Plant Physiol. (1980) 65, 1207–1210
0032-0889/80/65/1207/04/$00.50/0

Mitochondrial DNA from *Oenothera berteriana*

PURIFICATION AND PROPERTIES[1]

Received for publication July 7, 1979 and in revised form September 9, 1979

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ABSTRACT

Mitochondrial DNA (mt-DNA) from *Oenothera berteriana* tissue culture cells was isolated and characterized with respect to buoyant density in CsCl, melting point, contour length, and restriction fragments.

Because of the rather long purification procedure very few molecules retained their circularity. Only one distinct size class of molecules with a length of 100 kilobases was found. Restriction fragments were obtained with the enzymes, restrictionendonuclease I from *Serratia marcescens*, restrictionendonuclease III from *Haemophilus influenzae*, restrictionendonuclease I from *Bacillus amyloliquefaciens* H, and restrictionendonuclease I from *Escherichia coli* (Bohnert 1977 Exp Cell Res 106: 426–430); the added lengths of these fragments amounted to 180 to 190 kilobases. As in other higher plants, an intermolecular heterogeneity has to be postulated to explain the large number of restriction fragments. Unique to the mt-DNA from *Oenothera berteriana*, as compared to other higher plants, is the unusual high guanosine + cytosine content with 51% as determined by the buoyant density in CsCl of 1.710 grams/cubic centimeter and the melting point of 90 C.

So far, mt-DNA[2] from higher plants has been studied with only a limited choice of methods. Buoyant density in CsCl has been determined to be generally 1.706 g/cm^3 (16, 17, 25). Kinetic complexity of mt-DNA of peas as determined by reassociation curves was with 70×10^6 daltons in agreement with the electronmicroscopy of molecules from this species in which a single size class of 35 μm was observed. These data do not seem valid for the whole plant kingdom. In some species such as maize (16) and soybean (21), different size classes of circular molecules are observed, the largest being about the size of mt-DNA from the pea. These different mt-DNA molecules within the same species or even organism, as well as the mini- and maxicircles in *Trypanosoma* (2) could be an explanation for the complex data obtained with restriction analysis. Restriction fragment lengths of mt-DNA in higher plants amount to $130–210 \times 10^6$ daltons (12, 17), which is far larger than the longest molecule visualized by electronmicroscopy. It is not clear yet, whether there are different molecules of various sizes and varying restriction fragments or whether there is only one kind of molecules with a varying degree of methylation being the reason for the large number of restriction fragments, for example.

This paper includes data obtained mainly with these methods on mt-DNA from *Oenothera berteriana*.[3] *Oenothera* was chosen as an object because of its relatively well studied extrachromosomal inheritance patterns in cross-breedings of different *Oenothera* genetic varieties.

MATERIALS AND METHODS

Tissue cultures were established from surface-sterilized anthers of *O. berteriana* and grown on medium A_0 (14). Of approximately 500 anthers, three to four developed a small callus. These were transferred to fresh medium and allowed a growth time of about 25 days before being transferred again.

Mitochondria were isolated essentially as described by Kolodner and Tewari (8) through differential centrifugation. After disrupting the cells in a Braun multimix for 7 × 4 s in a 3-fold excess of extraction buffer (300 mM mannitol, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 50 mM Tris-HCl, 5 mM EDTA, 4 mM mercaptoethanol, 0.25 mM octanol [pH 7.4]), cell debris was removed by centrifugation for 15 min at 1,100g. Mitochondria were pelleted at 12,000g for 20 min, resuspended in DNase buffer (300 mM mannitol, 50 mM Tris-HCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$ [pH 7.4]), and incubated with 50 μg DNase I (EC 3.1.4.5) (Boehringer Mannheim) for 60 min at 4 C. Two hundred ml of extraction buffer were added, and the mitochondria were again pelleted. Two further washings removed the DNase sufficiently.

