

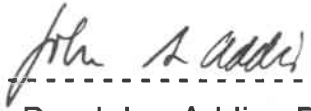
**Isolation and Preliminary Characterization of
Mitochondrial DNA From the Thatching Ant,
*Formica obscuripes***

Submitted in Partial Fulfillment of the Requirements for
Graduation with Honors to the
Department of Biology and Chemistry at
Carroll College, Helena, Montana

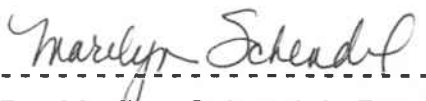
Deborah D. Dover
April 7, 1995




This thesis for honors recognition has been approved for the
Department of Biology and Chemistry by:



Dr. John Addis, Director



Dr. Marilyn Schendel, Reader



Dr. Art Westwell, Reader



Dr. Barry Ferst, Reader

April 7, 1995

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Abstract

A simple and relatively quick procedure was developed for the extraction of mitochondrial DNA from the thatching ant *Formica obscuripes*. DNA obtained using this procedure was analyzed both after digestion with restriction enzymes (Eco R1 and Bam H1) and without digestion by electrophoresis on agarose gels. Although the DNA isolated appeared to be larger than the usual upper size limit (approximately 20 kb) for mitochondrial DNA, the undigested and digested molecules behaved as would be expected of a circular molecule with one restriction enzyme recognition site (i.e., the undigested form migrated further into the gel than the digested form).

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Introduction and Literature Review

Interest in mitochondrial DNA (mtDNA) as a means of studying population genetics and population structure in animals from invertebrates to humans has grown greatly in the past fifteen years. Since mtDNA sequences mutate at a predictable rate (1), variation in mtDNA sequences between two individuals or populations can be translated into the time the two mtDNA types evolved from one another. Much work has been done to study the mtDNA of vertebrates, but relatively little to study that of invertebrates. This thesis describes the isolation of mtDNA from the thatching ant *Formica obscuripes* (Figure 1) and preliminary efforts to characterize it. What follows is a brief description of mitochondria and the biology of the thatching ant.

Mitochondria are the "powerhouses" of eukaryotic cells, producing energy for the cell by chemiosmosis. Mitochondria divide autonomously in the cell and contain double stranded, circular chromosomes which are approximately the size of bacterial chromosomes (15-20 kilobases) (1). In part because of these characteristics, it has been popularly speculated that mitochondria were once free-living bacterial cells (probably members of the purple bacteria) which entered into a symbiotic relationship with archaeobacteria about 1.5 billion years ago (1). The early mitochondria living inside the host archaeobacteria benefited by being protected from the elements

