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The Use of Restriction Endonucleases to Measure Mitochondrial DNA Sequence Relatedness in Natural Populations

III. Techniques and Potential Applications

Robert A. Lansman, Rosemary O. Shade, John F. Shapira, and John C. Avise

Department of Molecular and Population Genetics, University of Georgia, Athens, Georgia 30602, USA

Summary. Restriction endonucleases and agarose gel electrophoresis have been used to demonstrate extensive nucleotide sequence diversity in mitochondrial DNA (mtDNA) within and between conspecific populations of rodents and other mammals. Cleavage of mtDNA samples with a relatively small number of endonucleases provides information concerning the phylogenetic relatedness of individual organisms which cannot now be readily obtained by any other type of molecular analysis. This information is qualitatively different from that available from the study of nuclear genes or gene products because the mitochondrial genome is inherited intact from the female parent and is not altered by recombination or meiotic segregation.

The requirements for large tissue samples and laborious DNA purification procedures have imposed severe limitations on the kinds of population surveys in which this technique could be utilized. Here, we show that these difficulties can be overcome by using DNA-DNA hybridization to detect minute amounts of mtDNA in crude tissue fractions which can be more easily and rapidly prepared from very small amounts of tissue without the use of expensive and immobile laboratory equipment. The techniques are described in detail in an effort to make restriction analysis of mtDNA available to biologists who may be unfamiliar with current DNA technology.

Key words: Mitochondrial DNA – Restriction endonucleases – Population surveys – Maternal inheritance – Gel electrophoresis – Phylogeny

Offprint requests to: J. C. Avise

Introduction

Several characteristics of any new molecular technique strongly influence whether it is likely to gain wide acceptance and use in the field of population and evolutionary genetics. For analysis of the genetic relationships of natural animal or plant populations, an ideal technique would (1) be methodologically simple enough to permit assay of large numbers of samples; (2) involve reasonably inexpensive chemicals and equipment; (3) be applicable to many kinds of organisms, including those for which formal genetic crosses are impractical; (4) yield data which are precise with respect to the level of resolution demanded by the questions asked; and (5) yield data which can be interpreted simply and with a minimum number of assumptions.

In addition, the general scientific "climate", including the availability of information about new techniques, can strongly affect the incorporation of new approaches into population biology. For example, for many years before 1965 protein electrophoretic techniques were widely used by molecular biologists and biochemists. Not until Lewontin and Hubby (1966) and Harris (1966) systematically applied these techniques to estimate genic variation in populations did the molecular revolution in evolutionary biology really begin (reviewed by Lewontin 1974 and Nei 1975). Protein electrophoretic techniques closely satisfy several of the ideal characteristics listed above. Yet as noted by Selander (1976), in years prior to 1965, many "evolutionists were becoming apologists, denying that their field was moribund," and evolutionary biology "seemed threatened by the continuing rise and increasing dominance of molecular biology." The situation changed not because of any sudden technological breakthrough, but rather be-

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cause of a gradually changing scientific climate in population biology which, when coupled with the operationalism provided by Lewonton, Hubby and Harris, eventually welcomed molecular approaches. Selander (1976) concludes that "future historians will see molecular biology more as the salvation for than, as it first seemed, the nemesis of evolutionary biology."

We believe that a new revolution is occurring in molecular biology which will also have ramifications profoundly influencing population biology. The excitement stems from the recent discovery and purification of a number of Type II restriction endonucleases (Zabeau and Roberts 1979), each of which cleaves duplex DNA at a specific recognition site usually containing four, five, or six nucleotides (Boyer 1971, 1974). Restriction enzymes are finding a variety of applications in molecular biology ranging from study of chromosome structure and function to recombinant DNA technology (Zabeau and Roberts 1979). Regrettably they have as yet found limited use in population biology, but the potential appears great.

The first two papers in this series (Avise et al 1979a, b) dealt with specific applications of restriction enzyme technology to natural population analysis. We chose mitochondrial DNA (mtDNA) for study because it is small in size and easy to isolate. At least the great majority of mtDNA molecules appear to be maternally inherited (Avise et al. 1979a,b; Dawid and Blackler 1972; Hayashi et al. 1978; Hutchinson et al. 1974; Upholt and Dawid 1977). Also, mtDNA appears to evolve very rapidly (Brown et al. 1979). Therefore, restriction enzyme analyses of mtDNA may prove particularly valuable for estimating matriarchal relationships among conspecific organisms and closely related species.

The purpose of this paper is two-fold. First, it provides the first description of new approaches to sample preparation and analysis which considerably increase the range of organisms to which mtDNA restriction analysis can be applied, while at the same time decreasing sample preparation time and laboratory equipment required. While most of the procedures described are part of the general craft of many molecular geneticists, they are described here in a manner which we hope will make them available to biologists who have little or no experience in handling DNA. Secondly, we wish to describe in systematic fashion the approaches we have taken to analyzing the data obtainable by restriction analysis of mtDNA, and to discuss a few of the potential applications we feel can make good use of mtDNA population surveys.

Materials and Methods

A. Conventional mtDNA Approaches

Tissue Handling and Homogenization. Mammalian cells typically contain 1000 mtDNA molecules (1.7×10^{-2}- pg) and about 10 pg of nuclear DNA. The crucial step in the isolation of mtDNA is, therefore, the efficient separation of intact nuclei from the cytoplasm. Tissues must be used in which cells can be broken without disrupting the nuclear envelope. Virtually all previous studies of rodent mtDNA have utilized liver because it contains very little fibrous connective tissue. We have found that heart and kidney yield more mtDNA per gram than liver and we use all 3 organs. If at all possible, fresh rather than frozen tissue should be employed. We have compared the mtDNA yield from fresh tissue with that obtained from equivalent samples which were frozen by immersion in liquid N_2 and stored at -70°C. Freezing decreases yields by at least 50%. Highly variable yields have been obtained from tissue frozen and stored less carefully. We are usually unable to isolate intact mtDNA from birds or rodents which were frozen intact at -70°C.

Small fresh tissue samples (< 10 g) can be simply minced with a scissors prior to homogenization. Larger samples are more conveniently disrupted in a Waring blendor or Sorvall Omnimix. Final homogenization is carried out in 2 or 3 ml of MSB-Ca^{++} (Table 1) per gram of tissue using 8–10 strokes with a tight-fitting, motor-driven glass teflon homogenizer. Disodium EDTA (0.2 M, adjusted to pH 7.5 with NaOH) is then added to the homogenate to a final concentration of 10 mM. The presence of Ca^{++} during homogenization reduces nuclear breakage (Bogenhagen and Clayton 1974); EDTA minimizes the aggregation of mitochondria, and also inhibits nuclease activity during differential centrifugation. All subsequent operations are carried out at 0–4°C.

Centrifugation. Nuclei and debris are removed from the homogenate by centrifugation at 700 x g for 5 min in a swinging bucket rotor. Chromosomal contamination is significantly reduced if the supernatant from this spin is carfully decanted and the centrifugation repeated. Mitochondria are then pelleted from the second low speed supernatant by centrifugation for 20 min at 20,000 x g. The pellet is washed by resuspending it in 10 to 20 ml of MSB-EDTA (Table 1) and recentrifugation at 20,000 x g for 20 min.

The crude mitochondrial pellet obtained by this procedure is usually sufficiently free of nuclear DNA so that mtDNA can be obtained by CsCl-ethidium bromide gradient centrifugation. If the level of nuclear contamination makes collection of mtDNA from the gradients impossible (see below) an alternative procedure can be used. The low speed supernatant can be layered over a step gradient made up of 5 ml of 1.5 M sucrose and 10 ml of 1.0 M sucrose (both solutions containing 5 mM EDTA and 10 mM Tris, pH 7.5) in a cellulose nitrate Beckman SW27 centrifuge tube. After centrifugation for 30 min at 90,000 x g, mitochondria band at the 1.0–1.5 M sucrose interface. We freeze the tubes in a dry ice-ethanol bath, slice out the mitochondrial band with a razor blade, dilute with 3 volumes of MSB-EDTA and collect the mitochondria by centrifugation at 20,000 x g as above.

mtDNA Purification. Mitochondria from 1–10 g of tissue are resuspended in 3 ml of STE (Table 1) and lysed by the addition of 0.15 ml of 25% sodium dodecyl sulfate (SDS). The suspension should clarify significantly within a few minutes at room temperature or 1–2 min at 37°C. Solid CsCl is then added using 1.1 g per ml of lysate. After the addition of 0.2 ml of a 10 mg/ml solution of ethidium bromide (in STE), the refractive index of the lysate should be adjusted to 1.391–1.392 by adding either CsCl or STE. The lysate is then centrifuged for 30–40 h at 20°C in the Beckman SW50.1 rotor at 36,000 rpm (160,000 x g) using either cellulose nitrate or polyallomar tubes. The gradients can then be visualized under UV light. Covalently closed circular mtDNA should be visible as a sharp band approximately 0.5 cm below a variable-sized upper band which contains nuclear DNA.