The last mitochondrial pellet was taken up in 10 ml of lysis buffer (300 mM mannitol, 5 mM EDTA, 10 mM KH_2PO_4, 50 mM Tris-HCl [pH 7.4]). The mitochondria were lysed with Sarkosyl (final concentration 0.4%) for 10 min on ice. Solid CsCl was added up to 1 g/ml and gently dissolved. A CsCl cushion was carefully underlayered and spun at 33,000 rpm in a Ti-50 rotor (Beckman) for 14 h (26; R. G. Herrmann, personal communication).

After centrifugation 0.7-ml fractions were collected. Of each fraction a 1-μl aliquot was dried on a glass fiber filter disc and stained with ethidium bromide. The DNA containing fractions were pooled for dialysis against TE (1 mM EDTA, 10 mM Tris-HCl [pH 8]). Ribonuclease A (EC 3.1.4.22) (Boehringer Mannheim) was added into the dialysis bag to a concentration of 0.5 mg/ml and incubated for 60 min at 37 C. Proteinase K (Merck, Darmstadt) (preincubated for 1 h at 37 C) was added up to 0.05 mg/ml and left for 14 h at 37 C.

Two extractions with phenol-chloroform (50:50) were followed by one extraction with chloroform-isoamylethanol (24:1) and one

[1] A brief report on this work was presented at the International Chemical and Nuclear Corporation, University of California at Los Angeles, Symposium, 1979.

[2] Abbreviations: mt-DNA: mitochondrial DNA; Eca I: restrictionendonuclease I from *Enterobacter cloacae*; GC: guanosine + cytosine; kb: kilobases; AT: adenosine + thymine; Sma I: restrictionendonuclease I from *Serratia marcescens*; Hind III: restrictionendonuclease III from *Haemophilus influenzae*; Bam HI: restrictionendonuclease I from *Bacillus amyloliquefaciens* H; Eco RI: restrictionendonuclease I from *Escherichia coli*.

[3] New nomenclature (Dietrich, 1977): *Oenothera berteriana*: *Oenothera villaricae*; *Oenothera odorata*: *Oenothera picensis sspec. picensis*.

with ether. The mitochondrial DNA was finally collected in an ethidium bromide/CsCl equilibrium gradient. The dye was removed by four extractions with propanol (equilibrated with distilled water saturated with CsCl), and the DNA dialyzed against TE. The mt-DNA was precipitated with ethanol and taken up in TE at a concentration of 100 μg/ml. Analytical ultracentrifugation was performed as described by Steinemann (22). The melting curves were done in 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate) (13) in a Gilford spectrophotometer.

Mt-DNA molecules were spread on parlodion-coated grids and rotary shadowed according to Royer and Hollenberg (18).

Restriction enzymes (Boehringer Mannheim) were assayed in the standard buffers. Eca I was kindly provided by Dr. H. Mayer, Stöckheim.

Electrophoresis was performed on 12- x 14- x 0.2-cm vertical slab gels of 1% agarose (Seakem, TE) for 5 h at 40 mamp in 200 mM Na acetate, 200 mM NaCl, 20 mM EDTA, 400 mM Tris-acetate (pH 8.15).

RESULTS

Isolation of mt-DNA. In *Oenothera* special difficulties had to be overcome in order to obtain pure mt-DNA. Experiments to purify mt-DNA from differentiated plant material were unsuccessful due to the abundant polysaccharides and phenolic compounds of *Oenothera*.

The nuclear DNA in *Oenothera* has a buoyant density of 1.703 g/cm^3 (7). The chloroplast DNA of 1.697 g/cm^3 (7), and for all higher plants examined so far, the buoyant density of mt-DNA has been 1.706 g/cm^3 (17, 25). It seemed impossible to separate mt-DNA from whole-cell DNA in a CsCl density gradient, assuming that *Oenothera* mt-DNA had the buoyant density found in other higher plants. In all experiments, mitochondria were isolated first, DNase-treated, and lysed after washing. The procedure described above was finally adopted for giving the cleanest restriction patterns and the largest fraction of high mol wt molecules as observed in the electron microscope. The centrifugation of the mt-DNA into a CsCl cushion from the lysed mitochondria proved to be extremely useful. The yield of mt-DNA with this procedure was 150–180 μg/kg tissue culture cells.

