Restriction Enzyme Analysis of Mitochondrial DNA of Members of the Genus *Aspergillus* as an Aid in Taxonomy

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Restriction endonuclease cleavage patterns of mitochondrial DNA from seven species of the genus *Aspergillus* (*A. nidulans*, *A. wentii*, *A. awamori*, *A. niger*, *A. oryzae*, *A. tamaritii* and *A. echinulatus*) have been compared in order to test the phylogenetic relationships between these species. The fraction of restriction fragments common between all pairs of species from this set ranged from 0 to 1. The use of this approach for the taxonomy of the genus *Aspergillus* is discussed. The postulated phylogenetic relationships differ significantly in two cases from the classification of the genus presented by Raper & Fennell (1965).

INTRODUCTION

Classification and delineation of species in the genus *Aspergillus* presented by Thom & Raper (1945) and by Raper & Fennell (1965) has been based on differences in morphology and growth characteristics. According to Fennell (1977), the 18 groups of the genus *Aspergillus* are not accredited taxa and serve only as convenient slots for characterization of the strains. Because of the importance of the genus for industry, clinical medicine and food production, many physical and biochemical investigations on various species have been performed, and different approaches have been used in order to provide better means of classification and better understanding of the evolution of the genus *Aspergillus*. Cell wall composition (Bartnicki-Garcia, 1968), protein electrophoresis (Kulik & Brooks, 1970), analysis of pyrolysis products (Vincent & Kulik, 1973), physicochemical analysis of nucleic acids (Storck & Alexopoulos, 1970) and other methods have been employed, but it seems that the phylogenetic relationships between the groups of the genus *Aspergillus* are still to be established (Fennell, 1977).

In this paper we present the application of restriction enzyme digestions of mitochondrial DNA for investigation of different species of the genus *Aspergillus*. Mitochondrial DNA is a circular molecule, varying in length between 15 and 120×10^3 base pairs (kb) in different organisms. It codes for mitochondrial rRNAs, tRNAs and a small number of proteins (Linnane & Nagley, 1978). The small size of the mitochondrial DNA makes it suitable for restriction enzyme analysis, and comparison of the degree of base substitutions should provide more information about the phylogeny of the species as it reflects the primary events in evolution, i.e. changes in DNA sequence. A similar approach has been used for different species of the rat (Hayashi *et al.*, 1979) and maize (Timothy *et al.*, 1979) and for mammals (Brown *et al.*, 1980).

The aim of our work was not to present data for taxonomy of the whole genus, but to test whether restriction enzyme analysis of mitochondrial DNA could be used for this purpose, and if it could, how our results would fit into the delineation of *Aspergillus* species made by Raper & Fennell (1965). We therefore tested the following strains: *A. nidulans* from the *A. nidulans* group; *A. wentii* from the *A. wentii* group; *A. awamori* and *A. niger* from the *A. niger* group; *A. oryzae* and *A. tamaritii* from the *A. flavus* group. In addition, we have used, for comparison, the data of Earl *et al.* (1980) on *A. echinulatus* from the *A. nidulans* group.

METHODS

Strains. *Aspergillus nidulans* Eidam Wint. strain was from the collection of the Department of Genetics, University of Warsaw. All the other strains (*A. wentii*, *A. tamarii*, *A. oryzae*, *A. luchuensis* s. *awamori* and *A. niger*) were from the collection of the Institute for Fermentation Industry, Warsaw. We shall use the name *A. awamori* for *A. luchuensis* s. *awamori*. All strains were carefully checked for their identity according to Raper & Fennell (1965) and Thom & Raper (1945).

DNA preparation and analysis. Plasmids pBS78 and pH3 (Bartnik *et al.*, 1979; Küntzel *et al.*, 1980) containing cloned fragments of the mitochondrial genome of *A. nidulans* were from the collection of the Department of Genetics. Preparation of mitochondrial DNA from mycelial pads was performed as described by Stępień *et al.* (1978) and modified by Hahn *et al.* (1979). Digestion with restriction enzymes and agarose gel electrophoresis were performed as described by Bartnik *et al.* (1979). Hybridization experiments were performed according to Southern (1975). ³²P-labelled RNA was prepared by *in vitro* transcription of pBS78 and pH3 plasmids as described by Tyler *et al.* (1974), using guanosine 5'-[α-³²P]triphosphate (Amersham; 3000 Ci mmol⁻¹, 110 TBq mmol⁻¹). Autoradiography was performed in a Kodak X-omatic Regular Cassette with intensifying screens using Kodak X-omat R films. Restriction endonucleases *Eco*RI and *Hind*III were produced by POCH, Gliwice, Poland.