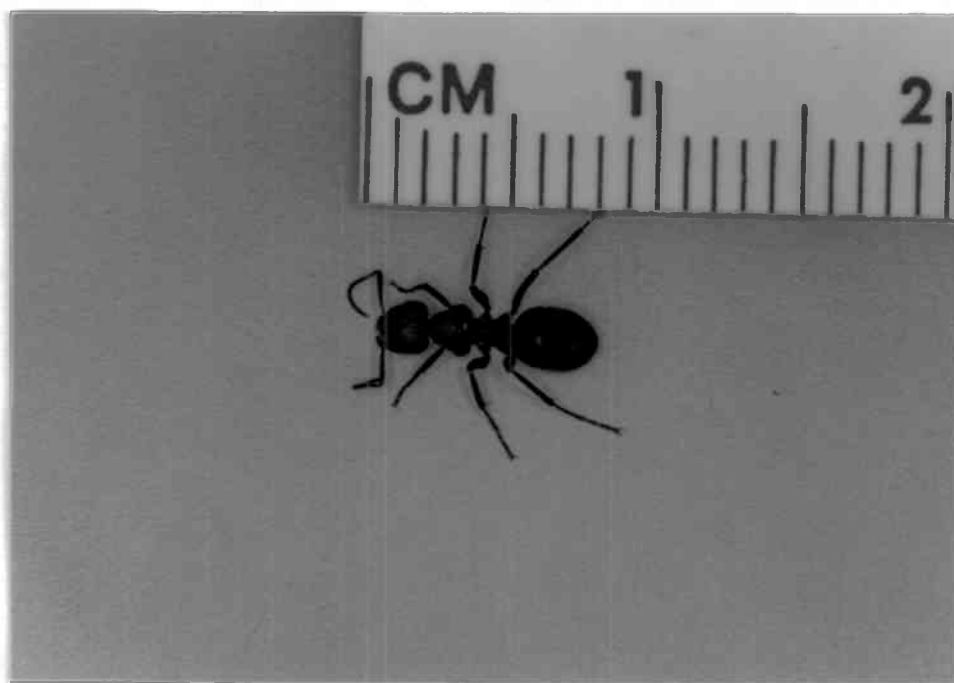


Figure 1. The thatching ant *Formica obscuripes*.

by the host, and the host benefited by use of the mitochondria's electron transport chain for more efficient ATP production (1).

Many of the components of the electron transfer chain are encoded for by mtDNA genes (1). The mammalian mtDNA genome is free of introns and contains the information for 13 proteins, 22 transfer RNAs, and two ribosomal RNAs (1). Additionally, the mtDNA has a control region which regulates mtDNA replication and transcription (1). The genetic code in mtDNA is somewhat different than it is in nuclear DNA; that is, 4 of the 64 possible codon sequences in mtDNA are translated into different amino acids than they would be in other genomes (1).

For population or phylogenetic studies, mtDNA is a valuable tool for two major reasons. First, mtDNA, are almost entirely maternally inherited through the cytoplasm of the mother's egg (2,11). Maternal inheritance is easier to trace phylogenetically than Mendelian inheritance and so lends itself well to evolution studies. Some evidence suggests that mtDNA can also be passed paternally (8,10,16) and that it can integrate with nuclear DNA (1). The effect of these factors on population evolution is being investigated (1,8,16). The other factor which makes mtDNA attractive to evolutionary biologists is that the mtDNA sequence changes at a specific rate which is higher than that of nuclear DNA. In spite of the greater number of mutations in mtDNA (1), the function of the genes is highly conserved because of a greater frequency of

mutation in the third (wobble) position of the codon. Anticodon pairing is less strict in mtDNA than in nuclear DNA; in that tRNA molecules will recognize any nucleotide in the wobble position (1). For this reason, mutations can occur in the wobble position without deleterious effects to the functioning of the mitochondrion (1). This accelerated rate of mutation is helpful in evolution studies because many differences will be apparent even among closely related populations (16,21).

Recent research in a number of organisms has uncovered some characteristics which must be taken into consideration when studying mtDNA. Although generally found to range in size from 15 to 20 kilobase pairs in size, larger genomes have been observed as well. For example, in the lizard *Cnemidophorus exsanguis*, the mtDNA genome measures 22.2 kilobases (13) and in the bark weevil, *Pissodes*, it measures from 30 to 36 kilobases (4). The larger size of these genomes seems to be due to repeated adenine- and thymine- rich sequences near the mtDNA control region. These findings are of interest because large DNA molecules would seem to be at a selective disadvantage since they would take longer to replicate than small ones (4).

Another finding of concern is heteroplasmy, the presence of more than one size or type of mtDNA in the same individual. This condition is due to variable lengths of repeating adenine and thymine regions in mtDNA molecules (4) or to biparental

inheritance of mtDNA (8). The possibility of heteroplasmy must be taken into account when analyzing the isolated mtDNA.

In order to analyze evolutionary changes in mtDNA, a battery of endonucleases is employed. These enzymes cleave the DNA at specific nucleotide sequences, giving DNA fragments of varying lengths. These segments can be separated using gel electrophoresis, forming bands which can be visualized with staining, and size estimated by comparing to size standards such as those prepared from phage Lambda DNA. Evolutionary changes can be recognized using this technique, understanding that as nucleotide sequence changes occur due to the molecular evolution of the DNA molecule over time, endonucleases will cleave at different locations along the DNA. These differences will be evidenced as different banding patterns in the gel (12).