Buoyant Density and Base Composition. Figure 1 shows a densitometer scan for an average DNA preparation after ultracentrifugation. A minor contamination with nuclear DNA can be observed even after DNase treatment of the intact mitochondria. As expected nuclear DNA and mt-DNA band very close in the density gradient. The buoyant density of mt-DNA from *O. berteriana* is different from the density of mt-DNA in all higher plants examined so far. With a buoyant density of 1.710 g/cm^3 this DNA is relatively GC-rich (51%) (19).

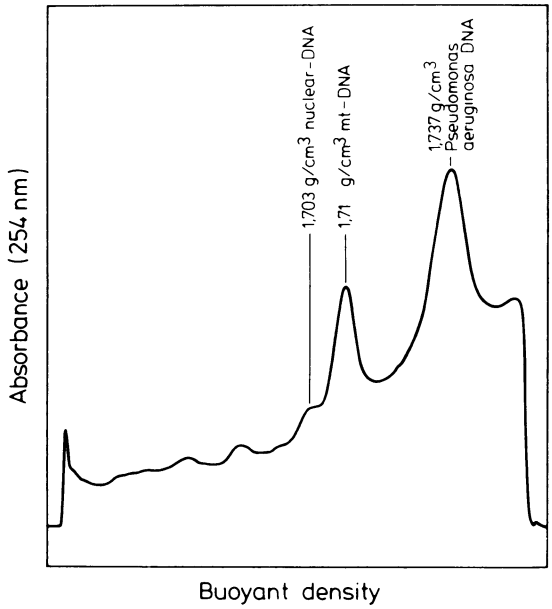

FIG. 1. Densitometer scan of mt-DNA from *O. berteriana* after centrifugation in a CsCl density gradient.

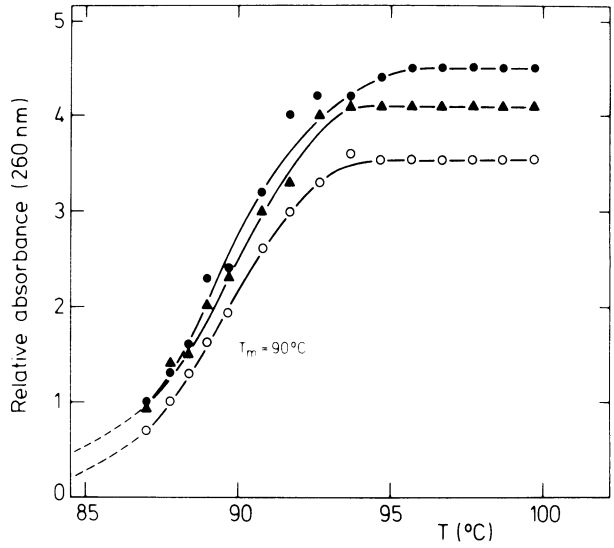

FIG. 2. Melting profiles of three preparations of mt-DNA from *O. berteriana*.

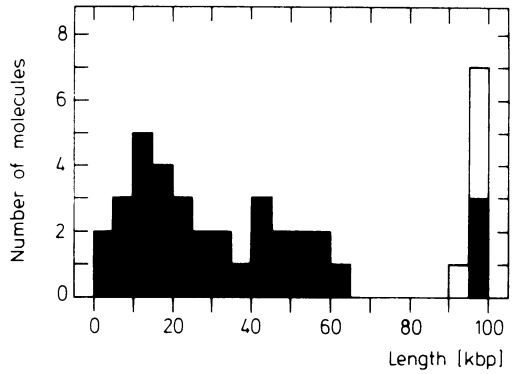

FIG. 3. Size distribution of the linear mt-DNA molecules (■) and of the circular molecules (□) measured.