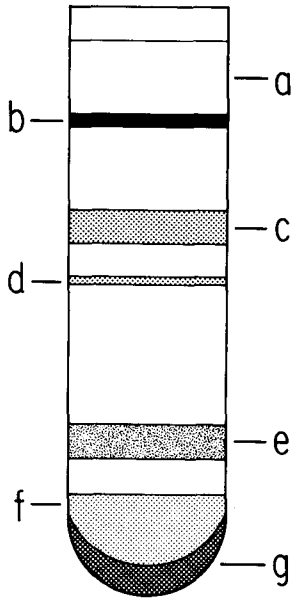

Fig. 1. Diagram of a CsCl-ethidium bromide gradient containing mitochondria lysed with SDS, visualized under UV illumination: **a** mineral oil overlayed on gradient to prevent tube collapse; **b** floating precipitate containing protein and SDS; **c** nuclear and relaxed forms of mtDNA; **d** covalently-closed-circular mtDNA; **e** glycogen (colorless, opalescent); **f** RNA; **g** RNA-containing pellet

The bottom third of the gradient contains RNA. A red "skin" of precipitated SDS and protein floats on top of the gradient as illustrated in Fig. 1.

The lower DNA band can be collected either by puncturing the bottom of the tube or by inserting a hypodermic through the side of the tube under UV illumination. Either technique yields a mtDNA fraction which is slightly contaminated with both RNA and nuclear DNA. Since neither contaminant interferes with restriction analysis, we do not further purify this fraction. Complete purification can be achieved if the DNA is rebanded in CsCl-ethidium bromide. We recommend that mtDNA to be used as a hybridization probe or for construction of recombinant plasmids be rebanded.

CsCl, ethidium bromide and excess EDTA are removed by dialysis against 1 M sodium acetate, 50 mM tris-HCl and 10 mM EDTA, pH 8.0 at room temperature until flourescence is no longer visible and then, against TE (Table 1) for at least 24 h and 4°C. The dialysed samples are stable for several months when stored at -20°C.

Recently we have adopted an alternative method for recovering samples from CsCl-ethidium bromide gradients which does not require dialysis (Davis et al. 1980). The sample volume is adjusted to 0.5 ml with STE. Samples are then extracted two or three times with butanol saturated with 5 M NaCl to remove ethidium bromide. The samples are then mixed along with 1.1 ml of H_2O and 3.2 ml of 95% ethanol in an ultracentrifuge tube (Beckman, SW50.1, polyallomar). After incubation at -70°C for 60 min, the DNA is pelleted by centrifugation at 30,000 x g for 30 min. The DNA is then resuspended in TE and used for restriction analysis.

Restriction Endonucleases and Digestion Procedures. At least 40 restriction endonucleases are now commercially available from several sources. We have used enzymes from 2 vendors, Bethesda Research Laboratories (BRL), Rockville, Maryland and New England BioLabs, Beverly, Massachusetts. The prices charged for most enzymes have dropped steadily over the past 3 years. It is now possible to carry out a reasonable restriction survey using enzymes such as *Eco*RI, *Bam*HI, *Hind*III, *Kpn*I, *Xba*I, *Xho*I and *Bst*EII which cost about 5 cents per digestion. Both of the above vendors supply complete instructions for the storage, dilution and digestion conditions used with each enzyme.

We now purify most of the enzymes we use by methods described by Greene et al. (1978). It is usually possible to obtain enough enzyme to last for many years in 4 or 5 days work. Investigators who have access to facilities appropriate for growing bacteria in 5 to 10 l cultures may want to consider purification of some enzymes. Bacterial strains are generally available from the investigators who have characterized each enzyme (see references in Roberts 1980) or from the American Type Culture Collection. Many of the strains we have used were kindly provided by Dr. R. J. Roberts.

We carry out digestions in 40 µl reaction mixtures containing the amount of DNA appropriate for the analysis to be performed and at least 1 unit of enzyme. When convenient we extend the time of digestion up to 3 or 4 h. The digestions are stopped by

Table 1. Composition of solutions used in mtDNA preparation and analysis

MSB	0.21	M	Mannitol
	0.07	M	Sucrose
	0.05	M	Tris-HCl, pH 7.5
MSB-Ca^{++}		MSB + 3 mM $CaCl_2$	
MSB-EDTA		MSB + 0.01 M EDTA, pH 7.5	
STE	0.1	M	NaCl
	0.05	M	Tris-HCl, pH 8.0
	0.01	M	EDTA, pH 8.0
2x TSM	0.06	M	Tris-HCl, pH 7.4
	0.3	M	NaCl
	3.0	mM	$MgCl_2$
TE	0.01	M	Tris-HCl, pH 8.0
	0.5	mM	EDTA, pH 8.0
TD	0.134	M	NaCl
	5	mM	KCl
	0.7	mM	Na_2HPO_4
	2.5	mM	Tris-HCl, pH 7.5
10x TEA	0.4	M	Tris base
	0.2	M	Na acetate
	0.02	M	Disodium EDTA
	0.18	M	NaCl
	Adjust to pH 8.05 with glacial acetic acid		
1x SSC	0.15	M	NaCl
	0.015	M	Na citrate
1x Denhardt's solution	0.02%	bovine serum albumin (Sigma, Fraction V)	
	0.02%	Ficoll (Sigma, Type 400)	
	0.02%	Polyvinyl pyrrolidone	
Prehybridization solution		5x SSC	
		5x Denhardt's solution	
	0.05	M	Na phosphate, pH 6.5
	1%	glycine[a]	
		250 µg/ml sonicated, denatured salmon sperm DNA	
Hybridization solution		5x SSC	
		1x Denhardt's solution	
	0.02	M	Na phosphate, pH 6.5
		50 µg/ml salmon sperm DNA	

[a]Required for diazotized paper filters only

the addition of 10 μl of a solution containing 50% glycerol, 0.02% bromphenol blue and 5% SDS. Stopped samples may be stored for weeks at 4°C.

Appropriate molecular weight standards must be included in each gel. We most frequently use a *Hin*dIII – digest of phage λ DNA which has seven bands ranging in size from 400 to 21,000 base pairs (Murray and Murray 1975). It is important to layer the standards in a buffer which is similar in ionic strength to the sample buffers, and to heat the standards to 65°C to dissociate the λ cohesive ends.

Agarose Slab Gel Electrophoresis. We use 13-well vertical slab gels which are 20 cm long, 14 cm wide and 3 mm thick. A complete double gel apparatus identical to the one we use can be purchased from Bio-Rad Laboratories, Richmond, CA (Model 221). A number of equivalent vertical and horizontal slab gel systems are commercially available. Since agarose does not adhere to the glass plates which form the gel sandwich, we pour a 10 ml polyacrylamide plug in the bottom of the sandwich which holds the gel in place. The stock solutions for the plug are the following: (1) acrylamide-bis: 28 g of acylamide, 2 g N,N'-methylene-bis-acrylamide, distilled H_2O to 100 ml.; (2) 10X TEA buffer (Helling et al. 1974 – see Table 1); (3) 10% ammonium persulfate in distilled H_2O. The plug mixture contains 3.3 ml acrylamide-bis, 1.0 ml 10X TEA (Table 1), 5.7 ml H_2O, 0.130 ml 10% ammonium persulfate, and 0.02 ml N,N,N',N'-tetramethylethylene-diamine (TEMED). The plug should be poured immediately after the addition of the TEMED and should polymerize in 2-3 min.

