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Nucleotide Composition of Nuclear and Mitochondrial Deoxyribonucleic Acid of Fungi

VICENTE D. VILLA AND R. STORCK
Department of Biology, Rice University, Houston, Texas 77001

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The buoyant density of nuclear and mitochondrial deoxyribonucleic acid (DNA) from 14 species of fungi was determined by CsCl density gradient equilibrium centrifugation. The buoyant density of both types of DNA was the same for all three *Mucorales* analyzed. The buoyant density of mitochondrial DNA was significantly lower than that of the nuclear DNA for nine species of *Ascomycetes* and two species of *Basidiomycetes*. No simple correlation could be obtained from the comparison of the two types of DNA. Mitochondrial DNA represented a very small proportion of total DNA. Heat-denatured mitochondrial DNA renatured more readily than nuclear DNA.

The mole per cent of guanine plus cytosine (%GC) of the deoxyribonucleic acid (DNA) found in fungal extracts was shown (27) to exhibit certain variations suggesting taxonomic and phylogenetic relationships. These relationships have been confirmed in a survey of about 700 additional species of *Eumycotina* (Storck and Alexopoulos, *in preparation*). The DNA molecules from most of these organisms were unimodally distributed in a CsCl density gradient. However, for a few species, a minor band with a buoyant density about 0.020 g/cc lower than that of the main band was sometimes detected. The recent demonstration that DNA isolated from purified mitochondria from *Saccharomyces* (4, 18, 25, 33), *Neurospora* (15, 21), and *Physarum* (8, 10) had on the average a buoyant density 0.015 g/cc lower than that of the homologous nuclear DNA (nu-DNA) suggested that this minor band might have been of mitochondrial origin. Among animals (9), the buoyant density of mitochondrial DNA (mit-DNA) was either the same or higher than that of the nu-DNA, whereas for plants it was always higher (2).

The findings listed above prompted us to isolate mitochondria from several fungal species in order to find out whether they contained DNA and whether the %GC of mit-DNA determined by CsCl density gradient equilibrium centrifugation (17) was always less than that of the corresponding nu-DNA.

In the present work, 14 species of fungi distributed among *Zygomycetes*, *Ascomycetes*, and *Basidiomycetes* were studied. In all cases, DNA could be isolated from purified mitochondria. Furthermore, our analyses of the %GC of both nu-DNA and mit-DNA demonstrated the following: (i) the compositional variation of mit-DNA, like that of nu-DNA, was minimal for organisms belonging to the same genus; (ii) the overall range of %GC for mit-DNA was less than for nu-DNA; (iii) the %GC of mit-DNA was either equal to that of its homologous nu-DNA or it was less, but it was never significantly higher; (iv) no simple correlation could be obtained from the comparison of nu-DNA and mit-DNA; (v) mit-DNA represented but a very small proportion of total DNA; and (vi) heat-denatured mit-DNA renatured more readily than nu-DNA.

MATERIALS AND METHODS

Organisms. Organisms were selected in order to cover a %GC range from 30 to 60%. Among the species studied, there were five for which the %GC of nu-DNA had been previously determined. These five species included *N. crassa* and *N. sitophila* in which mit-DNA had also been analyzed (15, 21).

Growth and harvest. The organisms were grown in the medium of Bartnicki-Garcia and Nickerson (1) at 25 C on a rotary shaker in baffled, long-necked, 2-liter Erlenmeyer flasks. Cultures totaling 6 liters were harvested by filtration. The mycelial pads were washed with 1 liter of cold 0.02 M tris(hydroxyl)-aminomethane (Tris), *p*H 7.9, containing 0.001 M ethylenediaminetetraacetate (EDTA) and 0.44 M sucrose.