Ants and other eusocial insects, like bees and wasps, seem well-suited for mtDNA research. Indeed, some research has already been done on the mtDNA of bees (6) and ants (17). The reason ants make such good subjects for mtDNA research has to do with the structure of the colony. According to Wilson (9,15), a generalized ant colony life cycle begins with a young virgin queen leaving the nest of her mother (the queen) and her sisters (sterile workers or other virgin queens). The young queen flies high above the nest and finds a mate, a winged male who dies shortly after copulation. The mated queen finds an agreeable nest site and digs the first nest cell. Here she lays her eggs and rears her young (all sterile

females), which become the first workers of the nest. With the help of the workers, the queen can confine herself to egg-laying, as the workers enlarge the nest, forage for food, and rear subsequent broods. After some seasons, depending on the species of ant, the colony will begin to produce virgin queens and males, and the cycle begins anew. Workers of the same colony should then theoretically have the same mtDNA, since ants of the same colony have the same maternal parent, the queen. Because of this fact, any desirable number of ants can be used for mtDNA isolation, yielding a single type of mtDNA.

Formica obscuripes, the thatching ant, was selected for this research because of its conspicuous nests (Figure 2), abundance on prairies and fields in Montana, and ease of collection. *Formica* ants are known to engage in some behaviors which must be taken into account when studying their mtDNA. They are known slave collectors, raiding nests of other species for pupae for food. Some of these pupae survive in underground chambers of the raiders and join the raiders' work force as workers (2). Also, more than one queen may be active in a single nest, a condition called polygyny (9,15). Such a situation could be evidenced by more than one kind of mtDNA isolated from workers of the same nest. In addition, *Formica obscuripes* will often build trail systems between nests which are close together. Ants observed on one nest at one time may be observed on another nest later (14). It is not clear whether these observations are examples of polydomous

colonies (one colony having separate nests) or of conspecific tolerance of nearby, unrelated colonies (14).

In this study, a procedure was developed for isolating mtDNA from *Formica obscuripes*. The procedure was based on the alkaline lysis procedure of Tamura and Aotsuka (18). Preliminary characterization of the mtDNA using endonuclease digestion was then carried out. Although this characterization was not complete, it revealed that the mtDNA genome of *Formica obscuripes* is unusually large.

Materials and Methods

Collection of Organisms

Collection of thatching ants was a simple process. Worker ants are abundant on the surface of their nests from late morning to early evening during the summer months. (Figs.1,2) They are most active when the temperature is warm (65-80° F) and are best collected when the nest is dry (not wet from rain or dew) so as to cause the least damage to the nest. Large numbers of ants can easily be scooped into a jar along with some nest material. The ants could be kept for up to a month stored either at 4° C or at room temperature if water and food (usually table sugar or bread crumbs) were provided.

Ants were collected from two different nests separated by approximately 500 feet. No trail systems between the two nests were evident. The nests are located on the Dover residence, approximately 8 miles south of Helena, Montana and in a vacant field behind the Dover residence (Figs. 3,4).

Characteristics

The ant's genus was determined using taxonomic keys in Hölldobler and Wilson (9) and species identification was made by entomologist Ron Lang from the U. S. Department of Agriculture, Bozeman, Montana. The worker ants from the different hills were physically indistinguishable, both measuring approximately 1 cm in length with red heads and thoraxes and black abdomens with 3 iridescent gray bands.



Figure 2. Nest of the thatching ant.



Figure 3. Habitat of the thatching ant, approximately 8 miles south of Helena, Montana.

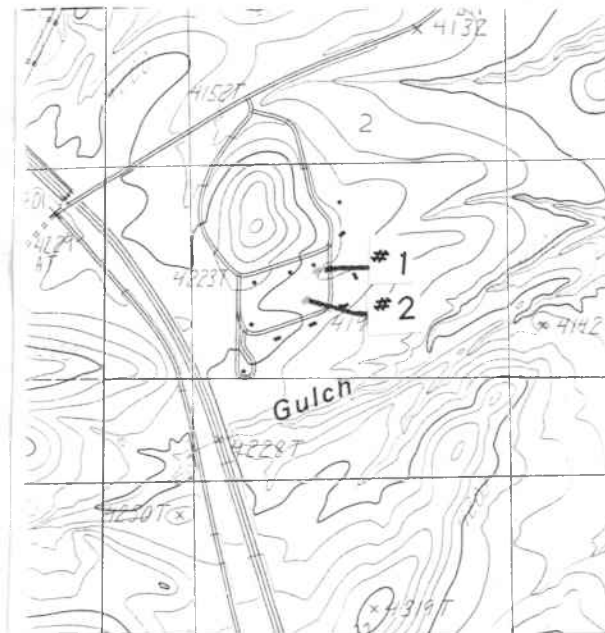


Figure 4. USGS topo map of the locations of the nests used in these experiments. T9N, R3W Section 2, SW4. East Helena Quad.

