# RESTRICTION ENDONUCLEASE FRAGMENT ANALYSIS OF MITOCHONDRIAL DNA FROM MALE FERTILE AND CYTOPLASMIC MALE STERILE ALFALFA (*MEDICAGO SATIVA* L.)

by

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B.A., Saint Louis University, 1981

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A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Genetics

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1986

Approved by:

Major Professor

## ACKNOWLEDGMENTS



I wish to thank my co-advisors, Drs. Edgar L. Sorensen and George H. Liang, for guidance to, for many helpful suggestions in working on, and for financial support throughout, this research project. I thank Dr. Lowell B. Johnson, the third member of my committee, for time and expertise ungrudgingly shared.

I wish also to thank Dr. Ray J. Rose, for introducing the isolation of mitochondrial DNA from alfalfa, Harold B. Rathburn, for generous help in carrying out restriction endonuclease fragment analysis of that DNA, and Steven Lee, for frequent technical assistance.

Finally, I express deep gratitude to my parents, Dr. and Mrs. Paul A. Gatschet, and other members of my family, for their unwavering support.

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ABSTRACT

Mitochondrial DNA (mtDNA) was isolated from leaves of male fertile alfalfa (Medicago sativa L.) cultivars and cytoplasmic male sterile (cms) and male fertile maintainer alfalfa lines, of Flemish and Turkistan germplasm sources. Restriction endonuclease fragment analysis was carried out on the isolated mtDNA with the objectives of testing this analysis as a method of:

1) classifying alfalfa according to germplasm source

2) distinguishing cms from male fertile maintainer alfalfa.

Early studies with the restriction endonucleases Xho I and Eco RI yielded clear mtDNA fragment patterns. Gels from a later study, however, did not yield clear patterns with Xho I; only lower molecular weight (generally, less than 7.4 kb) bands were revealed with Eco RI.

In this later study, in contrast to results of the early studies, the Eco RI fragment pattern of mtDNA from the Flemish cultivar (Apex) did not differ from that of the Turkistan cultivar (Lahontan). The Eco RI fragment patterns of mtDNA from the Flemish cms line (MN 1292-CMS) and the Turkistan cms line (MN White-CMS) were indistinguishable. The Eco RI fragment pattern of mtDNA from the Flemish maintainer line (MN 1292-MA), however, did differ slightly from that of the Turkistan maintainer line (MN White-MA);

both patterns differed from those of the cms lines.

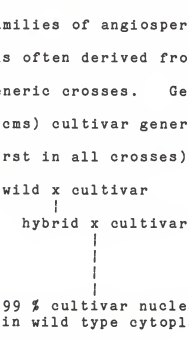
In the early studies, higher molecular weight bands were generated with Xho I and Eco RI. The Eco RI fragment pattern of mtDNA from the Flemish cultivar (Apex) differed from that of the Turkistan cultivar (Lahontan). For both Xho I and Eco RI, the fragment patterns of mtDNA from the Flemish cms line (MN 1292-CMS) differed from those of the Flemish maintainer line (MN 1292-MA).

The restriction endonuclease Xba I seemingly did not digest mtDNA from the alfalfa cultivar CUF 101.

Cytoplasmic Male Sterility

Male sterile plants fail to produce functional pollen grains. If male sterility is inherited according to Mendelian rules, it is classified as a nuclear trait. If male sterility is not inherited according to Mendelian rules, but is instead maternally transmitted, it is classified as a cytoplasmic trait (Laughnan and Gabay-Laughnan, 1983). Cytoplasmic male sterility (cms) is widely distributed throughout the plant kingdom. By 1972, cms had been reported in approximately 140 plant species of 47 genera from 20 families of angiosperms (Laser and Lernsten, 1972).

Cms is often derived from intraspecific, interspecific, or intergeneric crosses. Generation of a cytoplasmic male sterile (cms) cultivar generally is as follows (seed parent written first in all crosses):




Pollinating the resultant cms plants with wild type pollen often restores male fertility in the hybrid progeny and allows for the identification of nuclear restorer of fertility (Rf) genes that override the male sterile effect

of the cytoplasm even though they may produce no heritable change in the cytoplasm. In most cases of cms, nuclear Rf genes have been found (Hanson and Conde, 1985).

Edwardson (1970) notes that in a 1968 paper Lacadena proposed that in classifying types of cms, the term autoplasmic male sterility be used for those types in which the sterility apparently occurs spontaneously, homoplasmic male sterility for those types arising from intraspecific crosses, and alloplasmic male sterility for those types arising from interspecific or intergeneric crosses.

That cms often results from interspecific or wider crosses, and can be overridden by nuclear Rf genes, indicates that cms is a common manifestation of nuclear-cytoplasmic incompatibility (Hanson and Conde, 1985). Hanson and Conde (1985) cite a 1976 work of Grun suggesting that in some genera, cms was selected for as an outbreeding device. Among crop plants today, cms is utilized in the production of hybrid seed to avoid emasculation of the seed parent.

## CMS in Alfalfa

Davis and Greenblatt (1967) were the first to document evidence of cms in alfalfa (*Medicago sativa*). They screened more than 50,000 alfalfa plants for male sterility and eventually selected one completely male sterile and nine partially male sterile plants. Four of these plants had a male sterility that was transmitted solely through the

maternal cytoplasm. Bradner and Childers (1968), and Pederson and Stucker (1969) subsequently published evidence of cms in alfalfa.