Isolation and purification of mitochondria. The procedure of Luck and Reich (15) was used for the isolation of mitochondria. The crude mitochondrial fraction was divided into two equal parts, and one of these was treated with pancreatic deoxyribonuclease (once crystallized, Worthington Biochemical Corp.,

Freehold, N.J.) (15). A 1.5- to 2-ml amount of mitochondrial suspension containing between 75 and 100 mg of protein and corresponding to 3 liters of culture was layered on a 20-ml sucrose density gradient. This gradient was prepared by the successive layering of 5 ml of 0.02 M Tris (*p*H 7.9) containing 0.001 M EDTA and having the following sucrose concentrations: 2.2, 2.0, 1.9, and 0.97 M. Fractions (1 ml) were collected by puncturing the bottom of the centrifuge tube after 1 hr of centrifugation at 25,000 rev/min at 4 C in an SW 25 rotor of a Spinco model L ultracentrifuge. The protein content of each fraction was determined by the method of Layne (14) with the use of the Folin-Ciocalteau reagent. Cytochrome *c* oxidase contents were determined according to the method of Haidle and Storck (11). A centrifugation profile showing the distribution of cytochrome *c* oxidase specific activity (enzyme units per milligram of protein) is presented in Fig. 1 for mitochondria extracted from *N. crassa*. Similar profiles were obtained with the following organisms: *Cunninghamella echinulata*, *Gelasinospora autosteira*, and *Mucor rouxii*. The fractions with the highest specific activity, for example, 13 to 17 in the case of Fig. 1, and which correspond to a distinct turbid band, were pooled and the mitochondria were pelleted by a 90-min centrifugation at 30,000 rev/min at 4 C in the no. 30 rotor of a Spinco preparative ultracentrifuge. The cytochrome *c* oxidase specific activity was not determined for organisms not listed above. For these organisms, mitochondria were purified as described except that we used an SW 25.2 rotor which permitted us to layer twice as much material on 56 ml of the sucrose gradient. After centrifugation, the distinct turbid band of mitochondrial material was collected in one fraction which was directly centrifuged in the no. 30 rotor.

Extraction and purification of mit-DNA. The centrifugation pellet was suspended in 6 ml of Tris buffer (*p*H 7.9) containing 0.001 M EDTA, 2.5% (w/v) sodium dodecyl sulfate and 0.2% (w/v) sodium deoxycholate. The suspension was frozen and thawed and then was incubated at 37 C for 30 min. An equal volume of water-saturated phenol (Fisher Scientific Co., Pittsburgh, Pa.; certified, for chromatography), *p*H 5.5, containing 0.001 M EDTA was added. The mixture was shaken for 30 min and then centrifuged. The traces of phenol contaminating the aqueous phase were removed by shaking for 5 min with an equal volume of anhydrous ether. Two volumes of absolute ethyl alcohol were added to the aqueous phase after the removal of the ether by N_2 bubbling. The nucleic acid precipitation was completed after 1 hr at 0 C. The precipitate was collected by centrifugation and dissolved in 6 ml of a solution of saline and sodium citrate (SSC) (16). α-Amylase (B grade; Calbiochem, Los Angeles, Calif.) was added to a final concentration of 2 mg/ml. The mixture was incubated for 30 min at 37 C. Ribonuclease (five times crystallized; Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 50 μg/ml from a 2 mg/ml stock solution which had been previously heated at 80 C for 10 min to destroy possible contaminating deoxyribonuclease. After incubation at 37 C for 2 hr, Pronase (B grade, Calbiochem) was added to a final concentration of 50 μg/ml. The mixture was incubated for 2 hr at 37 C. The DNA preparation was dialyzed for 24 hr at 4 C against 2 liters of SSC. DNA concentration was estimated by optical density measurement at 260 mμ (OD_{260}) with 1.0 OD_{260} unit equivalent to 50 μg/ml.