Homogenization

All solutions used in this experiment except for the sodium dodecyl sulfate (SDS) and the potassium acetate (KAc) were prepared with double distilled water and then autoclaved at 121°C and 15 pounds of pressure for 15 minutes or sterile filtered using a Gelman Sciences Acrodisc disposable filter (0.45 µm). Glassware was double rinsed in distilled water and either autoclaved as described above or rinsed in 70% ethanol. Determinations of pH were made using a Fisher Accumet model 10 pH meter. All solutions were kept at 4°C or on ice during the procedure.

In order to isolate mtDNA from ants, the Tamura and Aotsuka (18) method upon which our isolation technique was based was altered in several ways. A larger starting mass (0.5 g-1.0 g) of ants was needed than the 50 mg used by Tamura and Aotsuka in order to yield visible bands, perhaps because worker ants have lower concentrations of mitochondria than *Drosophila*, the flying insect studied by Tamura and Aotsuka. Ants were euthanized by placing a paper towel soaked in ethyl acetate in the jar of ants and nesting material, closing the lid and waiting approximately 5-10 minutes. The dead ants were then chopped quickly by hand with razor blades, weighed on a Mettler AC 100 balance, and ground using a mortar and pestle with about 20% the total volume of ice cold homogenization buffer (HB, 0.25 M sucrose/30 mM Tris-HCl, pH 7.5/10 mM EDTA; 50 mg ants/mL HB).

The ground ants were transferred to a large (approximately 50 mL) test tube with the remaining volume of HB, and the large particles were allowed to settle out of suspension for 5-10 min. The supernatant was removed with a Pasteur pipette and transferred to a homogenization tube. This suspension underwent 10 cycles in a Bellco homogenizer operated at setting 8. The homogenate was then transferred into two glass 15 mL centrifuge tubes (Sorvall).

Isolation

The homogenate was centrifuged in a Sorvall RC-2B centrifuge with an SS-34 rotor at 3000 rpm (1075 x g) for 4 min to pellet cellular debris and nuclei. The large pellets yielded in this step were beige with brown to black particles massed at their bottoms. The supernatants were removed with Pasteur pipettes, taking care to leave as much of the floating lipid layer as possible behind with the pellet, and combined in another 15 mL tube. This tube was centrifuged at 10,000 rpm (11,950 x g) for 10 min in the Sorvall RC-2B centrifuge in order to pellet mitochondria. This pellet was again beige with few if any brown to black particles, typically with a volume of approximately 200 μ L.

The pellet was resuspended in 250 μ L Saline-Tris-EDTA (STE, 10mM Tris-HCl, pH 8.0/0.15 M NaCl/10 mM EDTA) and transferred to a 1.5 mL microcentrifuge tube. To the suspension, 500 μ L 1% sodium dodecyl sulfate (SDS)/0.2 M NaOH were added. The suspension was then vortexed with a

VWR vortexer at setting 5 and incubated on ice for 10 min. The SDS causes degradation of the mitochondrial membrane and selective denaturation of nuclear (linear) DNA while circular mitochondrial DNA remains intact (21). Next, 375 μ L KAc (3 M potassium acetate/5 M acetic acid) was added, the suspension vortexed, and divided between two microcentrifuge tubes. The tubes were incubated on ice 5 min then centrifuged at 12,000 x g for 5 min at 4° C. The KAc causes renaturation of the nuclear DNA, forming an insoluble precipitate which will pellet out of solution when centrifuged (21).

To the supernatant in each tube, equal volumes of phenol:chloroform (1 phenol: 1 chloroform: 24 isoamyl alcohol) were added. The solution was then vortexed and centrifuged at 12,000 x g for 3 min at room temperature. The aqueous phase was removed carefully with a Pasteur pipette, so as to exclude the phenol:chloroform and the white precipitate layer between the phases, and transferred into clean microcentrifuge tubes. If a white precipitate was visible between the aqueous and organic phases, the extraction was repeated.