Childers and McLennan (1960) had previously investigated a male sterility in alfalfa that was not transmitted through the cytoplasm. They studied a male sterile alfalfa designated 20 DRC and found that its completely male sterile character appeared to be controlled by three recessive nuclear genes inherited in a disomic manner. Degeneration of microspores in the anthers of 20 DRC was complete very soon after the quartet stage of meiosis and no trace of aborted pollen grains was observed. Barnes et al. (1972) note that McLennan and Childers later transferred this male sterility from the tetraploid to the diploid level and showed that the male sterility of 20 DRC was controlled by one gene (ms3), instead of three disomic genes.

Varying degrees of partial sterility occur in alfalfa cytoplasmic male steriles. Pollen production in the anther can range from a trace to near normal amounts and can vary both between and within plants. In contrast to the early degeneration of microspores in the anthers of 20 DRC, the time of pollen degeneration is much later in cytoplasmic male steriles and many functional pollen grains may in fact be produced. A low percentage of normal pollen grains, however, is associated with a lack of pollen dehiscence. A high frequency of aborted pollen grains may prevent the

buildup of enough internal pressure to completely rupture the anther wall and extrude the sticky pollen (Barnes and Garboucheva, 1973).

Apparently, in both nuclear (Childers and McLennan, 1960) and cytoplasmic (Davis and Greenblatt, 1967) male sterility in alfalfa, there is no detectable effect on the male sterile plant's female fertility.

Barnes et al. (1974) proposed a system to classify the amount of pollen shed by male sterile alfalfa, providing a pictorial description to help reduce variability among observers. Flowers, it was proposed, were to be tripped and classified for pollen production as follows: 1 = no pollen, 2 = trace of pollen, 3 = moderate amount of pollen, and 4 = much pollen. Barnes et al. (1972) note that for critical studies, the anthers from plants previously classified as 1 could be observed at 20X magnification and scored as 0 or 1: 0 = no pollen and 1 = few nondehisced pollen grains.

Assuming one nuclear Rf locus, tetraploid alfalfa nulliplex for nuclear Rf allele(s) and having a male sterile cytoplasm, would be completely male sterile. Plants simplex, duplex, triplex, or quadriplex for the nuclear Rf allele(s), despite having a male sterile cytoplasm, would be increasingly more fertile (Barnes and Garboucheva, 1973).

Barnes et al. (1972), expanding the terminology acknowledged by Leonard et al. (1968) for sorghums, recommended that the following nomenclature be used for cms

classification in alfalfa:

A = male sterile clone or seed propagated line

B = male fertile, nonrestoring maintainer clone or seed propagated line

C = male fertile pollen parent (pollenizer) for three-way hybrid; can be either a B or R line. The term "pollenizer" rather than "pollinator" is suggested so as not to confuse the source of pollen with the pollinating insect.

R = male fertile, pollen-restoring clone or seed line; includes most alfalfa plants

Utilization of cms in the seed parent is the most efficient pollen control method used in the production of alfalfa hybrids (Barnes et al., 1972). The most workable crossing plan is:

A (ms) x B
↓
AB (ms) x C
↓
ABC hybrid

Since pollen production is not correlated with forage yield in alfalfa hybrids (Pederson and Hill, 1972) and alfalfa is primarily a forage crop, the resulting ABC hybrid need not be male fertile to be valuable. The C line pollenizer then can be either a nonrestoring maintainer B line or a pollen-restoring R line.

In 1968, L. Teweles Seed Co. of Milwaukee, Wis. marketed in the Midwest the first commercial alfalfa hydrids

produced utilizing cms. On March 16, 1971, U.S. Patent No. 3,570,181 was issued to the L. Teweles Seed Co. on the hybrid-production process. This patent presumably precludes the use of cms in the commercial production of hybrid alfalfa varieties in the United States without payment of royalties (Childers and Barnes, 1972).

Thompson and Axtell (1978) tested whether factors conditioning cms in alfalfa could be asexually transferred to male fertile scions grafted onto male sterile stocks. They found that in no case was the male fertility of the scion flowers that had been grafted onto male sterile stocks changed to male sterility, but that cms was transferred to F1 scion progeny in three graft combinations. The graft transfer of cms in alfalfa is similar to the graft transfer of cms in petunia (Petunia hybrida). The transfer system in petunia is also characterized by first appearance of male sterility in F1 and S1 progeny of maintainer scions (Frankel, 1956; Edwardson and Corbett, 1961).

## Cytoplasmic Genomes and CMS

In theory, mitochondrial genomes, plastomes, and/or other maternally inherited cytoplasmic genomes could encode cytoplasmic factors conditioning cms. Maternal inheritance of organelles is a general rule of organelle transmission genetics in plants as well as animals. About one third of all plant genera investigated, however, have biparental

plastid inheritance (Birky, 1978). In angiosperms, evidence for regular biparental transmission of organelles is restricted to plastids (Galun et al., 1982).