We have found that 1.1% agarose gels provide the best resolution and most accurate molecular weights for DNA fragments between 300 and 16,000 base pairs. 1.1 g of agarose (BRL) is suspended in 90 ml distilled H_2O plus 10 ml of 10X TEA. The agarose is dissolved by boiling, then cooled to 60–65°C before it is poured into the gel sandwich. A well-forming comb is inserted and the gel is allowed to set for at least 45 min. The comb is carefully removed after the top of the gel has been immersed in electrophoresis buffer, 1X TEA. The samples are then layered under the buffer with a micropipettor. To maximize resolution and reproducibility, electrophoresis should be carried out at relatively low voltage (1.8 to 2.0 volts per cm) for approximately 16 h or until the bromphenol blue tracking dye reaches the agarose-polyacrylamide interface.

The gel is stained with 2 μg/ml ethidium bromide in electrophoresis buffer for 20 min. The stained gel is then examined on a short-wave length UV transilluminator (Model C–61), Ultraviolet Products, San Gabriel, CA, and photographed using Polaroid Type 55 P/N film, exposed for about 1 min through a Kodak #9 gelatin filter. The negative obtained has good contrast and resolution and can later provide an enlarged positive print which can be easily interpreted and measured.

B. New Techniques for mtDNA Restriction Analysis

Most previous restriction analyses, including our own, have been performed with highly purified mtDNA as described above. Relatively large samples of mtDNA are required because restriction fragments containing less than 10–20 ng of DNA cannot be directly visualized on gels. Since a 500 base pair (bp) fragment represents less than 3% of the 16,000 bp mitochondrial genome, at least 0.5 μg of mtDNA must be used per digestion to ensure that bands of this size will be detected. Our yields of mtDNA from fresh tissue are usually less than 3 μg per gram of tissue wet weight. Thus the liver, heart and kidneys of an adult mouse (*Mus musculus* or *Peromyscus maniculatus*), which weigh about 2 g, provide enough DNA for at best 10 digests. In many situations, it is necessary to perform complete analyses with much smaller tissue samples.

One approach is to label restricted DNA with ^{32}P prior to electrophoresis, and then detect fragments by autoradiography. The terminal phosphate at the 5' ends of a restriction fragment can be replaced using polynucleotide kinase with ^{32}P derived from γ-^{32}P-ATP (Berkner and Folk 1980; Chaconas and van de Sande 1980). Alternatively, nucleotides at the 3' ends of restriction fragments can be replaced or filled-in with γ-^{32}P-deoxynucleotide triphosphates using DNA polymerase. The latter technique has been used in a series of studies of primate mtDNA (Drouin and Symons 1979; Brown 1980). We have used the 3' to 5' exonuclease activity of T4 DNA polymerase to remove 3' nucleotides from restriction fragments. If dNTP's are then added to the reaction, the 3' nucleotides are filled in. By using 1 μCi of α-^{32}P-dATP in the reaction, more than 10^5 cpm of label can be incorporated into 20 ng of DNA, depending on the number and base composition of the ends generated by the restriction digest. The details of this reaction are described by Challberg and Englund (1980).

Some problems may be encountered in these direct labeling procedures. It is difficult to separate labeled DNA from unincorporated ^{32}P-labeled substrates. At least two techniques have been described which serve this purpose (Davis et al. 1980; Neal and Florini 1973). In our hands, neither technique consistently yields clean DNA in good yield. A second limitation is that the results obtained are dependent on the purity of mtDNA in the samples. Contaminating nuclear DNA (and RNA in the case of the kinase reaction) are labeled to the same extent as mtDNA. This problem can be overcome when the mtDNA is first purified in CsCl-ethidium bromide gradients. We now use these "end-labeling" procedures only with purified mtDNA samples.

Because of these difficulties, in some of our studies of mtDNA from mammalian tissue we employ yet another approach to mtDNA restriction analysis. This procedure involves rapid isolation of a mtDNA-containing tissue fraction which we call CNA for "cytoplasmic nucleic acids" and the use of "Southern blots" for the detection of mtDNA restriction fragments. In the Southern (1975) procedure, the fragments are not visually detected. Instead they are denatured and transferred out of the gel and bound to nitrocellulose or diazotized paper filters. The filters are then incubated under DNA-hybridization conditions with radioactively-labeled mtDNA sequences. An autoradiograph of a filter reveals positions where the labeled mtDNA "probe" has hybridized to the fragments in the sample. The amount of DNA in a given fragment which can be detected by this technique depends on the specific radioactivity of the hybridization probe. Since specific activities in excess of 10^8 counts per min (cpm) per μg of DNA are relatively easy to obtain, and since 100 cpm are sufficient to produce a clearly visible band in an autoradiograph exposed for 24 h, a fragment containing 10 picograms of DNA is detectable. Thus the amount of DNA required per digest is reduced by a factor more than 100 below that necessary for direct visualization in the gel.

In the following sections we provide a detailed description of the techniques outlined above, beginning with the preparation of hybridization probes and the isolation of the CNA fraction. Southern transfer and hybridization procedures are also described. We conclude the discussion of techniques by presenting the results and analyses of experiments which illustrate the versatility of this approach to restriction analysis.

Choice of Hybridization Probes. We have constructed recombinant plasmids which contain the complete mitochondrial genomes of each of 2 rodent species: *Mus musculus* (pSL1178) and *Peromyscus maniculatus* (pSL400). These plasmids will be described in detail elsewhere. We believe there are two basic advantages in using plasmid DNA, as opposed to highly purified mtDNA, as hybridization probe. First, the plasmids can be readily purified in milligram quantities from moderate-sized *E.coli* cultures. Repetitive purification of mtDNA is not necessary. Se-

cond, the use of plasmids eliminates the possibility of artifacts resulting from hybridization of highly repeated nuclear DNA sequences. We are currently constructing plasmids containing mtDNA from other animals including insects and reptiles. We will provide *E.coli* cultures containing these plasmids and our DNA purification protocol to any investigators who wish to use them for population surveys.

Preparation of Hybridization Probes. *E.coli* DNA polymerase I will utilize double-stranded DNA containing a single strand break (nick) as a primer-template, degrading the nicked strand in a 5' to 3' direction and replacing it with nascent DNA. Rigby et al. (1977) and Maniatis et al. (1975) showed that nick-translation carried out in the presence of α-^{32}P-labeled deoxynucleotide triphosphates can produce DNA with specific activities in excess of 2 x 10^8 cpm per μg. When nick-translated DNA is used as a hybridization probe, picogram quantities of homologous-sequence DNA fragments can be easily detected. The technique is relatively simple, rapid and can be made highly reproducible. We recommend that investigators unfamiliar with the technique begin by using the "nick-translation kit" available from Bethesda Research Laboratories, following the instructions which are included. The only reagent not included is the α-^{32}P-nucleotide triphosphate which can be obtained from several vendors. We use α-labeled dATP from Amersham with a specific activity > 350 Ci/mMole.

We routinely label 2 μg of plasmid DNA using 70 μCi of α-^{32}P-dATP which has been dried under vacuum in a 1.5 ml Eppendorf microfuge tube. The reaction is carried out in 0.1 ml for 90 min exactly as described in the BRL protocol except for one modification. Higher specific activities are obtained if the last 10-fold dilution of the DNase 1 is omitted so that 10-fold more enzyme is used. The reaction is terminated by the addition of 0.1 ml of phenol which has been equilibrated with STE buffer.The mixture is vortexed, then centrifuged for 5 min in an Eppendorf microfuge. The upper aqueous phase is removed and retained; the phenol phase is reextracted with 0.1 ml of STE. The pooled aqueous phases are then passed through a 10 ml column of Sephadex G-50-fine (Pharmacia) in the same buffer. The column is poured in a disposable 10 ml pipet, plugged at the tip with siliconized glass wool and fitted with a disposable plastic valve (Bio-Rad). The labeled DNA comes through the column in the void volume well separated from a retarded peak containing unincorporated label. The separation can be followed easily with a Geiger counter. Fractions containing DNA are collected, pooled and stored at -20°C. The column separation reduces background during hybridization and allows immediate determination of the specific activity of the labeled DNA.