Phenol:chloroform extraction removes protein contaminants.

The aqueous phase from the two tubes was pooled into one tube to which two volumes of absolute ethanol were added. The tube was vortexed, incubated at room temperature for 15 min, and then centrifuged at 12,000 x g for 5 min at room temperature. The ethanol precipitates DNA. The alcohol was discarded carefully to leave the pellet attached to the side of the microcentrifuge tube. The pellet was washed with 70%

ethanol and then allowed to air dry for 5-10 min. The white-beige pellet obtained was generally very small, at no time larger than the head of a pin.

The next step involved removing any lingering RNA contaminants from the mtDNA pellet. The pellet was resuspended in 50 μ L Tris-EDTA (TE, 10 mM Tris-HCl, pH 8/1 mM EDTA) to which 1 μ L DNase-free RNase (Boehringer Mannheim) was added. The sample was allowed to incubate in a 37° C water bath for 30 min. Two volumes of phenol:chloroform were then added, and the tube was vortexed and centrifuged at 12,000 x g for 2 min to inactivate and remove the RNase. To the aqueous phase, 2 volumes of absolute ethanol were added. The solution was vortexed, incubated at room temperature for 15 min, and centrifuged at 12,000 x g for 5 min. The pellet was washed with 70% ethanol and air dried 5-10 min. After this step, the pellet was either faintly visible, or not visible at all.

Restriction Endonuclease Digestion

The sample now was ready for restriction endonuclease digestion. Endonucleases Eco R1 and Bam H1 were used in this experiment. All enzymes, buffer concentrates, and bovine serum albumen (BSA) were obtained from Sigma. For Eco R1 digestion, the pellet was resuspended in 35 μ L TE to which 5 μ L of a 1:20 dilution of buffer concentrate, 5 μ L of a 1:20 dilution of BSA, and 5 μ L of a 1:60 dilution of Eco R1 were added. Eco R1 is an enzyme which recognizes the nucleotide

sequence GAATTC and cuts between G and A. The tube was allowed to incubate in a water bath at 37° C for one hr. Bam H1 digestion was carried out in much the same way. The pellet was resuspended in 35 μ L TE and 5 μ L BSA, 5 μ L of a 1:20 dilution of Bam H1, and 5 μ L of concentrate buffer were added. The tube was allowed to incubate at 37°C for one hr. Bam H1 recognizes the nucleotide sequence GGATCC and cleaves between the Gs.

To insure that the endonuclease was functioning properly, phage Lambda DNA (Sigma) was usually digested as well. Lambda DNA (35 μ L diluted stock) was combined with 5 μ L BSA (1:20), 5 μ L digestion buffer (1:20), and 5 μ L Eco R1 or Bam H1 and incubated in a water bath at 37° C for one hour.

Electrophoresis

The DNA was analyzed using agarose gel electrophoresis. Negatively charged DNA fragments will move through an agarose gel matrix in response to an applied voltage. Larger DNA fragments move more slowly than smaller ones, and linear DNA moves more slowly than circular DNA of equal mass.

Agarose gels (0.9%) were prepared immediately before electrophoresis. To 20 mL of Tris-acetate-EDTA (TAE, 40 mM Tris-acetate/2 mM EDTA) buffer, 0.18 g of electrophoresis grade agarose (Sigma) was added and heated on a stir plate until the agarose was completely dissolved. The molten agarose solution was removed from the heat and allowed to cool for approximately 2 min and then poured into a 5 cm x 7.5

cm casting tray which had been sealed at both ends with masking tape and had an eight well gel comb in place. Once the gel was completely solidified, the comb and tape were removed from the casting tray, and the gel (still in the casting tray) was submerged in electrophoresis buffer approximately 1 cm beneath the surface of the electrophoresis buffer in the horizontal electrophoresis apparatus with the well end of the gel placed near the cathode end of the apparatus.