Mitochondrial genomes of higher plants are larger and more variable than plastomes (Levings, 1983). The size of the Chinese cabbage (Brassica campestris) mitochondrial genome, the first mitochondrial genome of a higher plant for which a complete restriction map was reported, is 218 kilobase pairs (kb), a size at the lower end of the range in sizes of higher plant mitochondrial genomes (Palmer and Shields, 1984). The complexity of mitochondrial genomes in the Cucurbitaceae family is estimated to range from 220 megadaltons (Md) (350 kb) for watermelon to 1600 Md (2500 kb) for muskmelon (Ward et al., 1981). Plastomes, in contrast, range in size from 120 to 217 kb (Palmer, 1985). Pring and Lonsdale (1985) have reviewed the molecular biology of the mitochondrial genomes of higher plants.

In maize accumulating evidence indicates that mitochondria encode factors that condition cms (Leaver and Gray, 1982). Three male sterile maize cytoplasms-- T, S, and C --were originally identified on the basis of differing patterns of fertility restoration by nuclear restorer of fertility (Rf) genes (Laughnan and Gabay-Laughnan, 1983). These cytoplasms can now be distinguished from each other and from male fertile cytoplasms (N cytoplasms; plants having a N cytoplasm and a nuclear background devoid of the

Rf genes, yet produce functional pollen) by electrophoretic analysis of mitochondrial DNA (mtDNA) species (Kemble et al., 1980) and mitochondrial translation products (Forde et al., 1980).

Restriction endonuclease fragment analysis of mtDNA has also been used to distinguish these cytoplasms. When restriction endonuclease fragments of mtDNA from T, S, C, and N cytoplasms are fractionated by agarose gel electrophoresis, each of the four cytoplasms is differentiated by a characteristic pattern (Pring and Levings, 1978; Borck and Walbot, 1982). Though many of the mtDNA bands are common to all four, each cytoplasm is charaterized by specific bands.

Restriction endonuclease fragment analysis of mtDNA has been used to distinguish male sterile from male fertile cytoplasms in a number of other higher plants. Differences between the restriction endonuclease fragment patterns of mtDNA from male sterile versus male fertile cytoplasms have been reported for wheat (Quetier and Vedel, 1977; Ricard et al., 1986), tobacco (Belliard et al., 1979; Galun et al., 1982), sorghum (Pring et al., 1980; Pring et al., 1982), petunia (Kool et al., 1982; Boeshore et al., 1983), sugar beet (Powling, 1982; Mikami et al., 1984), rape (Vedel et al., 1982; Chetrit et al., 1985), faba beans (Boutry and Briquet, 1982), and sunflower (Leroy et al., 1985).

More compelling evidence for mitochondrial involvement in maize cms comes from studies of T cytoplasm anther cells.

Loss of internal mitochondrial structure in the tapetum and middle layer by tetrad and later stages is the first indication of abnormality in anthers of maize plants carrying the T cytoplasm (Warmke and Lee, 1977). These plants, and more specifically their mitochondria, are sensitive to very low levels of the host-specific toxin of the fungus Helminthosporium maydis race T, the pathogen responsible for the 1970 epidemic of southern corn leaf blight disease in the U.S.A. Efforts to separate toxin sensitivity and T type cms by tissue culture regeneration of T cytoplasm maize generally have been unsuccessful (Hanson and Conde, 1985).

Additional evidence is provided by studies of spontaneous cytoplasmic reversions to fertility of S cytoplasm maize. Such reversions are correlated with the virtual disappearance of the free mitochondrial plasmid-like DNAs, S-1 (6.2 kb) and S-2 (5.2 kb) and the integration of S-1 and/or S-2 into the main chromosomal mtDNA (Levings et al., 1980; Hanson and Conde, 1985).

Though there is little evidence to implicate plastomes in encoding factors determining cms, differences in the two dimensional electrophoresis patterns of restriction endonuclease digests of chloroplast DNA (cpDNA) from cms lines and their male fertile maintainers in maize, wheat, and rape have provided some evidence for plastome involvement in cms (Li and Liu, 1983).

There is good evidence to suggest that a defective

cytoplasmic viral genome may transmit type 447 cms of faba beans (Vicia faba). Restored lines remain fertile even if subsequently pollinated with non-restoring maintainers (Bond et al., 1966), the presence of RNA containing cytoplasmic spherical bodies strictly correlates with this cms (Scalla et al., 1981), and cms is expressed in plants that have received the cytoplasmic RNA via a dodder bridge (Grill and Garger, 1981).

Cytoplasmic Genomes of Alfalfa

A few studies on cytoplasmic genomes of alfalfa have been reported, though no molecular analysis of a cytoplasmic genome of cms alfalfa has been reported. Using electrophoretic analysis of mtDNA, Nikiforova and Negruk (1983) found several plasmid-like molecules in Medicago sativa mtDNA preparations, different from those from mtDNA preparations of Vicia species.

Smith et al. (1986) published evidence for biparental inheritance of plastids in alfalfa. Their study indicates that plastid transmission is more frequent through pollen than through egg cells in alfalfa.