To verify this observation, melting profiles were performed (Fig. 2). The melting point of 90 C in 1 × SSC corresponds to a GC content of about 50.5% (13).

Electronmicroscopy. Mt-DNA from higher plants contains the longest mt-DNA molecules observed so far. Most authors found lengths from 90–120 kb in the various species (8, 17). This also holds true for *O. berteriana*. After the rather long purification procedure, few molecules retained their full length. However, the longest molecules have very similar lengths of 100 kb (Fig. 3). This was the only length indicating any dominance. Circular molecules were also found in mt-DNA from *O. berteriana*, but generally associated with electron-dense particles (1). No smaller circular molecules could be observed as were found to exist in maize (15) and in soybean (21).

Restriction Enzyme Analysis. Restriction fragment analysis of mt-DNA was done for two reasons: (*a*) to find out whether the heterogeneity observed in higher plants applies to *Oenothera* as well; and (*b*) to prepare for an analysis of the inheritance pattern and the stability of *O. berteriana* and *Oenothera odorata* mito-

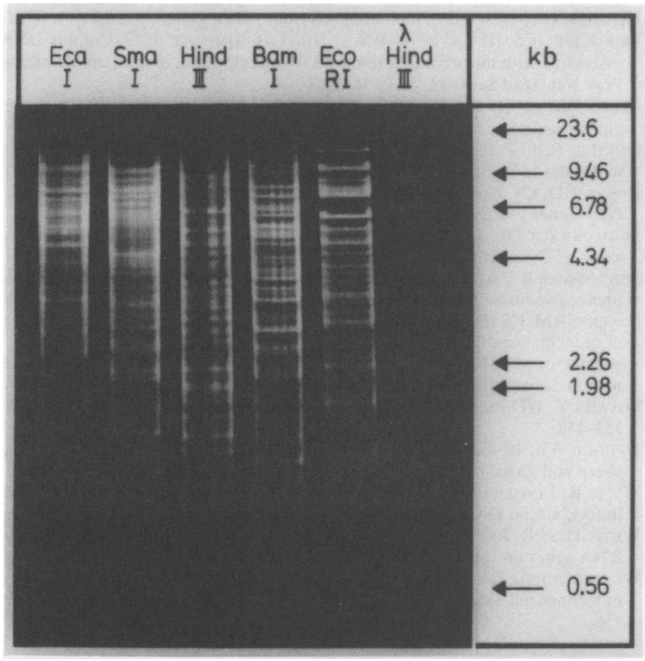

FIG. 4. Gel electrophoresis pattern of restriction fragments generated from mt-DNA of *O. berteriana* with the enzymes indicated. Hind III fragments of mt-DNA were used as length standards.

chondrial DNA in various genetic varieties of these two species (20). Closely related races of *Podospora anserina* (4, 5) and of *Zea mays* (16), different members of the same strain in rats (9), and even individuals of sheep and goats (24) show different restriction fragment patterns.

Mt-DNA from *O. berteriana* was analyzed with various restriction enzymes. Figure 4 shows the fragments generated with the enzymes Sma I, Hind III, Bam HI, Eco RI, and the enzyme Eca I, which recognizes a heptanucleotide. Table I lists the fragments obtained with the enzymes Eco RI, Bam HI, and Sma I. The sum of the fragment lengths amounts to 180–190 kb. Of the fragments generated by Sma I, a 26-kb fragment seems to exist only in incomplete digestion and could be observed only in some preparations. It is unlikely that the mt-DNA preparations lacking this 26-kb fragment have a larger amount of broken DNA, as the restriction fragment patterns of these extractions show no irregularities with other restriction enzymes.

DISCUSSION

The major part of the work reported in this paper was used to develop a method for isolating mt-DNA from *O. berteriana* in spite of its similar density to the nuclear DNA. Mt-DNA in *Oenothera* contains molecules of uniform length of 100 kb. No smaller circular molecules could be observed in *Oenothera*, and as the employed purification procedures are rather selective for shorter molecules, it is concluded that they do not occur in this species, as they do in soybean and maize (11).

