mixing of the two mitochondrial types and, consequently, their competition.

Finally, our report opens the way for the study of the complex mitochondrial heredity of the basidiomycetes by using the point mutations as molecular markers. Moreover, this RFLP analysis revealed important mitochondrial genome rearrangements whose mechanisms and physiological roles are unknown but appealing subjects for further investigations in accordance with their wide distribution in the natural isolates.

ACKNOWLEDGMENTS

This work was supported by grants from the Conseil Scientifique de l'Université de Bordeaux II, the Conseil Régional d'Aquitaine, and the Institut National de la Recherche Agronomique.

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