RESULTS AND DISCUSSION

Two different kinds of test were performed. First, mitochondrial DNA from each strain was digested to completion with a mixture of *Eco*RI and *Hind*III restriction enzymes and the resulting DNA fragments were resolved by agarose gel electrophoresis, photographed and their molecular weights were established using restriction fragments of bacteriophage λ DNA as standards (Figs 1 and 2). Second, the DNA from gels was transferred to nitrocellulose filters by the method of Southern (1975) and hybridized to two different radioactive probes – RNA obtained by *in vitro* transcription of plasmid pH3 or pBS78 containing the genes for 16S and 23S mitochondrial rRNA, respectively (Lazarus *et al.*, 1980). This approach enabled us to confirm the identity of some DNA bands in the size regions of 1.6–1.9 kb and of 4 kb.

Table 1 presents the comparison of fractions of common bands between all the species tested. This fraction (*F*) was calculated according to the formula:

$$F = 2c_{XY}/(u_X + u_Y + 2c_{XY})$$

where c_{XY} represents the number of bands common to strains X and Y, and u_X and u_Y represent numbers of unique bands. The fraction of conserved bands provides information on phylogenetic relationships between species.

Our data confirm a close relationship of the species *A. echinulatus* and *A. nidulans*, both belonging to the *A. nidulans* group. Two members of the *A. flavus* group, *A. oryzae* and *A. tamarii*, also show a close homology. Unexpectedly, *A. wentii* shows an identical restriction pattern with *A. tamarii*; this result indicates an extremely close phylogenetic relationship between the two species. All members of the *A. flavus* and *A. nidulans* groups tested in this work have about 25% of the bands in common and show less homology to *A. niger* and *A. awamori* (both from the *A. niger* group). The phylogenetic distance between the *A. nidulans* and *A. flavus* groups seems to be less than the distances between *A. flavus* and *A. niger* and between *A. niger* and *A. awamori*. The last result is striking, as *A. niger* and *A. awamori* are classified together within the *A. niger* group and one might expect more homology between the two species.

It might be of interest to calculate sequence divergence i.e. the degree of base substitutions per base pair in different mitochondrial DNAs. It is, however, important to point out that the procedure of Upholt (1977) for calculating sequence divergence can be applied here only with several reservations. First, it seems that the rate of base substitutions is not equal in all parts of the mitochondrial genome (Brown *et al.*, 1980). Second, some of the observed changes in restriction patterns might be due to insertions, as was shown for *A. echinulatus* by Earl *et al.* (1980), and as is indicated by the differences in the molecular weights of the mitochondrial genomes studied. Third, we present here restriction patterns and not restriction maps; thus there is a possibility of misinterpretation in calculating common bands versus

Fig. 1. *Eco*RI and *Hind*III restriction endonuclease digestion patterns of mitochondrial DNA from various aspergilli (a) and their gel transfer hybridization to probes representing 16S rRNA (b) and 23S rRNA (c) from *A. nidulans*. Lane 1, *A. nidulans*; 2, *A. tamarii*; 3, *A. oryzae*; 4, *A. wentii*; 5, *A. awamori*; 6, *A. niger*.

Fig. 2. A schematic diagram and molecular weights (in kb) of fragments of mitochondrial DNA from various species of aspergilli generated by *Eco*RI and *Hind*III restriction endonucleases. [Data for *A. echinulatus* are from Earl *et al.* (1980).] *a*, Bands hybridizing to the radioactive probe representing 16S rRNA; *b*, bands hybridizing to the radioactive probe representing 23S rRNA; *c*, bands showing weak hybridization to the probe (4.0 kb fragment of *A. nidulans* mitochondrial DNA containing the 16S rRNA gene and its flanking sequences) probably due to small regions of homology between different fragments of mitochondrial DNA; ?, hybridization could only be seen on the negative.