Using restriction endonuclease fragment analysis, Rose et al. (1986) studied the cpDNA and mtDNA of Regen S alfalfa regenerated from protoplasts and found evidence for two parental cpDNAs. Summing restriction fragments, they estimated the size of the alfalfa cpDNA to be about 127 kb,

and the size of the alfalfa mtDNA to be about 350 kb. Electrophoresis of undigested mtDNA did not reveal any low molecular weight mtDNA species.

Objectives

Turkistan and Flemish germplasms are numbered among the nine distinct sources of alfalfa germplasm introduced into the United States. Another of the sources, Medicago falcata, an alfalfa species characterized by orange to yellow flowers and straight to sickle-shaped pods, readily crosses with M. sativa. Some of the alfalfa sources contain varying amounts of both M. sativa and M. falcata germplasm. Turkistan alfalfas appear to contain primarily, and Flemish alfalfas only, M. sativa germplasm (Barnes et al., 1977).

As discussed previously, restriction endonuclease fragment analysis of mtDNA has been used to distinguish male sterile from male fertile cytoplasms in a number of higher plants. The objectives of this work were to test restriction endonuclease fragment analysis of mtDNA from male fertile alfalfa cultivars and cms and male fertile maintainer alfalfa lines, of Flemish and Turkistan germplasm sources as a method of:

1) classifying alfalfa according to germplasm source

2) distinguishing cms from male fertile maintainer alfalfa.

## MATERIALS AND METHODS

### Alfalfa Cultivars and Lines

After early studies (Appendix 2), two alfalfa cultivars, one Flemish (Apex) and one Turkistan (Lahontan) (Barnes et al., 1977), were grown from locally available seeds. In addition, four alfalfa lines--a Flemish cms line (MN 1292-CMS) and its maintainer (MN 1292-MA), and a Turkistan cms line (MN White-CMS) and its maintainer (MN White-MA)--were grown from seeds released by the USDA Agricultural Research Service, Washington, D.C., and the Minnesota Agricultural Experiment Station, St. Paul, Minnesota. Five pots each of Apex, Lahontan, MN 1292-CMS, MN 1292-MA, MN White-CMS, and MN White-MA were planted. Plants were thinned after reaching the trifoliolate leaf stage so that each pot contained five to nine plants. The seeds were germinated and the seedlings established at 25 °C under constant, 100 to 150 Microeinsteins / m*m*sec photoperiods (PAR) (light intensity measured just above the level of the pot tops).

### Pollen Production Classification

The pollen production of each cultivar and line was estimated by scoring four to twelve plants following the simple procedure of Barnes et al. (1974), except that in scoring each plant, instead of tripping "several flowers of

each of two racemes onto a red plastic pot label," often, several flowers of only one raceme were tripped (often only one raceme having several flowers in bloom was present on a plant); also, a 5 X or 7 X magnifier was not used. Flowers from three to four plants of the cms lines were tripped onto a microscope slide. Material shed was stained with acetocarmine and observed at 160 X.

The plants were cut back and transferred to a growth chamber set at 21 °C, with 12-hour, 85 to 100 Microeinsteins / m*m*sec photoperiods (PAR) (light intensity measured just above the level of the pot tops). After being allowed to grow to a mature height (alfalfa is a long-day plant and generally does not flower under 12-hour photoperiods), the USDA plants were cut back and allowed to grow to a slightly lesser height. The Apex and Lahontan plants were grown similarly, except that they were allowed to grow three weeks longer before being cut back and were then sprayed twice (12 and 16 days after this cutting back) with Cygon insecticide containing spreader-sticker.

Isolation of MtDNA

MtDNA was isolated from 5-g samples of alfalfa green leaves following a differential centrifugation procedure derived with only slight modifications from the one of Rose et al. (1986). The isolation procedure followed is listed in detail in Appendix 1.

Analysis of MtDNA

MtDNA isolated from the alfalfa cultivars and lines was digested using the restriction endonucleases Xho I (Feb. 1986 Sigma Chem. Co. Cat. No. R 6379) and Eco RI (Feb. 1986 Sigma Chem. Co. Cat. No. R 2627). The mtDNA fragments generated were electrophoresed through 0.8 % agarose gels (11 x 14 cm) in a Bethesda Research Laboratories (BRL) Model H5 Horizontal Gel Electrophoresis Apparatus at 52 volts (4 volts/cm) (and about 25, decreasing to about 22 milliamps) for eight hours. The digests of mtDNA were electrophoresed in lane arrangements facilitating comparisons, e.g., digests of mtDNA from MN 1292-CMS and MN White-CMS were electrophoresed in adjacent lanes. The analysis procedure followed for Eco RI is listed in detail in Appendix 1. An identical procedure (except for the restriction endonuclease) was followed for Xho I.

## RESULTS

### Pollen Production Classification

Five of ten plants of the alfalfa cms line MN White-CMS were classified 2, i.e., producing a trace of pollen. All eight MN 1292-CMS plants scored were classified 1, i.e., producing no pollen (Table 1). Under observation at 160 X of material shed from MN 1292-CMS flowers, some pollen grains were found. Most appeared normal, although only a few grains were free of non-pollen anther material. As expected from pollen production classification, more pollen grains from flowers of MN White-CMS were found at 160 X observation. Most appeared normal and more were free of non-pollen anther material.