Extraction and purification of total DNA. Mycelial pads were washed with a saline-EDTA solution (0.1 M EDTA, 0.15 M NaCl). The mycelial mats were

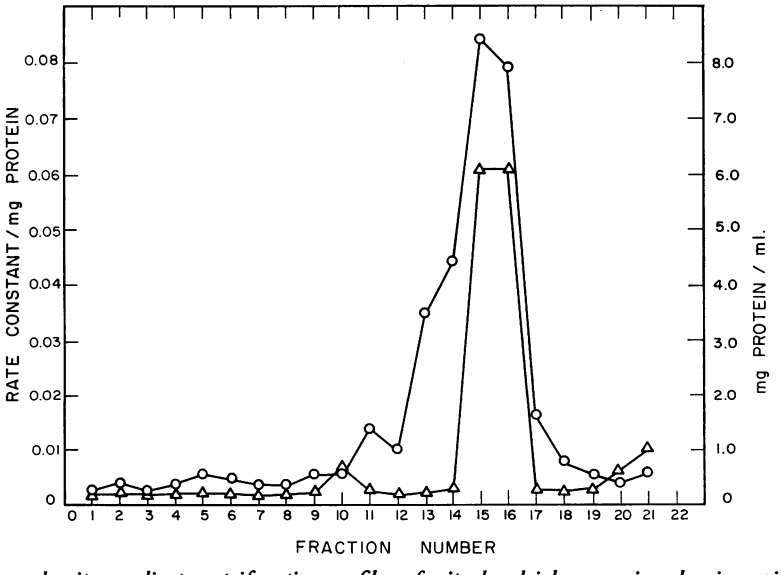

FIG. 1. *Sucrose density gradient centrifugation profiles of mitochondrial suspension showing coincidence of the distribution of protein* (△) *and of cytochrome oxidase activity* (○).

frozen with liquid N_2 in a mortar. After the N_2 had boiled off, grinding was carried out for 1 min until caking of the resulting powder began to occur. More N_2 was added, and grinding was continued until microscopic observation showed that approximately 90% of the filaments had been ruptured. The dry frozen powder was transferred to a long-necked, round-bottom, distillation flask and covered with a solution of saline-EDTA. The procedure for DNA extraction was similar to that described by Cheng and Sueoka (3), but included in addition incubations with α-amylase and Pronase as described for mit-DNA preparation. On completion of the incubations, an equal volume of a mixture of isoamyl alcohol, chloroform, and phenol (2:48:50) was added, and shaking was carried out for 30 min to remove the enzymes and the other residual proteins. The aqueous phase separated by centrifugation was deproteinized as above. The final clear aqueous solution was precipitated with 2 volumes of absolute ethyl alcohol. The precipitate was collected either by spooling onto a glass stirring rod or by allowing it to settle after mixing of the aqueous and ethyl alcohol phases. Further purification of DNA was obtained by using the isopropyl alcohol precipitation technique described by Marmur (16).

Determination of GC content. The method of Meselson, Stahl, and Vinograd (17) was used for the determination of the buoyant density of the DNA. The conversion into %GC was calculated according to Schildkraut, Marmur, and Doty (23). SP8 bacteriophage DNA was used as a reference. All DNA buoyant densities were related to that of *Escherichia coli*, which was taken to be 1.710 g/cc (23).

RESULTS

In most instances, total DNA preparations exhibited at equilibrium a unimodal distribution. Conversely, DNA preparations obtained from mitochondria untreated with deoxyribonuclease prior to extraction were often contaminated by a small amount of nu-DNA. This is illustrated in Fig. 2a for *Gelasinospora cerealis* by the presence of a small peak with a buoyant density of 1.713 g/cc, which is equal to that of nu-DNA and is located between the SP8 DNA (1.743 g/cc) and the mit-DNA (1.701 g/cc) bands. As shown in Fig. 2b, deoxyribonuclease treatment of mitochondria yielded a satellite-free mit-DNA preparation.