Table 1. Values of *F* (fraction of common bands between two tested species) and *p* (sequence divergence) calculated for seven species of aspergilli

Species compared	<i>F</i>	<i>p</i> (%)
<i>A. nidulans</i> – <i>A. tamaritii</i>	0.24	8.6
<i>A. nidulans</i> – <i>A. wentii</i>	0.24	8.6
<i>A. nidulans</i> – <i>A. oryzae</i>	0.23	8.9
<i>A. nidulans</i> – <i>A. awamori</i>	0	—
<i>A. nidulans</i> – <i>A. niger</i>	0.25	8.3
<i>A. nidulans</i> – <i>A. echinulatus</i>	0.78	1.4
<i>A. tamaritii</i> – <i>A. wentii</i>	1.0	0
<i>A. tamaritii</i> – <i>A. oryzae</i>	0.83	1.1
<i>A. tamaritii</i> – <i>A. awamori</i>	0.07	16.5
<i>A. tamaritii</i> – <i>A. niger</i>	0.15	11.7
<i>A. tamaritii</i> – <i>A. echinulatus</i>	0.26	8.1
<i>A. wentii</i> – <i>A. oryzae</i>	0.83	1.1
<i>A. wentii</i> – <i>A. awamori</i>	0.07	16.5
<i>A. wentii</i> – <i>A. niger</i>	0.15	11.7
<i>A. wentii</i> – <i>A. echinulatus</i>	0.26	8.1
<i>A. oryzae</i> – <i>A. awamori</i>	0.07	16.1
<i>A. oryzae</i> – <i>A. niger</i>	0.14	11.9
<i>A. oryzae</i> – <i>A. echinulatus</i>	0.22	9.1
<i>A. awamori</i> – <i>A. niger</i>	0.14	11.9
<i>A. awamori</i> – <i>A. echinulatus</i>	0.07	16.1
<i>A. niger</i> – <i>A. echinulatus</i>	0.24	8.6

Fig. 3. A hypothetical tree of phylogenetic relationships between the tested species of the genus *Aspergillus*.

unique bands. For the above reasons, the values of sequence divergence (p) presented in Table 1 should be treated as estimates only.

It has been established for mammals that the rate of evolution of mitochondrial DNA is 10 times faster than that of nuclear DNA (Brown *et al.*, 1979). In that study, sequence divergence values were correlated with data from the study of fossils. In contrast to this, the rate of evolution of fungal mitochondrial DNA is not known. However, if we assume that the rate of evolution of mitochondrial genomes from the genus *Aspergillus* is constant, then the values of sequence divergence are proportional to the time in evolution when the two compared species separated. On this assumption, we have constructed a hypothetical tree of phylogenetic relationships between the members of the genus *Aspergillus* tested in our laboratory (Fig. 3).

Some comments on the credibility of the results obtained should be made. Molecular weights of DNA fragments are usually calculated with an accuracy of about 5%, but it is important to point out that the mitochondrial DNA molecule is circular, and that identifying two bands as being identical can often be done not only by comparing their molecular weights, but also by comparing their relative positions with respect to other bands, i.e. the band pattern. Additional information can be obtained from hybridization data or from comparison of restriction patterns obtained with different mixtures of enzymes. We tested such patterns using digestion of mitochondrial DNAs with *Hind*III alone (data not shown), and the results confirmed our conclusions.

It is difficult to estimate the error of our calculation of the fraction F (and of sequence divergence p , as p is a function of F). This is due to the fact that when F approaches 1, the accuracy reaches its maximum. When F is significantly lower than 1, the possibility of misinterpretation of c_{xy} is greater, and so is the error. If we assume misinterpretation of two bands, then the error is 0.07. Any such error would not affect our conclusions on the phylogenetic relationships between the tested species.

All the species examined were very carefully checked for their identity using the methods described by Raper & Fennell (1965). However, we consider that investigations on other members of this group are needed in order to establish the divergence within the genus *Aspergillus*; unfortunately, we have no such strains in our collection.

We believe our data clearly show that the use of restriction enzyme analysis of mitochondrial DNA would be a very useful method in the taxonomy of the genus *Aspergillus*. In our small sample of seven species the values of F (the fraction of common bands) from the array of comparisons varied from 0 to 1 in an almost continuous fashion. This means that in the genus *Aspergillus* both close relationships and relatively great phylogenetic distances can be estimated using this method. We hope, therefore, that this approach will be continued and will provide a useful aid for the taxonomy of the genus *Aspergillus*.

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