A certain heterogeneity in the given population of mt-DNA molecules would be necessary as explanation for the much larger complexity deduced from the sum of the restriction fragment lengths. These amounted to 180–190 kb, almost twice the contour length of an individual molecule.

This intermolecular heterogeneity seems to occur between molecules of approximately the same size. Experiments to separate DNA bands of lower mol wt from the isolated mt-DNA on low percentage agarose gels were unsuccessful; the undigested DNA migrated in one band of high mol wt.

Whether the large number of restriction fragments is due to actual differences in sequence or just degree of modification still remains unsolved.

Unusual for higher plants is the GC content of mt-DNA from *Oenothera*. The buoyant density in CsCl with 1.710 g/cm^3 and the melting point correspond to a slightly higher GC content than mt-DNA has in all other plants examined until now. Whether this can be explained through changes in an AT-rich region, as was shown for the mt-DNA of different species of the genus *Drosophila* (27), remains to be shown.

Table I. *Restriction Fragment Lengths of mt-DNA from O. berteriana*

Fragment lengths were obtained from three different DNA preparations (for the enzyme Sma I, four preparations). Very faint bands present in much less than stoichiometric amounts where omitted from the measurements.

Fragment No	EcoRI	Bam HI	Sma I
		kb	
1	14.6	19.5	20.2
2	10.9	15.3	16.3
3	10.6	14.0	14.6
4	9.4	11.3	13.5
5	8.8	10.3	11.0
6	8.3	8.5	10.3
7	7.9	7.8	9.4
8	7.2	7.05	8.5
9	6.1	6.7	8.1
10	5.5	6.05	7.7
11	5.3	5.6	7.1
12	5.0	5.4	6.65
13	4.8	5.1	6.3
14	4.6	4.75	6.0
15	4.35	4.3	5.35
16	4.15	4.0	4.6
17	4.0	3.9	4.15
18	3.9	3.7	4.0
19	3.7	3.5	3.3
20	3.55	3.55	3.1
21	3.45	3.2	2.8
22	3.3	2.95	2.6
23	3.15	2.7	2.52
24	3.0	2.55	2.25
25	2.85	2.42	2.15
26	2.75	2.22	1.85
27	2.6	2.14	1.77
28	2.5	1.9	1.54
29	2.35	1.75	1.25
30	2.3	1.55	1.15
31	2.25	1.45	1.05
32	2.1	1.35	0.94
33	1.8	1.1	0.8
34	1.7	1.05	
35	1.55	0.97	
36	1.45	0.85	
37	1.38	0.75	
38	1.32	0.7	
39	1.1	0.62	
40	1.02	0.57	
41	0.88	0.52	
42	0.72		
43	0.65		
44	0.58		
Total	180.7	183.61	192.82

Acknowledgments—I am greatly indebted to Prof. Dr. B. Schwemmle for the initiation of this work, to Prof. Dr. R. G. Herrmann (Universität Düsseldorf) for kindly communicating his experience with *Oenothera*, and to Mrs. U. Christner for excellent technical experience.