Pollen production was more variable in the male fertile alfalfa cultivars, with classifications ranging from 1 (no pollen) for two of eleven Apex plants, to 4 (much pollen) for eight of twelve Lahontan plants. Pollen production classifications of the maintainer lines MN 1292-MA and MN White-MA supported their male fertility; both lines had a mode classification of 3, i.e., producing a moderate amount of pollen (Table 1).

### Analysis of MtDNA

In the later study, after an electrophoresis of Xho I digests of mtDNA from each of the cultivars and lines

(except MN White-MA--mtDNA from MN White-MA was not digested with Xho I), no clear fragment patterns resulted. While acceptably clear Eco RI fragment patterns of mtDNA from the alfalfa cultivars and lines were generated, only lower molecular weight (generally, less than 7.4 kb) bands were generated.

In contrast to results of the early studies (Fig. 2), the Eco RI fragment pattern of mtDNA from Apex, the Flemish cultivar, did not differ from the pattern of mtDNA from Lahontan, the Turkistan cultivar. The Eco RI fragment patterns of mtDNA from MN 1292-CMS, the Flemish cms line, and MN White-CMS, the Turkistan cms line, were indistinguishable. The Eco RI fragment pattern of mtDNA from MN 1292-MA, the Flemish maintainer line, however, did differ from the pattern of mtDNA from MN White-MA, the Turkistan maintainer line, in some small (less than 3.5 kb) bands (Fig. 1).

The Eco RI fragment pattern of mtDNA from both cms lines differed from the patterns of mtDNA from the maintainer lines (Fig. 1).

## DISCUSSION

### Pollen Production Classification

Classifying the cultivars and lines for pollen production (Table 1) was done simply to check their male fertility. The number of plants sampled was too small to estimate precisely the pollen production of each cultivar and line. That the cms lines produced a small amount of pollen was not unexpected. As noted previously, varying degrees of partial sterility occur in alfalfa cytoplasmic male steriles.

The variable pollen production of the male fertile alfalfa cultivars could be attributed to diversity in cytoplasmic and/or nuclear genetic information. If it is the result in part of cytoplasmic genetic diversity, harvesting leaves from five or more plants (Appendix 1-Step 1 of Isolation of MtDNA) was appropriate to help ensure that the restriction endonuclease fragment patterns of mtDNA obtained were characteristic of the cultivar or line under study, and not simply of one plant.

### Analysis of MtDNA

In the later study, no clear fragment patterns resulted after electrophoresis of Xho I digests of mtDNA. Possibly mtDNA as isolated needed further purification in order to be efficiently digested by this enzyme. Alternately, high

buffer of a different composition may be a better medium for Xho I digestion of the mtDNA.

The Xho I fragment pattern of mtDNA from Regen S alfalfa documented by Rose et al. (1986) contained about fifteen bands of a size greater than 5.6 kb, while the Eco RI pattern had about eight. Also, most of the about fifteen Xho I bands were of a size greater than most of the about eight Eco RI bands. If the higher molecular weight mtDNA sections necessary for the generation of higher molecular weight Xho I bands were seriously diminished in mtDNA as isolated in the later study, clarity of Xho I fragment patterns could not be expected.

Higher molecular weight (generally, greater than 7.4 kb) bands were lacking in the Eco RI fragment patterns of mtDNA from the alfalfa cultivars and lines (Fig. 1). Addition of an extra shake and centrifugation routine (Appendix 1, Step 17 of Isolation of MtDNA) in the second and third phenol-chloroform extractions (submitting the bulk of each lysis solution to a total of five shake and centrifugation routines, versus generally three in the early studies) may have contributed to breakage of mtDNA sections and, hence, to the lack of higher molecular weight bands.

These shake and centrifugation routines were added to reduce contamination of the mtDNA in the aqueous layers with a cloudy residue, a residue that persisted even after digestion with Eco RI of a mtDNA sample from Lahontan

alfalfa (Fig. 1, well of lane B). However, bands greater in size than 7.4 kb, similar in size to the bands in Fig. 2, were present in early studies (Appendix 2) after analysis of mtDNA isolated following a procedure with four (submitting the bulk of each lysis solution to four shake and centrifugation routines) phenol-choroform extractions (data not shown). This makes it unlikely that addition of a shake and centrifugation routine in the phenol-chloroform extractions of the isolation procedure in the later study followed was responsible, at least wholly, for the lack of higher molecular weight Eco RI bands.

MtDNA from the alfalfa plants of the early studies that was successfully isolated and analyzed may have had fewer depurinated sites and/or ribonucleotides. Pring and Lonsdale (1985) report that depurinated sites and ribonucleotides are found in mammalian mtDNAs. They suggest that their presence in the mtDNAs of higher plants may account for the low proportion of circular DNA species that have been found in higher plant mitochondria, the circular species being fragmented into linear species during isolation of the mtDNAs.

That mtDNA digests of the later study were contaminated with nucleases is also a possibility. Because of their size, the higher molecular weight mtDNA fragments would be expected to be cleaved preferentially by nucleases.