Satellite DNA peaks were never observed in preparations, not treated with deoxyribonuclease, from *C. echinulata*, *Mucor fragilis*, and *M. rouxii*, and the buoyant density was the same for total DNA and mit-DNA preparations. Since it had been reported (4, 24, 25) that mit-DNA renatured more readily than nu-DNA, the DNA preparations obtained from *M. rouxii* were analyzed for their ease of renaturation (Fig. 3 and 4). A solution of nu-DNA was heated at 100 C for 10 min and immersed in an ice bath. When it was

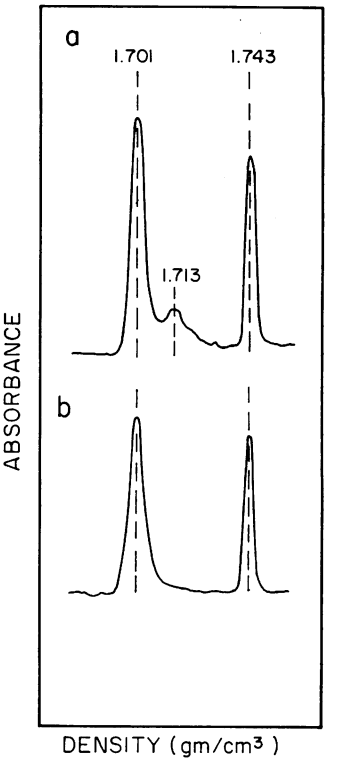

FIG. 2. *Microdensitometric tracings of Gelasinospora cerealis DNA photographed after 20-hr centrifugation at 44,000 rev/min on an analytical ultracentrifuge. (a) DNA preparation obtained from mitochondria untreated with deoxyribonuclease prior to extraction showing contamination of mit-DNA (ρ = 1.701 g/cc) by nu-DNA (ρ = 1.713 g/cc). (b) The same preparation after treatment of intact mitochondria with deoxyribonuclease. The DNA from SP8 bacteriophage (ρ = 1.743 g/cc) was used as reference.*

chilled, it was mixed with a solution of native nu-DNA and the mixture was centrifuged. As shown in Fig. 3a, two peaks were present at equilibrium. The peak with a buoyant density of 1.714 g/cc corresponds to denatured nu-DNA and the peak with a buoyant density of 1.698 g/cc to native nu-DNA. The difference in buoyant density observed (0.016 g/cc) is close to the expected value of 0.015 g/cc (23). As shown in Fig. 3b, only one peak with a buoyant density of 1.714 g/cc was present with heat-denatured nu-DNA alone. A similar profile was observed (Fig. 3c) when the DNA solution in 2 × SSC was incubated at 60 C for 2 hr (26) after denaturation at 100 C for 10 min.

As illustrated in Fig. 4, heat denaturation of mit-DNA from *M. rouxii* caused an increase in buoyant density from 1.697 to 1.713 g/cc (Fig.

4a). However, if the mit-DNA solution in 2 × SSC was incubated at 60 C for 2 hr after heating at 100 C, it yielded a band with a buoyant density of 1.701 g/cc (Fig. 4b), which is only 0.004 g/cc higher than the buoyant density of native mit-DNA (Fig. 4c).

The results of the analyses of %GC in nu-DNA and mit-DNA are listed, respectively, in columns 1 and 2 of Table 1. Each value represents an average of at least two determinations of the buoyant density on the same DNA sample; in the case of *M. rouxii* and *N. crassa* the values represent an average of two determinations performed on DNA preparations extracted from two different cultures. No significant difference was found