Before loading the wells with sample, 2 μL of a 10X loading buffer (20% Ficoll 400/0.1 M Na_2EDTA , pH 8/1.0% SDS/0.25% bromophenol blue) was added. The Ficoll causes the sample to sink into the gel wells instead of floating into the electrophoresis buffer. The bromophenol blue acted as a tracking dye. Each well was loaded with 15 μL of sample, care being taken not to spill sample into neighboring lanes. Samples were electrophoresed with empty lanes between them to prevent contamination from DNA in neighboring lanes.

Gels were electrophoresed at 86 V for 90 to 100 min and removed from the electrophoresis apparatus for staining.

Staining

In order to visualize the DNA in the gel, the gel was stained with ethidium bromide. The gel was pushed gently out of the casting tray into a staining tray containing 100 mL distilled water and 100 μL 1000X ethidium bromide (0.001% final concentration). Staining was carried out at 4° C for 24

hr. The gel was destained in distilled water for approximately 10 min prior to viewing and being photographed with a Foto-Dyne UV 15 transilluminator and a FCR-10 camera system (Foto-Dyne).

Results

In an initial experiment, mtDNA was isolated from Nest #1 ants and electrophoresed (Fig. 5) One clearly-visible band was apparent from the DNA which had not been digested with endonucleases.

Next, Eco R1-digested mtDNA from Nest #1 was analyzed. Two bands were evident (Figure 6). The approximate molecular weights of the mtDNA bands, (assuming that the DNA in the bands were linear rather than circular), were 29,607 base pairs and 19,171 base pairs, large for mtDNA.

An experiment was next performed using ants from Nest #2 in which half the isolated DNA was digested with Eco R1 and the other half (which was inadvertently exposed briefly to the endonuclease) was to remain undigested. Commercially supplied Eco R1 digested Lambda DNA (Sigma) was used as a size marker in this experiment. Three identical bands were present in both ant DNA lanes (Figure 7). The following possibilities were considered: (1.) Brief exposure to the Eco R1 had caused cleavage of the sample DNA even without proper exposure time and incubation temperature; (2.) Eco R1 was not working to cleave any DNA, (3.) the enzyme was functioning properly, but the ant DNA lacked Eco R1 cleavage sites, or (4.) the sites were inaccessible to the endonuclease because of the conformation of the mtDNA molecule (3). A gel was prepared to see whether the endonuclease was working properly. Lambda DNA was digested with Eco R1 and run on a gel

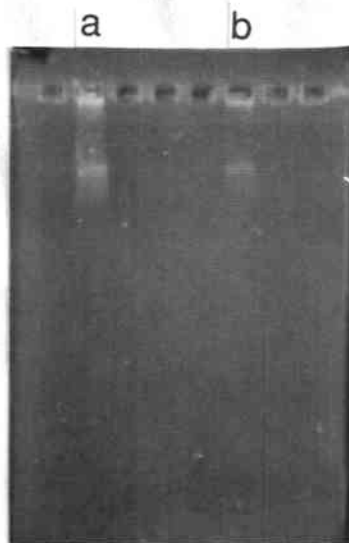


Figure 5. mt DNA from Nest #1 ants (1% agarose gel). (a) Full strength, (b) 2/3 strength.



Figure 6. mt DNA digested with Eco R1 from Nest #1 (0.9% agarose gel). (a) Nest #1 mtDNA, (b) Lambda DNA.



Figure 7. Undigested mtDNA and Eco R1-digested mtDNA from Nest #2 (0.9% agarose gel). The lanes are (a) Lambda DNA, (b) ant mtDNA exposed briefly to Eco R1, and (c) Eco R1-digested ant mtDNA.

alongside the commercially prepared Eco R1-digested Lambda DNA that had been used all along. The same banding patterns appeared in both lanes. It was concluded that the Eco R1 was functioning properly to cleave at least Lambda DNA.

To determine whether the brief exposure to the endonuclease had caused digestion of the mtDNA, undigested mtDNA from Nest #2 was electrophoresed, and two bands were present (Figure 8).

The conclusion drawn at this point was that the DNA isolated thus far either had no sites for Eco R1 cleavage or had them, but they were inaccessible to the enzyme, a likely probability since it has been reported that up to 20 times the amount of endonuclease used in this experiment can be required to cleave mtDNA (3).