Rapid Isolation of Cytoplasmic Nucleic Acids. To purify the CNA fraction from 0.5 to 2 g of tissue, we mince and homogenize tissue as usual, but in 5 ml of 2X TSM buffer (Goldenberg and Raskas 1979-Table 1). The homogenate is then made 0.2% nonidet P40 (BRL) by the addition of 0.1 ml of a 10% solution, and incubated on ice for 5 min. The detergent causes dissolution of all cellular membranes except the nuclear envelope. The intact nuclei are removed by centrifugation in 15 ml conical tubes for 5 min at 1500 x g. The cytoplasmic supernatant is decanted into a 30 ml Corex tube, 5 ml of STE-saturated phenol is added and the tube is then incubated at 65°C for 5 min with periodic thorough mixing. The phases are separated by centrifugation for 10 min at 6000 x g in a swinging bucket rotor. The upper aqueous layer is removed with a pipette and reextracted with 5 ml of phenol. Nucleic acids are then precipitated from the aqueous phase by the addition of 10 ml (2 volumes) of 95% ethanol and incubation at -70°C for 30 min or -20°C for 2 h. The flocculant precipitate is collected by centrifugation at 6000 x g for 15 min. The tube is then rinsed once with 67% ethanol, without disturbing the pellet, to remove traces of phenol and salt. The ethanol is evaporated at room temperature and the pellet dissolved in 1 ml of TE buffer. Though samples prepared in this way contain large amounts of RNA, which prevent visualization of small mtDNA restriction fragments, the DNA is readily digestible with restriction endonucleases and the fragments are detectable by hybridization as described below.

Hybridization. In order to detect mtDNA restriction fragments by hybridization, the DNA must be transferred from agarose gels either to nitrocellulose filters (BA-83, Schleicher and Schuell, Keene, NH), as described by Southern (1975), or to diazotized paper, as described by Wahl et al (1979). In either case, the gel is first incubated in 250 ml of 0.25 M HCl for 15–30 min at room temperature to allow partial depurination of the DNA. The DNA is then denatured by incubating the gel for 15 min in 1.5 M NaCl, 0.5 M NaOH. For transfer to nitrocellulose, the gel is neutralized in 3.0 M NaCl, 0.5 M tris-HCl, pH 7.0 and placed on top of 3 or 4 sheets of Whatman 3 mm paper cut slightly larger than the gel and saturated with 3 M NaCl and 0.3 M sodium citrate (20X SSC). Plastic spacers, 3 mm thick, are then placed around the gel and a sheet of nitrocellulose, cut the size of the gel and wetted with 2X SSC, is laid on top with care taken to prevent air bubbles from being trapped between the filter and the gel. Strips of Whatman 3 mm paper 1 inch wide are soaked in 2X SSC and placed over the edges of the filter and the spacers. Then a stack of 2 wet (2X SSC) and 3 dry sheets of 3 mm paper, 2 or 3 inches of paper towels and a glass plate are placed on top of the filter. Over a period of about 3 h the 20X SSC is wicked up through the gel and filter, leaving the single stranded DNA adhering to the filter. To be sure that the transfer is complete, the gel can be restained with ethidium bromide and examined under UV light. If an efficient transfer has occurred, only trace amounts of DNA will remain in the gel. The nitrocellulose filter is rinsed in 2X SSC, placed on a sheet of filter paper and dried at room temperature for 2 h at 60°C for 30 min. The filter is then baked for 2 h at 80°C in a vacuum oven. The baked filters can be used immediately for hybridization or stored for as long as 6 months.

Diazotized paper can either be purchased (Transabind, Scheicher and Schuell: ABM cellulose paper, BRL) or synthesized according to Alwine et al. (1977) or a much simpler modification devised by Seed (unpublished results). The advantages of diazotized paper are that it binds low molecular weight DNA more efficiently than nitrocellulose, that hybridized DNA can be removed and the filter reused, and that it is less fragile and easier to handle than nitrocellulose. The disadvantages are the time required to make the paper (or the considerable expense of purchasing it) and the higher backgrounds which result from nonspecific adsorption of probe to the paper. The gel handling and blotting procedures used with diazotized paper are described in detail by Wahl et al. (1979). The crucial difference between this procedure and the one described above is that the transfer buffer is 1 M sodium acetate, pH 4.0.

Both nitrocellulose and diazotized paper filters can be hybridized by the procedure of Wahl et al. (1979). We have modified this procedure by omitting formamide in the prehybridization and hybridization solutions (see Table 1), and by carrying out both steps at 60°C rather than 42°C. The prehybridization is carried out by sealing the filter in a plastic bag (Sears, SEAL-AND-SAVE) along with 20 ml of prehybridization solution. After a 16 h incubation, the bag is opened and the solution is replaced by 9 ml of hybridization solution. An aliquot of the DNA to be used as probe, containing between 2 and 5 x 10^6 cpm, is denatured in a boiling water bath for 5 min, then rapidly cooled in ice-water. The probe is mixed with 1 ml of hybridization solution and added to the bag. Air bubbles are forced out of the bag by drawing it gently across a straightedge before resealing.

After hybridization for 16 h at 60°C, the filters must be thoroughly washed to remove nonspecifically adsorbed probe. We cut a corner of the bag and rinse the filter with about 50 ml of 2X SSC using a disposable syringe and hypodermic fitted with polyethylene tubing. The filter is then removed and incubated with at least 5 changes of 200 ml of 2X SSC, 0.1% SDS at 60°C over a period of at least 2 h. Finally the filter is rinsed with 2X SSC, thoroughly air dried, and laid directly on Dupont Cronex 2 DC or Kodak Royal Xomat X-ray film for autoradiography. In most cases, adequate exposures are obtained with nitrocellulose filters in 48 h at -70°C without intensifying screens. Since hybridization to diazotized filters appears to be more efficient, exposures of 12 to 24 h are usually adequate.

Throughout the transfer, preincubation, hybridization and wash procedures, filters should be handled only with foreceps or rubber gloves. When 2 or more filters are processed together, they should not be allowed to remain in contact with each other. We have preequilibrated and washed as many as 6 filters simultaneously by sandwiching them between sheets of polypropylene screen (Tetco, Elmsford, NY). High backgrounds, smearing and cross-contamination can result from careless handling of filters.

Results

Gel phenotypes generated by essentially the same procedures as the "conventional DNA approaches" described above have previously been published by several authors (Avise et al. 1979a; Brown and Goodman 1979; Shaw and Langley 1977). For this reason, we will concentrate here on a presentation of results from the newer hybridization analysis.

Figures 2 and 3 are examples of the kind of results obtained by hybridization analysis of mtDNA and illustrate some of the problems involved. Figure 2 is a photograph of a 1.1% agarose gel containing digestions of CNA preparations from 6 *Peromyscus maniculatus* samples with the restriction endonucleases *Hpa*II and *Hin*dIII. Most of the DNA bands visible in the gel are phage λ fragments; 0.5 μg of λ DNA was included in each digestion to provide an indication of the completeness of each digestion and to point out electrophoretic distortions which can occur. In this case substoichiometric bands near the top of lanes 1,2, and 3 indicate that the λ DNA, and probably the mtDNA, in these samples were not digested to completion. The λ *Hin*dIII also provides molecular weight standards. The only mtDNA bands visible in the gel are the 6000 bp *Hpa*II fragments in lanes 7 through 12. The intense fluorescence at the bottom of each lane is ethidium-stained RNA which is present in all CNA preparations.

The DNA in this gel was transferred to a nitrocellulose filter as described in Materials and Methods. When the gel was restained with ethidium bromide after the transfer, the only DNA fragments visible in the gel were the 21,000 bp *Hin*dIII bands in lanes 1 through 6. The depurination reaction, which is intended to break down