LITERATURE CITED

1. BOHNERT HJ 1977 Size and structure of mitochondrial DNA from *Physarum polycephalum*. Exp Cell Res 106: 426–430
2. BORST P, JH HOEIJMAKERS 1979 Kinetoplast DNA. Plasmid 2: 20–40
3. BRENNICKE A (1979) Restriction analysis of mt DNA from *Oenothera berteriana*. J Supramol Struct Suppl 3: 141
4. CUMMINGS DJ, L BELCOUR, C GRANDCHAMP 1979 Mitochondrial DNA from *Podospora anserina*. Mol Gen Genet 171: 229–238
5. CUMMINGS DJ, L BELCOUR, C GRANDCHAMP 1979 Mitochondrial DNA from *Podospora anserina*. Mol Gen Genet 171: 239–250
6. DIETRICH W 1977 The South American species of *Oenothera* sect. Oenothera (Raimannia, Renneria; Onagraceae). Ann Missouri Bot Gard 64: 425–626
7. HERRMANN RG, H-J BOHNERT, KV KOWALLIK, JM SCHMITT 1975 Size, confirmation and purity of chloroplast DNA of some higher plants. Biochim Biophys Acta 378: 305–317
8. KOLODNER R, KK TEWARI 1972 Physicochemical characterization of mitochondrial DNA from pea leaves. Proc Nat Acad Sci USA 69: 1830–1834
9. KROON AM, WM DE VOS, H BAKKER 1978 The heterogeneity of rat-liver mitochondrial DNA. Biochim Biophys Acta 519: 269–273
10. LEVINGS CS III, DR PRING 1976 Restriction analysis of mitochondrial DNA from normal and Texas cytoplasmic male-sterile *Maize*. Science 193: 158–160
11. LEVINGS CS III, DR PRING 1979 Organization and information content of mitochondrial DNA's of higher plants. J Supramol Struct Suppl 3: 134
12. LEVINGS CS III, DR PRING 1979b Mitochondrial DNA of higher plants and genetic engineering. *In* JK Setlow, A Hollaender, eds, Genetic Engineering, Vol 1. Plenum Publ Co, New York
13. MARMUR J, P DOTY 1962 Determination of the base composition of DNA from its thermal denaturation temperature. J Mol Biol 5: 109–118
14. NITSCH J, C NITSCH 1969 Haploid plants from pollen grains. Science 163: 85–87
15. PRING DR, CS III LEVINGS, WWL HU, DH TIMOTHY 1977 Unique DNA associated with mitochondria in the "S"-type cytoplasm of male-sterile *Maize*. Proc Nat Acad Sci USA 74: 2904–2908
16. PRING DR, CS III LEVINGS 1978 Heterogeneity of maize cytoplasmic genomes among male-sterile cytoplasms. Genetics 89: 121–136
17. QUETIER F, F VEDEL 1977 Heterogeneous population of mitochondrial DNA molecules in higher plants. Nature 268: 365–368
18. ROYER HD, CP HOLLENBERG 1977 *Saccharomyces cerevisiae* 2-μm DNA. Mol Gen Genet 150: 271–284
19. SCHILDKRAUT DL, J MARMUR, P DOTY 1962 Determination of the base composition of DNA from its buoyant density in CsCl. J Mol Biol 4: 430–443
20. SCHWEMMLE B 1962 Über den Einfluß von Kern, Plasma und Plastiden auf die photoperiodische Reaktion einiger *Raimannia Oenotheren*. Planta 58: 619–646
21. SYNENKI RM, CS III LEVINGS, DM SHAH 1978 Physicochemical charakterization of mitochondrial DNA from soybean. Plant Physiol 61: 460–464
22. STEINEMANN M 1978 Co-replication of satellite DNA of *Chironomus melanotus* with mainband DNA during polytenization. Chromosoma 66: 127–139
23. SUYAMA Y, WD BONNER 1965 DNA from plant mitochondria. Plant Physiol 41: 383–388
24. UPHOLT WB, IB DAWID 1977 Mapping of mitochondrial DNA of individual sheep and goats: rapid evolution in the D loop region. Cell 11: 571–583
25. WELLS R, J INGLE 1970 The constancy of the buoyant density of chloroplast and mitochondrial DNA's in a range of higher plants. Plant Physiol 46: 178–179
26. WHITFIELD PR, RG HERRMANN, W BOTTOMLEY 1978 Mapping of the ribosomal RNA genes on spinach chloroplast DNA. Nucl Acids Res 5: 1741–1751
27. WOLSTENHOLME DR, J GODDARD, C FAURON 1979 Structure and replication of mitochondrial DNA from the genus *Drosophila*. J Supramol Struct Suppl 3: 136