The next step was to try to cleave the DNA with a different restriction enzyme. Bam H1 was selected, the banding patterns of mtDNA digested with endonuclease and undigested mtDNA were compared (Figure 9). Two bands could be seen in the lane containing undigested DNA, and a barely perceptible band was visible in the original gel in the lane containing the Bam H1 digest (bands difficult to see in the photo). The significance of the band in the Bam H1 lane is that the band migrated less into the gel than did the uncut bands, as a linear DNA fragment would. Recall that circular DNA migrates faster during electrophoresis than linear DNA of equal size. Though the band is faint, it is fairly certain that at

least one cleavage site for Bam H1 exists in this ant genome. Although accurate sizing is not possible from this gel, the location of the band in the gel indicates that this piece of DNA is very large.



Figure 8. Undigested mtDNA from Nest #2 ants (0.9% agarose gel). The lanes are (a) Lambda DNA and (b) Nest #2 mtDNA.

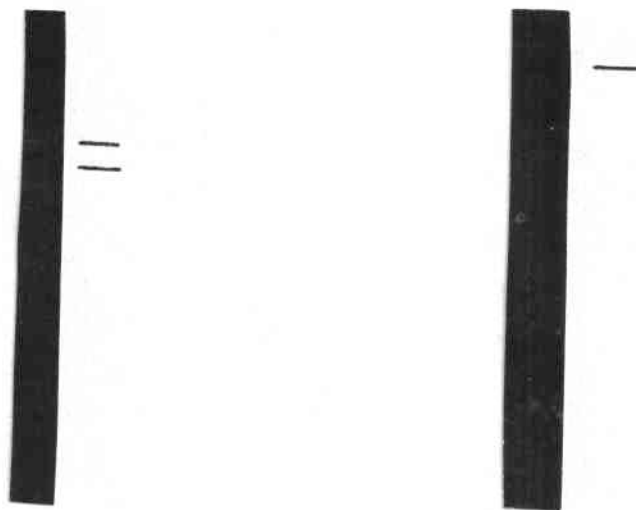


Figure 9. Undigested mtDNA and Bam H1 digested mtDNA from nest #2 ants (0.9% agarose gel). The lanes are (Right, barely perceptible) Bam H1-digested ant DNA, (Left) uncut ant DNA.

Discussion and Conclusions

The experiment with Nest #1 ants gave promising results: a single mtDNA type was isolated in the first, undigested sample, and two bands were observed in the digested sample. These results seemed to indicate that the single circular mtDNA type was cleaved by the Eco R1 in 2 locations, yielding two fragments measuring 29,607 and 19,171 base pairs.

For Nest #2 ants, however, multiple mtDNA sizes were observed even in the undigested samples. Some uncertainty remains about the origin of these multiple bands. The variation of sizes could be attributed to three major factors: (1.) polygyny (9, 13); (2.) slave use by *Formica obscuripes* (2), and (3.) heteroplasmy (4, 8).

Species of *Formica* often have more than one queen per nest, offering opportunity for more than one type of mtDNA to be present in the workers of the same nest (9, 13). Polygyny would be one way to explain the multiple bands, but could only be proven by taking the nest apart, capturing the queen(s), and extracting their mtDNA. Species of *Formica* are also notorious takers of slaves, raiding the nests of other species for pupae to be used as food. Some of these pupae develop in underground chambers of the raiders' nest and then serve as slave workers in the colony (2). Presence of slave workers of different maternal origin interspersed with the other workers could also explain the multiple kinds of mtDNA isolated.

Indeed, some ants in the colonies surveyed did differ markedly in appearance from the majority of the workers. These unusual ants were smaller and darker in coloration than the regular large, brightly-colored workers, but knowing that they could be slaves rather than regular workers, these ants were separated out and not used for these experiments. To have been included in the sample, slaves would have had to have been very similar in appearance to the colony members. The third possibility would be heteroplasmy, that is the presence of different sizes of mtDNA within the same individual due to varying lengths of repeating DNA segments within the non-essential portion of the genome. Mitochondrial DNA exhibiting heteroplasmy can be nearly twice the size of most mtDNA (>20,000 base pairs) (4). This phenomenon is thought to be caused by a number of factors including paternal leakage of mtDNA, maternal age effects, and large mtDNA molecules not being at a selective disadvantage over smaller mtDNA fragments (4, 8). Heteroplasmy has been observed in three species of bark weevil (4), and would seem to be more frequently encountered in invertebrates than in vertebrates. The ants used in this survey displayed from one to three distinct sizes of mtDNA. These results point most prominently toward heteroplasmy (perhaps in combination with one or more of the other above factors). The observation of large sizes of mtDNA with the multiple lengths of mtDNA isolated from a single nest would tend to indicate heteroplasmy rather than polygyny since large mtDNA size is indicative of the former