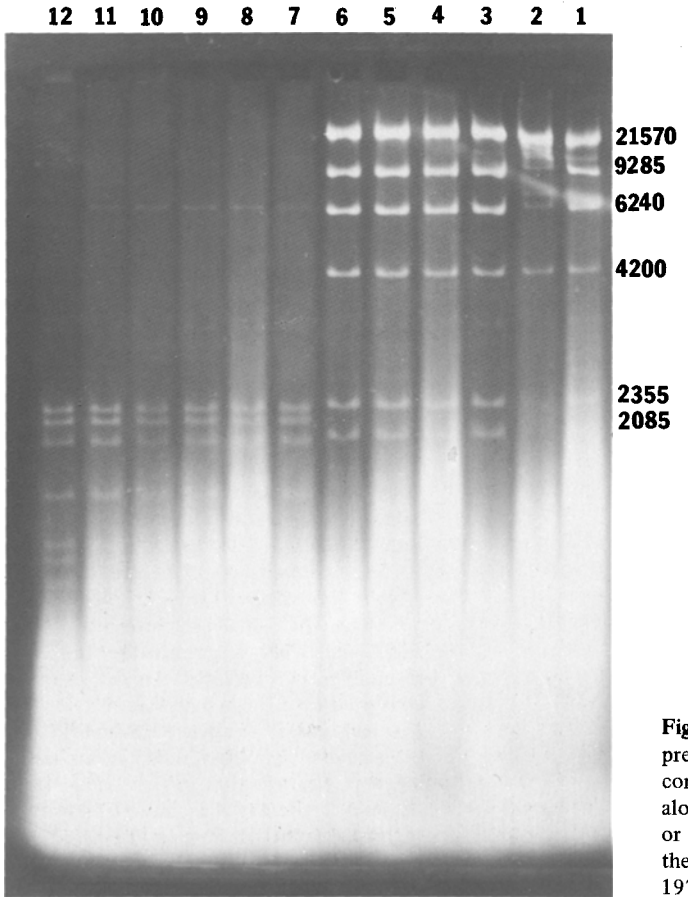

Fig. 2. An ethidium bromide stained 1.1% agarose gel of CNA prepared from 6 *Peromyscus maniculatus* samples. Each sample consists of 2% of the CNA fraction from a single liver digested along with 0.5 μg of phage λ DNA by either *Hpa*II (lanes 1–6) or *Hin*dIII (lanes 7–12). The molecular weights indicated are the 6 largest bands in the λ-*Hin*dIII digest (Murray and Murray 1975)

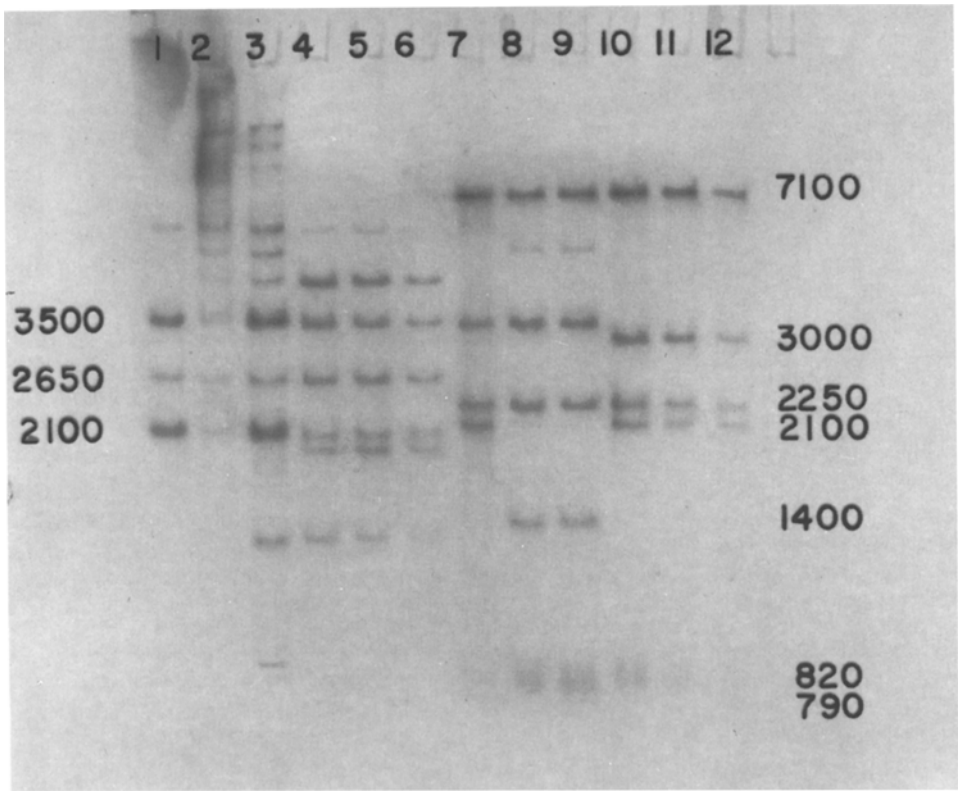

Fig. 3. Detection of *P. maniculatus* mtDNA fragments by hybridization after transfer to nitrocellulose. DNA from the gel shown in Fig. 2 was transferred as described in the text to nitrocellulose and hybridized with nick-translated pSL400 DNA. The filter was exposed for 48 h at room temperature to Cronex 2DC film. Lane number *1*, sample number 43, origin Texas, digested by *Hin*dIII; *2*, 44, Texas, *Hin*dIII; *3*, 45, Texas, *Hin*dIII; *4*, 46, Arizona, *Hin*dIII; *5*, 47, Arizona, *Hin*dIII; *6*, 48, Arizona, *Hin*dIII; *7*, 43, *Hpa*II; *8*, 44, *Hpa*II; *9*, 45, *Hpa*II; *10*, 46, *Hpa*II; *11*, 47, *Hpa*II; *12*, 48, *Hpa*II. The molecular weights, indicated in bp, are for the prominant *Hin*dIII bands in lane 1 and all of the visible *Hpa*II bands except the 3200 bp band in lanes 7, 8, and 9.

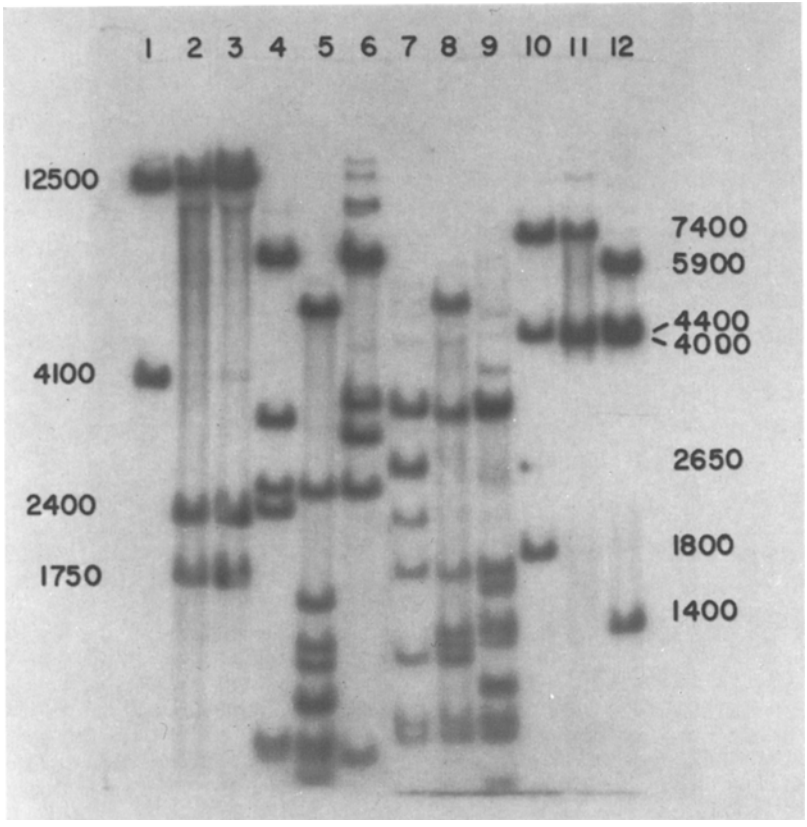

Fig. 4. Detection of *P. maniculatus* mt DNA fragments by hybridization after transfer to diazotized paper as described in the text. Lane number *1*, sample number 5829, origin Oregon, digested by *Bgl*II; *2*, 229, Vermont, *Bgl*II; *3*, 67, California, *Bgl*II; *4*, 5829, *Hpa*II; *5*, 229, *Hpa*II; *6*, 67, *Hpa*II; *7*, 5829, *Hin*cII; *8*, 229, *Hin*cII; *9*, 67, *Hin*cII; *10*, 5829, *Xba*I; *11*, 229, *Xba*I; *12*, 67, *Xba*I. Molecular weights of all *Bgl*II and *Xba*I fragments are indicated in bp. The two *Bgl*II patterns can be interconverted by a single base substitution. The *Xba* patterns in samples 5829 and 67 can be generated by single substitutions creating new cleavage sites in the 229 pattern. At least 2 substitutions must be involved to explain the differences observed between each of the *Hin*cII and *Hpa*II patterns

high molecular weight fragments into smaller, more easily transferred molecules, was apparently not extensive enough to allow complete transfer of this band. The filter was then hybridized as described using 3 x 10^6 cpm of pSL400 DNA which had been labeled by nick translation, using 70 μCi of α-^{32}P-dATP, to a specific activity of 5 x 10^7 cpm per μg. An autoradiograph of the filter, exposed for 48 h is shown in Fig. 3.