Considering the first objective of this work, i.e.,

testing restriction endonuclease fragment analysis of mtDNA as a method of classifying alfalfa according to germplasm source, the results of this study were inconclusive. Again, higher molecular weight (generally, greater than 7.4 kb) bands were lacking in the later study. Had they been present, though, it would have been possible to determine more conclusively whether or not there is a correletion between the Eco RI fragment patterns of mtDNA and alfalfa germplasm source, i.e., in this study, Flemish or Turkistan germplasm source. Some of the data of the early studies are relevant here and are discussed in Appendix 2.

Using other restriction endonucleases in addition to Eco RI to generate fragment patterns of mtDNA would be helpful in determining the feasibility of classifying alfalfa according to germplasm source by restriction endonuclease fragment analysis of mtDNA. Comparison of fragment patterns of mtDNA from other alfalfa germplasms, with sampling from a larger number of representative cultivars and lines, also would be helpful.

Considering the second objective of this work, i.e., testing restriction endonuclease fragment analysis of mtDNA as a method of distinguishing cms from male fertile maintainer alfalfa, in both early studies (Appendix 2) and in the later study (Fig. 1), Eco RI restriction endonuclease fragment analysis of mtDNA allowed cms alfalfa to be distinguished from male fertile maintainer alfalfa. In the

early studies, analysis of mtDNA with Xho I also allowed MN 1292-CMS alfalfa to be clearly distinguished from MN 1292-MA alfalfa (Appendix 2).

The results of this work then suggest that restriction endonuclease fragment analysis of mtDNA may be a useful method for distinguishing cms from male fertile maintainer alfalfa. Use of other restiction endonucleases and cms and male fertile maintainer alfalfa lines of other germplasm sources would help to determine more conclusively the utility of restriction endonuclease fragment analysis of mtDNA as a method of distinguishing male sterile from male fertile cytoplasms.

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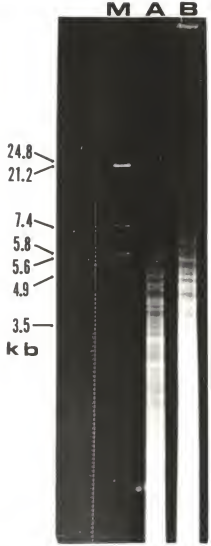
Table 1. Pollen production classification.

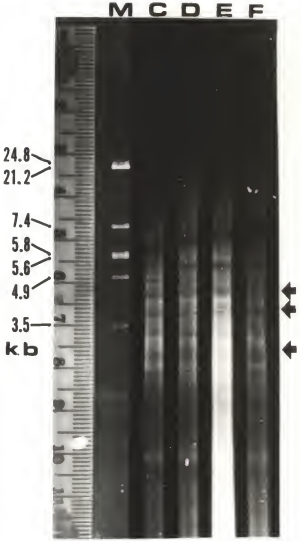
| | Apex | Lahontan |
|---|---|---|
| | 3 | 3 |
| | 1 | 4 |
| | 3 | 4 |
| | 3 | 4 |
| | 2 | 4 |
| | 2 | 4 |
| | 2 | 4 |
| | 3 | 3 |
| | 3 | 4 |
| | 3 | 3 |
| | 1 | 2 |
| | | 4 |
| average: | 2.36 | 3.58 |
| mode: | 3 | 4 |
| range: | 1 - 3 | 2 - 4 |

| | MN 1292-MA | MN 1292-CMS | MN White-CMS | MN White-MA |
|---|---|---|---|---|
| | 3 | 1 | 2 | 2 |
| | 4 | 1 | 2 | 4 |
| | 3 | 1 | 1 | 3 |
| | 3 | 1 | 2 | 2 |
| | | 1 | 1 | 3 |
| | | 1 | 2 | 3 |
| | | 1 | 2 | 2 |
| | | 1 | 1 | 3 |
| | | | 1 | 3 |
| | | | 1 | |
| average: | 3.25 | 1.00 | 1.50 | 2.78 |
| mode: | 3 | 1 | 1 & 2 | 3 |
| range: | 3 - 4 | - | 1 - 2 | 2 - 4 |

1 = no pollen, 2 = trace of pollen, 3 = moderate amount of pollen, and 4 = much pollen.

Figure 1. Eco RI fragment patterns of mtDNA from Apex (A), Lahontan (B), MN 1292-MA (C), MN 1292-CMS (D), MN White-CMS (E), and MN White-MA (F) alfalfa following electrophoresis through 0.8% agarose gels. The location and size in kilobase pairs (kb) of Eco RI fragments of phage lambda DNA (M) are indicated, as are differences in the Eco RI fragment patterns of mtDNA from the MA lines (third arrow) and the MA versus the CMS lines (all three arrows).


M A B
24.8
21.2
7.4
5.8
5.6
4.9
3.5
kb


M C D E F
24.8
21.2
7.4
5.8
5.6
4.9
3.5
kb

APPENDIX 1

## Isolation and Analysis of MtDNA from Alfalfa Leaves

### Isolation of MtDNA (see Rose et al., 1986)

Stock Solutions (see Maniatis et al., 1982)

0.1 M TES, pH 7.2 (1 liter)
Dissolve 22.93 g TES in c. 800 ml distilled H20 (dH20). While stirring, adjust the pH to 7.2 by adding NaOH (pellets & aq); add dH20 to a liter, maintaining the pH at 7.2 by adding NaOH (aq). TES (aq) readily supports growth of micro-organisms; sterilize not long after each opening by autoclaving.