(4,9,13). Another possibility, which cannot be ruled out at this time is that some nuclear DNA contaminated the mtDNA preparation.

This procedure, created to isolate ant mtDNA, worked well with samples of between 0.5 g and 1.0 g of starting material, yielding relatively clear bands after electrophoresis. The use of ethidium bromide as a highly-sensitive staining agent was necessary to visualize the minute quantities of DNA isolated in this procedure (an accurate estimate of this amount of DNA is not available at this time). The use of an agarose concentration of 0.9% rather than a 1% or 1.2% provided greater resolution of bands. In future experiments an even lower concentration of agarose could be used to achieve better migration of bands into the gel and improve resolution further.

Some preliminary experiments focused on finding a simpler method of mtDNA purification than the Tamura and Aotsuka method provided. None of these experiments were successful in purifying mtDNA better than the alkaline lysis method described in this paper. Several experiments were performed using the SpinBind Mini-prep System (FMC) which consists of a microcentrifuge tube fitted with a small filter which should bind DNA. Stonefly mtDNA, partially purified by Curtis Horton by the Tamura and Aotsuka method during the summer of 1993 was used in an initial experiment to determine whether the SpinBind Mini-prep System could be used to purify the mtDNA further. A preparation which had

been put through the SpinBind system was electrophoresed together with the untreated preparation. Similar bands were observed in both lanes, the only difference being the SpinBind band was fainter. Next mtDNA was extracted from ants and run through the SpinBind system. No bands were visible in this gel.

A means was also sought to extract the uncut mtDNA from bands in the gels, cutting it with endonucleases, and running it out on a second gel for greater resolution and purity. Uncut mtDNA was electrophoresed on a 1% low melting point agarose (Sigma) gel. The two resulting bands were excised out of the gel with a razor blade and heated separately to 65° C to melt the agarose. TE buffer was added to dilute the agarose to 0.4%. The DNA in the two tubes next underwent Eco R1 digestion and were stored at -20° C overnight. The following day, the Eco R1-digested material was heated to 65° C, and the mtDNA was extracted with two volumes of absolute ethanol as described earlier in "Materials and Methods." The pellet after centrifugation was quite large and could not be resuspended in the TE for electrophoresis until heated to 65° C, indicating that agarose was still present in the sample. To the tube, 1 μ L loading buffer was added, and the gel was loaded with some difficulty since the agarose kept resolidifying. After staining, no bands were visible.

In still another experiment for removing DNA from gels, Ultra-Free-MC Filter Units (Millipore) were tried. Uncut Lambda DNA was electrophoresed on a 1% agarose gel and

stained with ethidium bromide. The band was cut out of the gel and macerated with a razor blade, then run through the Ultra-Free MC Filter Units according to the instructions provided. The resulting filtrate was then extracted with absolute ethanol, resuspended in 15 μ L TE, combined with 1 μ L electrophoresis loading buffer, and electrophoresed on a 1% agarose gel. Again, no bands were visible upon staining.

Despite these setbacks, promising results were achieved with the Nest #1 ants, from which a single type of mtDNA was isolated and apparently cleaved in two locations by Eco R1. Many questions remain about the results obtained from Nest #2 ants, and further work should be pursued to answer them. Future studies might include utilization of restriction endonucleases other than Eco R1 (e.g. further work with Bam H1), quantification of DNA concentrations in bands, development of methods to prevent unwanted loss of mtDNA during purification, and determination of the precise nature of the multiple bands encountered in this experiment. Surely the work with Hymenopteran mtDNA will continue as a key to unlocking their evolutionary history and as a means to better understand the nature of the ever-changing mtDNA molecule.

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