The *Hpa*II digestions in lanes 7 through 12 are nearly complete. The sum of the molecular weights of the intense bands in each lane equals 16,000 ± 500 bp. Three different digestion phenotypes are apparent. The 6-band pattern seen in lane 7 can be converted to the 7-band pattern in lanes 8 and 9 by a single nucleotide substitution in the 2100 bp fragment visible in lane 7 which crates a new cleavage site and the 1410 and 720 bp fragments seen in lanes 8 and 9. A second nucleotide substitution can explain the differences between lane 7 and the pattern observed in lanes 10, 11, and 12. In this case, a substitution near the end of the 3200 bp fragment in lane 7 creates a new cleavage site giving rise to a 3000 bp band and a 200 bp fragment which would not be detected because fragments of this size do not bind to nitrocellulose (Southern 1975). Clearly, other explanations of the differences between the three phenotypes are possible, but any other model would predict a larger number of sequence differences between the samples. Throughout our analysis we choose the most conservative possible interpretation so that our estimates of divergence between samples are always minimal.

Interpretation of the *Hin*dIII digestions on this autoradiograph is more difficult. The digestion obtained with samples in lanes 1, 2, and 3 is obviously incomplete since no set of intense bands is visible which total 16,000 bp in size. This result is expected from the appearance of the λ DNA fragments in this digest. Complete digestion was obtained in lanes 4, 5 and 6. The 6 major bands in these patterns have a total molecular weight of 15,300 bp. However, we know from previous work with purified mtDNA samples of *P. maniculatus* that this *Hin*dIII digestion phenotype contains a 340 bp band which does not appear in the autoradiograph. This failure to obtain reproducible hybridization of fragments less than 500 bp using nitrocellulose is one of the compelling reasons for using diazotized paper transfer techniques.

Figure 4 is an example of the kind of results which can be obtained by transfer to diazotized paper. Each well contains a digest of 1/50th of the CNA prepared from a single *P. maniculatus* liver (< 40 ng of mtDNA). After digestion and electrophoresis, the fragments were transferred and hybridized using approximately 2 x 10^6 cpm of pSL400 DNA using our modification of the procedure of Wahl et al. (1979). The filter was exposed to X-ray film for 12 h at room temperature without intensifying screens. The results illustrate two major advantages of this transfer and hybridization system. First, low molecular weight DNA fragments are efficiently bound to diazotized paper. The fragments at the bottom of the *Hinc*II digest in lane 9 and the *Hpa*II digest in lane 5 have molecular weights of approximately 350 bp. When they are present, fragments smaller than 150 bp can be detected. Second, the overall efficiency of the transfer and hybridization appear to be increased enough so that exposure times can be reduced by at least a factor of 4 compared to those required when equivalent samples are transferred and hybridized by the original procedure of Southern (1975).

The only problem we have encountered with diazotized paper transfers is illustrated by the digest in lane 10. This sample contains a 2650 bp fragment, only part of which is bound to the filter. We do not know whether this is caused by a failure to obtain uniform diazotization of the paper, or by a localized failure of the transfer. Because of the possibility that fragments in a given digest may not be hybridized efficiently, digests in which the observed fragments do not have the expected total molecular weight should be repeated.

Data Analyses and Interpretations

Before discussing some possible applications of mtDNA surveys, it will be useful to summarize by means of example certain characteristics of the data obtained by these restriction techniques. One of the largest data sets currently available describes variation in mitochondrial DNA's among 87 pocket gophers (*Geomys pinetis*) collected throughout the range of the species in the southeastern United States (Avise et al. 1979b). Each restriction enzyme produced for each animal, an mtDNA gel profile consisting of between three and nine digestion fragments (see examples in Fig. 5). These digestion fragments constitute the raw data base.

An obvious index of the relative genetic similarity between various pairs of organisms is the proportion of fragments shared in their mtDNA digestion profiles. Clearly, more information can be obtained by including larger numbers of restriction enzymes in a survey, and by counting the total fraction of shared fragments, $\hat{F}$:

$$\hat{F} = 2N_{XY} / (N_X + N_Y) \quad , \tag{1}$$

where N_X and N_Y are the number of fragments in organisms X and Y, respectively, and N_{XY} is the number of fragments shared by the two organisms. Upholt (1977) was the first to show that if certain assumptions are met F is related to p, the number of base substitutions per nucleotide (or the percentage of nucleotides) separating a given pair of organisms. Upholt's formula is

$$p = 1 - \left[\frac{-F + (F^2 + 8F)^{1/2}}{2}\right]^{1/n} \quad , \tag{2}$$

where n is the number of base pairs recognized per cleavage site. Values of p must be calculated separately for sets of restriction enzymes differing in n. Other workers have since elaborated and refined Upholt's basic approach. For example, Nei and Li (1979) present formulas which were derived in a somewhat different fashion, but they yield virtually identical values of p when applied to most data sets.

The derivations of these formulas depend upon several assumptions: (1) all fragment changes arise solely by base substitution; (2) the frequencies and distributions of cleavage sequences in the DNA are similar to those expected in random sequences of same-base composition; (3) all fragments can be observed, and non-homologous fragments of similar molecular weight are not scored as identical. Most available information suggests that the first two assumptions are usually valid, at least to a first approximation (Upholt 1977; Upholt and Dawid 1977; Nei and Li 1979).

The probability that the third assumption will be valid for analysis of a particular data set can be increased by choosing for study restriction enzymes which individually produce a relatively small and hence readily scorable number of fragments. The expected number of fragments in a digest of mtDNA with m_t nucleotide pairs is given by $m_t a$, where

$$a = (g/2)^{n_1} \; [(1-g)/2]^{n_2} \qquad (3)$$

(Nei and Li 1979), g is the G-C content of the mtDNA, n_1 and n_2 are the number of guanines (G) plus cytosines (C) and the number of adenines (A) plus thymines (T) in the restriction site, respectively, and $n_1 + n_2 = n$. For example, if $g = 0.5$ and $m_t = 16{,}000$, the number of digestion fragments produced by *Eco*RI (GAATTC) is expected to be 3.9, and the number of fragments produced by *Alu*I (AGCT) should be about 62.5. *Alu*I (and other enzymes with $n = 4$ ("four-base enzymes")) would produce many fragments of mtDNA smaller than 100–500 bp, not always detectable by the electrophoretic techniques described. Furthermore, with more total bands per digest, the probability of scoring non-homologous fragments as identical increases severely. For these reasons, we recommend that for routine population surveys of animal mtDNA several different enzymes with $n = 5$ or 6 should be used (see also Nei and Li 1979).

In the case of *Geomys*, a total of six "five-base" and "six-base" restriction enzymes were employed. Using the statistical procedures outlined above, we estimated the number of mtDNA base substitution per nucleotide (p) differentiating individual animals. Values of p ranged from 0.000 to a high of 0.047. Genetic distances displayed a clear geographic pattern. Gophers collected within local geographic areas invariably exhibited p's near zero, and the largest genetic distances were generally observed between gophers collected from more distant portions of the species' range (Avise et al. 1979b).

Most of the few other available estimates of mtDNA divergence also involve various mammal populations. Results are summarized in Table 2. Typically, conspecific organisms exhibit less than about 3% sequence divergence, whereas organisms belonging to closely related species have shown mean values of between 0.08 and 0.25 base substitutions per nucleotide. Presumably, the general increase in p with probable level of evolutionary divergence reflects relative times since organisms last shared a common female parent. Since mitochondria are maternally inherited, the process of speciation (the development of reproductive isolation) should be of no direct significance to mtDNA differentiation.

The quantitative values which constitute the body of such distance matrices can be further analyzed by any of a number of agglomerative procedures for estimating dendrograms and phylogenetic trees (Fitch and Margoliash 1967; Farris 1972; Prager and Wilson 1978; Sneath and Sokal 1973). A discussion of these procedures is beyond the scope of this report.