0.2 M EDTA, pH 7.2 (1 liter)
Dissolve 74.45 g EDTA.2Na in c. 800 ml dH20. Stir vigorously. While stirring, adjust the pH to 7.2 by adding NaOH (pellets & aq); add dH20 to a liter, maintaining the pH at 7.2 by adding NaOH (aq). Sterilize periodically by autoclaving.

1.0 M MgCl2 (100 ml)
Dissolve 20.33 g MgCl2.6H20 in c. 75 ml dH20. After the MgCl2 has dissolved, add dH20 to 100 ml. MgCl2 is extremely hydroscopic and should be measured and handled with this in mind. Sterilize periodically by autoclaving.

1.0 M Trizma Base, pH 8.0 (1 liter)
Dissolve 121.1 g Trizma Base in 800 ml dH20. While stirring, adjust the pH to 8.0 by adding concentrated HCl (c. 40 ml) and HCl (aq); add dH20 to a liter, maintaining the pH at 8.0 by adding HCl (aq). Sterilize periodically by autoclaving.

0.5 M EDTA, pH 8.0 (1 liter)
Dissolve 186.1 g EDTA.2Na in 800 ml dH20. Stir vigorously. While stirring, adjust the pH to 8.0 by adding NaOH (c. 20 g pellets, and aq); add dH20 to a liter, maintaining the pH at 8.0 by adding NaOH (aq). Sterilize periodically by autoclaving.

TE Buffer (1 liter)
0.05 M Trizma Base (50 ml 1.0 M Trizma Base, pH 8.0)
0.01 M EDTA (20 ml 0.5 M EDTA, pH 8.0)
Add dH20 to a liter. Sterilize periodically by autoclaving.

Sarkosyl Buffer (100 ml)

0.05 M Trizma Base (5 ml 1.0 M Trizma Base, pH 8.0)
0.01 M EDTA (2 ml 0.5 M EDTA, pH 8.0)
10 % N-Lauroylsarcosine Sodium Salt (10 g)
Add dH20 to 100 ml. Sterilize periodically by autoclaving.

2.0 M Ammonium Acetate (100 ml)

Dissolve 15.5 g ammonium acetate in c. 75 ml autoclaved demineralized distilled H20 (a.ddH20). After the ammonium acetate has dissolved, add a.ddH20 to 100 ml. Do not autoclave; use sterile equipment in mixing.

TE-Saturated Phenol

Redistill phenol (180 C). Add 8-hydroxyquinoline to 0.1 %. Saturate phenol with and an equal volume of TE buffer. Store refrigerated (4 C).

Chloroform

Commercial preparations with ethanol or octanol added (to c. 1%) as a preservative are acceptable. Isoamyl alcohol need not be added.

1 mg/ 100 ul tRNA Solution

Dissolve tRNA (Feb. 1986 Sigma Chem. Cat. No. R 1753) in a.ddH20 to a concentration of 1 mg/ 100 ul. Store frozen.

100% Ethanol

70% Ethanol

Isolation Solutions

Grinding Buffer (400 ml) [200 ml]

0.5 M Mannitol (36.44 g) [18.22 g]
0.05% L-Cys (Hydrochl. monohyd.) (200 mg) [100 mg]
0.01 M TES (40 ml 0.1 M TES, pH 7.2) [20 ml]
0.005 M EDTA (10 ml 0.2 M EDTA, pH 7.2) [5 ml]
0.2% BSA (Fraction V, f. a. free) (0.8 g) [0.4 g]
Make up grinding buffer shortly before step 1. Sprinkle BSA on the solution surface and stir it in, after dH20 has been added to the total vol.(400 or 200 ml).

Sucrose Buffer (170 ml) [90 ml]

0.6 M Sucrose (34.92 g) [18.49 g]
0.01 M TES (17 ml 0.1 M TES, pH 7.2) [9 ml]
0.02 M EDTA (17 ml 0.2 M EDTA, pH 7.2) [9 ml]
Add dH20 to total vol. (170 or 90 ml). For convenience, make up sucrose buffer w/ grinding buffer shortly before step 1, too.

Isolation

Note: Mortars, pestles, filtering clothes, funnels, centrifuge tubes, graduated cylinders, pipets, brushes, flasks, and test tubes should all be sterilized. Flasks, pipets, and test tubes used after lysis of mitochondria should also be siliconized (such materials need not be siliconized fresh before every extraction, only periodically as needed). For ungraduated pipets, use short, large bore Pasteur pipets.

1. Collect four [two] 5.0 g leaf (w/ little petiole) tissue samples from alfalfa plants (5+ plants / sample, collecting less than 1.0 g/ plant) grown in a growth chamber at 21 °C under 12 hour, 85 - 100 Microeinsteins / m*m*sec photoperiods (PAR) (light intensity measured just above the level of the pot tops). Wear plastic gloves to avoid contaminating samples w/ nucleases from skin, through step 2.