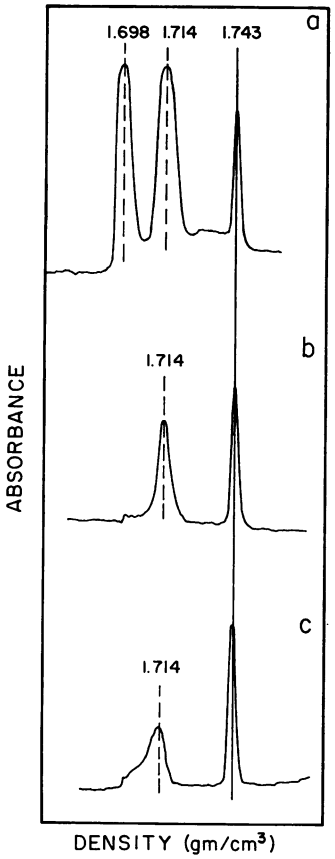

FIG. 3. *Microdensitometer tracings of Mucor rouxii nu-DNA photographed after 20-hr centrifugation at 44,000 rev/min on an analytical ultracentrifuge. (a) A mixture of heat-denatured nu-DNA (ρ = 1.714 g/cc) and native nu-DNA (ρ = 1.698 g/cc). (b) Heat-denatured nu-DNA without mixing with native nu-DNA. (c) Heat denatured nu-DNA after 2-hr incubation at 60 C in 2 × SSC. The buoyant density, 1.714 g/cc is the same as in (b). The DNA from SP8 bacteriophage (ρ = 1.743 g/cc) was used as reference.*

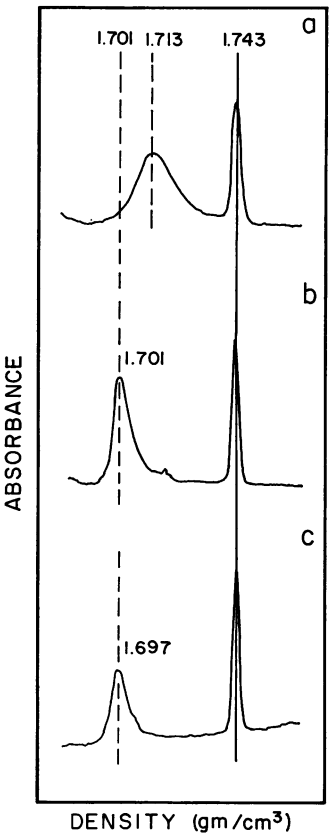

FIG. 4. *Microdensitometer tracings of Mucor rouxii mit-DNA photographed after 20-hr centrifugation at 44,000 rev/min on an analytical ultracentrifuge. (a) Heat-denatured mit-DNA (ρ = 1.713 g/cc), (b) mit-DNA (ρ = 1.701 g/cc) renatured by incubation at 60 C for 2 hr, in 2 × SSC after heating at 100 C for 10 min, (c) native mit-DNA (ρ = 1.697 g/cc). The DNA from SP8 bacteriophage (ρ = 1.743 g/cc) was used as a reference. Comparison of Fig. 4 and 3 shows that mit-DNA renatured more readily than nu-DNA.*

between the buoyant density of mit-DNA extracted from deoxyribonuclease-treated and untreated mitochondrial preparations. The values for *N. crassa* and *N. sitophila* are the same as those reported in the literature (15, 21).

To permit a comparison, values obtained from the literature for microbes and animals are listed in Table 2.

DISCUSSION

A comparison of the values listed in Table 1 with those for *Physarum polycephalum* and *Saccharomyces* (Table 2) indicated that among fungi the %GC range for mit-DNA (22 to 43) was smaller than for nu-DNA (34 to 61). How-

TABLE 1. *Guanine plus cytosine content in moles per cent of nuclear and mitochondrial DNA of fungi*

Organism	(1) Nuclear DNA	(2) Mitochondrial DNA	(1) − (2)
Zygomycetes			
Cunninghamella echinulata	34	34	0
Mucor fragilis	39	38	+1
M. rouxii	37	38	−1
Ascomycetes			
Ceratocystis ulmi	56	40	+16
Gelasinospora autosteria	54	41	+13
G. calospora	55	41	+14
G. cerealis	55	42	+13
G. tetrasperma	—	44	—
Neurospora crassa	54	42	+12
N. sitophila	55	43	+12
Sordaria macrospora	54	42	+12
Chaetonium globosum	58	34	+24
Basidiomycetes			
Daedalea confragosa	57	31	+26
Schizophyllum commune	61	28	+33

ever, if Tables 1 and 2 are combined, these ranges respectively became: 22 to 56 and 26 to 61. Therefore, it must be concluded that the compositional diversity of mit-DNA did not differ much from that of nu-DNA.