An alternative approach to data analysis can provide much additional information. The entire mtDNA digestion profile produced by each restriction enzyme for each animal may be considered a qualitative phenotype (Avise et al. 1979a). Because these phenotypes are composed of several mtDNA fragments, they are unlikely to arise independently in the evolutionary process by convergence from unrelated phenotypes. If several enzymes are employed in a survey, each animal is characterized by a "composite phenotype" whose elements are the multiband phenotypes produced by each enzyme. Among 87 *Geomys pinetis* assayed, we observed 23 different composite phenotpyes. Gophers sharing a given composite phenotype belong to an mtDNA "clone" as assayed by our techniques. The geographic distributions of 19 of these clones are circled in Fig. 6.

The composite phenotypes characterizing these clones can readily be interconnected into a most-parsimonious phylogenetic network which then represents an estimate of matriarchal phylogeny. The network is initially unrooted. In Fig. 6 the unoriented network for *Geomys* has been superimposed over the geographic sources of collections.

Certain single-enzyme phenotypes (such as *Bgl*II-N and *Bgl*II-P in Fig. 5) appear to be related by single base substitutions that result in loss or creation of a cleavage site. The evolutionary loss of a cleavage site results in the disappearance of two fragments and the appearance of a new fragment whose molecular weight equals the sum of the lost fragments. For most other digestion phenotypes, it is more difficult to be sure how many substitutions are involved, but it must be at least two. In Fig. 6, solid lines crossing branches of the *Geomys* network indicate the number of digestion phenotype changes occurring along the pathway, and the number of solid

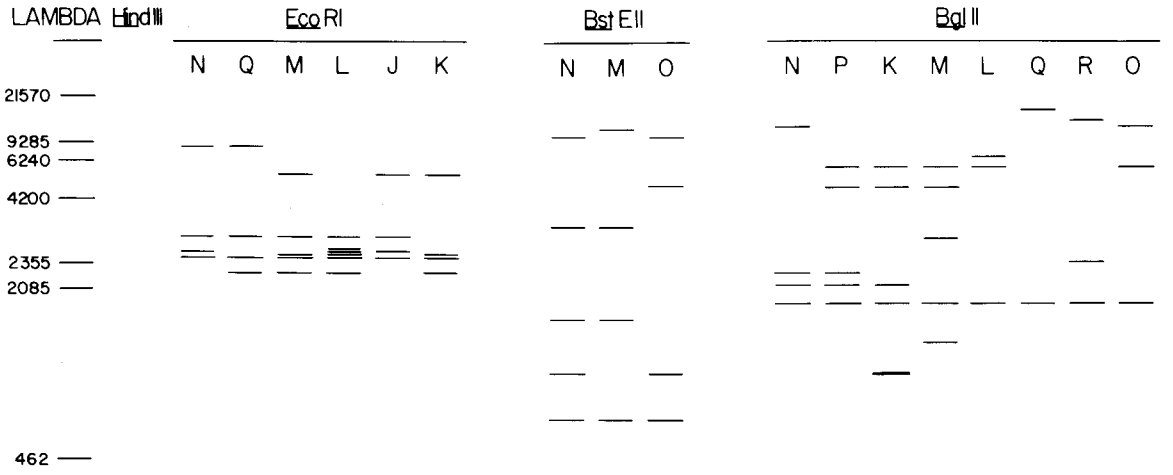

Fig. 5. Diagrammatic representation of all the *Eco*RI, *Bst*EII, and *Hin*dIII fragments observed in 87 samples of the pocket gopher *Geomys pinetis* collected throughout the range of the species. The samples are those described in Avise et al. (1979b). The positions and molecular sizes of the λ-*Hin*dIII fragments are indicated to the left

Table 2. Estimated genetic distances in base substitutions per nucleotide (p) between mammal mtDNA's digested with several restriction enzymes

Comparison	Organism	Animals surveyed	Restriction enzymes	p	Source
Conspecifics	*Geomys pinetis*	87	6	0.02	Avise et al. 1979b
	Peromyscus polionotus	36	6	0.01	Avise et al. 1979a
	Peromyscus maniculatus	10	6	0.03	Avise et al. 1979a
	Ovis aries	2	3	0.01[a]	Upholt and Dawid 1977
	Capra hircus	3	3	0.01[a]	Upholt and Dawid 1977
	Homo sapiens	21	11	< 0.01[a]	Brown et al. 1979 Brown and Goodman 1979 Brown 1980
Sibling species	*P. polionotus – P. maniculatus*	46	6	0.15	Avise et al. 1979a
Non-sibling species	*Peromyscus*	50	6	≈ 0.21	Avise et al. 1979a
	Ovis – Capra	5	3	≈ 0.08[a]	Upholt and Dawid 1977
	Homo – Papio-Macaca – Cercopithecus	5	11	≈ 0.25[a]	Brown et al. 1979

[a]Some or all of these values were estimated from maps of cleavage sites. All other values in the table were derived from fragment comparisons

plus dashed lines represents the *minimum* number of base substitutions required to account for these changes.

A more precise determination of the minimum number of base substitutions which separate two restriction phenotypes can be obtained if cleavage sites are mapped relative to one another in the mitochondrial genome. When the positions of cleavage sites and the sizes of adjacent fragments are known, it is possible to say exactly how many sites must be altered in order to interconvert two restriction patterns. By analyzing "site" conservation, rather than "fragment" conservation, it is also possible to quantitate sequence variation among more widely divergent organisms (Upholt 1977; Kaplan and Langley 1979; Nei and Li 1979; Gotoh et al. 1979; Brown et al. 1979). We are currently in the process of comparing the information obtained from fragment and site analysis of sequence variation within the species *Peromyscus maniculatus*. It is our preliminary impression that the increased precision available from site analysis does not greatly increase the value of the restriction data in analyzing population structure. On the other hand, it is clear that mapping and site analysis do provide information about the molecular nature of mtDNA evolution.

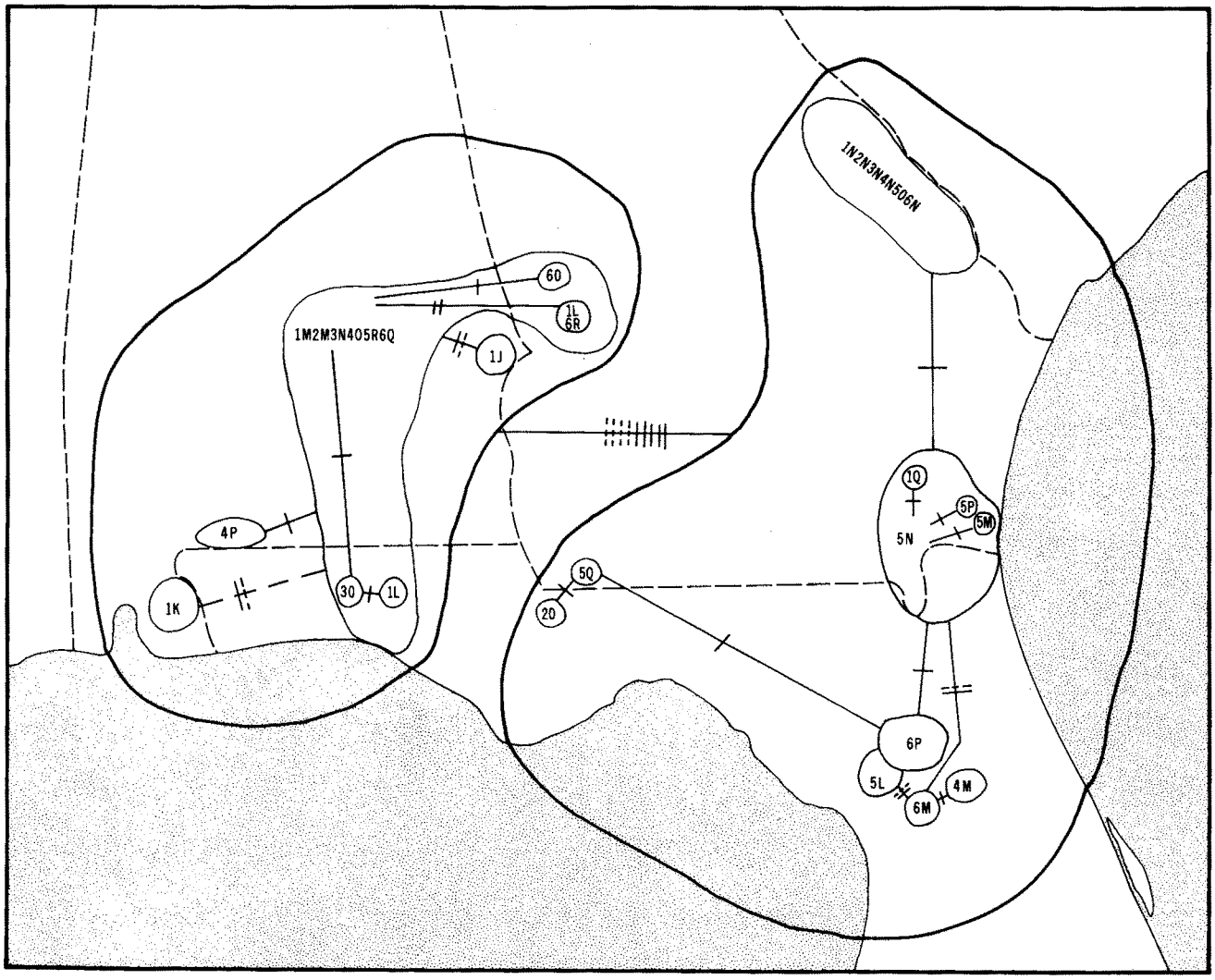

Fig. 6. Geographic distribution of 19 composite mtDNA digestion phenotypes observed in *G. pinetis*. The numbers refer to the 6 enzymes used in the survey: 1, *Eco*RI; 2, *Bam*HI; 3, *Bst*EII; 4, *Hin*dIII; 5, *Hin*cII; 6, *Bgl*II. The letters refer to the digestion patterns illustrated in Fig. 5 and in Fig. 1 of Avise et al. (1979b). The complete pattern designations are indicated for two phenotypes, examples of the "eastern" and "western" forms of the species. Only the individual pattern designations which differ from those in the two examples are indicated for the other samples

Potential Applications

Restriction assays of mtDNA share with most other molecular approaches, such as immunological or electrophoretic protein surveys, possibilities for assaying genetic diversity within and among natural populations. However, mtDNA surveys also offer unique opportunities not shared by other existing methodologies. These unique advantages stem directly from the mode of mtDNA inheritance, which is by asexual transmission through females.

Matriarchal Population Structure

Mitochondrial DNA heredity is the female analogue of male "surname heredity" in most human societies. Both male and female progeny inherit their mother's mtDNA, but only daughters subsequently transmit mtDNA to future generations. Thus any movements of males among populations will have only transitory (single generation) effects on the geographic distribution of mtDNA genotypes within a bisexual species. As a consequence, mtDNA variation can be used to examine the evolutionary history of female movement and survival in a species.

The natural history literature is replete with examples in which individual movement and dispersal from place of birth are highly asymmetric by sex, and it is usually the females which appear relatively sedentary. For example, in an intensive telemetry and observational study of white-tailed deer (*Odocoileus virginianus*), 80% of yearling bucks emigrated from birth site, while 87% of yearling does were sedentary (Hawkins and Klimstra 1970). In several studies of field mice (*Peromyscus maniculatus and P. leucopus*), young males dispersed con-

siderably farther than females (Stickel 1968). In a particularly detailed, long-term observational study of ground squirrels (*Spermophilus beldingi*), Sherman and Morton (1979) conclude "Perhaps the most interesting aspect of Beldings' ground squirrel demography is their pattern of dispersal, which is also asymmetric by sex. Most females are sedentary from birth, spending their lives among near and distant female relatives. Today, granddaughters and great-granddaughters of females marked when our studies began still occupy ancestral homesites. In contrast, juvenile males permanently disperse soon after weaning and establish burrows ten to twenty times farther from their natal burrows than their sisters ... Subsequent to mating, adult males also disperse ..."

Many mobile, social species also exhibit a pattern of asymmetric movement by sex which may have evolutionary consequences similar to those in more sedentary species. The females in lion prides comprise a closed "sisterhood" of closely related adults (Schaller 1972; Wilson 1975). Young females usually remain in the pride, but young males almost invariably leave the pride in which they were born, later to replace males of other groups. Packer (1979) summarizes information on intergroup movements in a total of 31 social mammalian species. In 81% of the cases, it is primarily males which transfer among groups.

In all of these instances, most nuclear gene flow among populations probably occurs through males. Populations could be highly structured with respect to female – transmitted traits (such as mtDNA), yet much less differentiated with respect to frequencies of nuclear alleles and their products. Thus mtDNA population surveys may prove particularly valuable for augmenting nuclear gene studies in species for which unequal movement by sex is suspected.

The maternal inheritance of mtDNA should prove useful in other evolutionary situations as well. For example, many parthenogenetic vertebrates and invertebrates were derived from past hybridizations of bisexual species which are still extant. It is of interest to know the female parental specie(s) involved in the original hybridization(s). Brown and Wright (1979) have employed restricition assays of mtDNA to determine that the bisexual lizard *Cnemidophorus tigris* was the maternal parent species for each of two parthenogenetic derivatives, *C. neomexicanus* and *C. tesselatus.* The extensively studied Australian grasshopper *Warramaba virgo* is another parthenogenetic species, derived by hybridization of two extant bisexual species. As recently as 1978, White wrote "we are never likely to know which species was the female parent". These particular species have not yet been examined for mtDNA composition. Nonetheless, it is interesting how rapidly the development of new approaches can offer real hope for solution of problems which only a few years earlier seemed totally intractable.

Individual Organisms as OTU's

Mitochondrial DNA genotypes are transmitted intact (barring mutation) from female to progeny, unaltered by the processes of segregation and recombination characteristic of nuclear genotypes. Because mtDNA genotypes are perfectly heritable, each individual organism carries within its mitochondrial genome relatively complete and unambiguous information about the female lineage to which it belongs. Thus in a population or systematic survey, individuals can be considered the basic unit of analysis (operational taxonomic unit or OTU). This contrasts with nuclear gene analyses, which are typically based on *population* allele frequencies.

These considerations may be of significance for studies in which the maternal phylogeny of individual organisms is of interest. We will illustrate by discussing a current problem in population management which has recently come to our attention. This problem involves determining movement patterns and matriarchal relationships in marine turtles (such as *Caretta caretta* and *Chelonia mydas*).

Marine turtles nest along beaches, and nesting-site faithfulness (females nesting at beach of hatching) appears very strong in at least some species (Carr and Carr 1972). Turtles spend most of their lives at sea and may move considerable distances. Turtes also mate at sea. Although virtually nothing is known about the mating system, it is likely that many matings take place among turtles hatched from different beaches. Therefore it is conceivable that populations over major portions of a species range will prove to be relatively homogeneous in frequencies of alleles at nuclear loci, yet higly structured and differentiated in mtDNA composition. If nesting assemblages can be distinquished by mtDNA analyses, it should in principle be possible to reconstruct migration routes by capturing individual turtles at sea and assigning them to beach of origin by mtDNA genotype.

Conclusions

It may be useful to briefly conclude our discussion by weighing these mtDNA techniques against the criteria advanced in the introduction as ideal characteristics for any new molecular approach. Restriction assays of mtDNA are sufficiently simple to permit assay of at least tens of samples in reasonably short time. However, many chemicals and pieces of equipment are required, and some of these can be quite expensive. The techniques are applicable to any animals from which sufficient quantities of mtDNA can be obtained. The major appeal of the techniques concerns the nature of the data obtained. The digestion profiles are complex and qualitative (minimizing problems of evolutionary convergence), yet almost perfectly heritable through females. A great

deal of assayable sequence heterogeneity exists within species assayed thus far.

It appears that many species have population and demographic characteristics ideally suited for analyses by any genetic characters such as mtDNA inherited strictly through females. If this review encourages a broader or more rapid application of mtDNA approaches to problems in population biology, it will have served its purpose.

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Note Added in Proof

Several publications have appeared since the original submission of this manuscript which will be of interest to readers. A variety of techniques used in DNA analysis are described in two new volumes, 65 and 68, of the series *Methods in Enzymology* (Academic Press, Grossman and Moldave, eds). These volumes are a convenient source of reference material relevant to many of the techniques we describe. Giles et al. (1980) have used hybridization techniques and rapid isolation of mtDNA from platelets to perform restriction analysis using human blood. The techniques used are similar to those we describe. Finally, restriction polymorphisms in nuclear DNA are being analyzed by hybridization techniques (Wyman and White 1980) These experiments should lead to results of great interest to evolutionary biology.