The composition heterogeneity of mit-DNA, like that of nu-DNA, tended to be minimal for genera, with the exception of *Tetrahymena* and *Saccharomyces*. For *S. cerevisiae* the extreme values indicated in Table 2 correspond to mitochondrial mutants having an altered respiratory activity (18). Such a modification in buoyant density could be due, according to Mounolou et al. (18), to a change in the extent of methylation or the presence of unknown components rather than to a change in %GC. Henceforth, it appears that the taxonomic value of nucleotide composition of mit-DNA is as good as that of nu-DNA.

When the %GC of mit-DNA was the same as that of nu-DNA, as in the case of *Mucorales* (Table 1) and some animal species (Table 2), mit-DNA could, however, as shown here for *M. rouxii*, be distinguished from its nuclear counterpart by its ability to renature more readily after heat denaturation. This readiness to renature appears to be characteristic of all mit-DNA preparations (2). This property, together with the results of nearest-neighbor frequency analyses of mit-DNA from *P. polycephalum* (5), has led to the suggestion (5) that mit-DNA is similar to procaryotic DNA. This suggestion could be taken to mean that mitochondria have a parasitic origin. Although this is a highly speculative matter, it is nevertheless important to note that the sedimentation coefficients of mitochondrial ribosomes and ribosomal ribonucleic acid (RNA) from *N. crassa* (13, 22) were different from their respective cytoplasmic homologues but were similar, respectively, to those of bacterial ribosomes (31) and bacterial ribosomal RNA (32). Likewise, the base composition of mitochondrial ribosomal RNA was different from that of cytoplasmic ribosomal RNA in *N. crassa* (13, 22). In *M. rouxii* (Villa and Storck, *in preparation*), the nucleotide com-

TABLE 2. *Guanine plus cytosine content in moles per cent of nuclear and mitochondrial DNA from microorganisms and animals as reported in the literature*[a]

Organism	(1) Nuclear DNA	(2) Mitochondrial DNA	(1) − (2)	Reference[b]
Physarum polycephalum	41	27	+14	8
P. polycephalum	38	27	+11	10
Saccharomyces cerevisiae	38	22	+16	24
S. cerevisiae	41	26	+15	33
Wild type	42	28	+14	18
Nuclear mutant	42	28	+14	18
Nuclear mutant	42	28	+14	18
Mitochondrial mutant	42	36	+6	18
Mitochondrial mutant	40	24	+16	18
Wild type	40	25	+15	4
S. carlsbergensis	40	24	+16	19
S. carlsbergensis	40	25	+15	4
Tetrahymena pyriformis Syngens	26	26	0	29
T. pyriformis Syngens	33	27	+6	29
T. pyriformis Syngens	28	27	+1	29
T. pyriformis Syngens	26	26	0	29
T. pyriformis Syngens	31	25	+6	29
Strain	29	25	+4	29
Paramecium aurelia	30	43	−13	30
Euglena gracilis	48	32	+16	7
Rana pipiens	43	43	0	6
Xenopus	43	45	−2	6
Chicken liver	39	48	+9	20
Mouse	40	40	0	26
Rat liver	44	42	+2	24
Guinea pig liver	41	41	0	4
Lamb heart	45	55	−10	12
Beef liver	44	56	−12	2

[a] When the authors gave only buoyant density values, these were converted into %GC by use of the equation of Schildkraut et al. (23).

[b] The reference numbers correspond to those found in the Literature Cited section.

position of total mitochondrial RNA was found to differ from that of total cytoplasmic RNA.

Although the number of analyses of mit-DNA presented here is limited, it is nevertheless relevant to stress the fact that for *Zygomycetes* there was no difference between the %GC of mit-DNA and nu-DNA, whereas average differences of 15 and 29% were found for *Ascomycetes* and *Basidiomycetes*, respectively. It appears possible, therefore, that when more results become available the analysis of the base composition of mit-DNA, as well as that of nu-DNA, might have a phylogenetic significance.

ACKNOWLEDGMENTS

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