2. In a cold room (4 °C), homogenize each sample ((25 ml grinding buffer + c. 2.5 g of sample)/ mortar (Coors 60316, 17) (2 mortars total, washed well between homogenizations of different samples). Grind each thoroughly until a fine slurry is obtained (1 - 2 min.). Filter each homogenate through five presoaked (10 ml grinding buffer) filtering layers (4 cheesecloth over 1 Miracloth (Chicopee Mills)) into a 50 ml centrifuge tube (2/sample) on ice. Rinse (20 ml grinding buffer/sample) each mortar, filter rinses, and wring cheesecloth layers (only gently press Miracloth), adding each rinse filtrate to a tube containing its corresponding previously collected filtrate.

Note: Keep grinding and sucrose buffers, as well as preparations in 50 ml centrifuge tubes, on ice. All centrifugations utilizing 50 ml centrifuge tubes are to be performed at 0 - 4 °C in a IEC (or Sorvall) centrifuge using a No. 410 (r = 4.23 in.) rotor (or SS-34 rotor (r = 4.25 in.) for Sorvall centrifuge).

3. Centrifuge filtrates at 3 K rpm (1,080 x g) for 10 min.

4. Centrifuge resulting supernatants at 12 K rpm (17,300 x g) for 20 min. After centrifugation, discard supernatants.

5. With a small camel's hair paint brush, gently resuspend each pellet in 1 ml grinding buffer. After resuspending, combine the two resuspensions of each sample into one and add 8 ml grinding buffer (total 10 ml/sample).

6. Make up 30 ul 1 mg/ 100 ul DNase I (Feb. 1986 Sigma Chem. Cat. No. D 5025) solution (e.g. 0.30 mg DNase I + 29.7 ul a.ddH20). After mixing, add 5 ul DNase I solution to each of four [two] 50 ml tubes on ice each containing 100 ul 1.0 M MgCl2.

7. Centrifuge resuspensions of step 5 at 3 K rpm (1,080 x g) for 10 min. Add resulting supernatants to 50 ml tubes on ice each containing 100 ul 1.0 M MgCl2 (0.01 M final conc.) and 5 ul DNase I solution (10 ug DNase I/ g tissue fr. wt.), and mix. Keep on ice for 1 hour.

8. After 1 hour, pipet DNase I-treated solutions onto 20 ml sucrose buffer in 50 ml centrifuge tubes on ice, balance with grinding buffer, and centrifuge at 12 K rpm (17,300 x g) for 20 min. After centrifugation, discard supernatants, taking care to pour off all of each supernatant.

9. With a small, thoroughly washed camel's hair paint brush, very gently, but thoroughly resuspend each pellet in 1 ml sucrose buffer. After resuspending, add 9 ml sucrose buffer to each (total 10 ml/ sample), and mix. Centrifuge at 12 K rpm (17,300 x g) for 10 min. After centrifugation. discard supernatants, again taking care to pour off all of each supernatant.

10. Repeat step 9 once.

11. Make up 210 ul [110 ul] 1 mg/ 100 ul Proteinase K (1985/1986 Boehringer Mannheim Biochem. Cat. No. 161 519) solution (e.g. 2.1 mg Proteinase K + 208 ul a.ddH20) [e.g. 1.1 mg + 109 ul]. After mixing, add 50 ul Proteinase K solution to each of four [two] 25 ml flasks each containing 0.5 ml sarkosyl buffer.

12. Again with a small, thoroughly washed camel's hair paint brush, gently, but thoroughly resuspend each pellet resulting from step 10 in 1 ml TE buffer. Pipet each resuspension into 25 ml flasks each containing 0.5 ml sarkosyl buffer and 50 ul Proteinase K solution. Rinse each 50 ml tube with 1 ml TE buffer and add rinse to its resuspension in 25 ml flask (0.05 M Trizma Base, pH 8.0, 0.01 M EDTA, pH 8.0, 2% N-Lauroylsarcosine Sodium Salt, and 0.02% Proteinase K, final concentrations neglecting pellet volume).

13. After mixing solutions in 25 ml flasks and capping flasks with aluminum foil, incubate mixtures with gentle mixing (c. 20 rpm) at 37 °C for 1 hour.

14. After incubation, gently pipet each lysate into a 16 x 100 mm Kimax tube. Rinse each flask with 300 ul 2.0 M

ammonium acetate, adding each rinse to its lysate. Gently add 3 ml TE-saterated phenol (settled after first being shaken, and at room temp.) to each.

Note: "Gently shake" is accomplished by holding the tubes with a hand at each end, parallel to the floor, and mixing the contents with a slow exaggerated movement up over the head, down to the waist, etc.

15. Gently shake the mixtures for 2 min. (c. 15 waist-head-waist movements). Add 3 ml chloroform to each and again gently shake each for 2 min. Centrifuge the resulting mixtures at room temp. and 4.3 K rpm (2,200 x g) for 10 min.

Note: To reduce protein contamination of the final aqueous layer, leave a 0.3+ mm aqueous layer at the first removal, and a 0.2 - 0.3 mm aqueous layer on the phenol-chloroform phase at each subsequent removal.

16. Pipet off and add each top aqueous layer to another fresh Kimax tube. Re-extract each remaining phenol-chloroform phase with 225 ul TE buffer and 25 ul 2.0 M ammonium acetate, gently shaking the mixtures with added TE buffer and 2.0 M ammonium acetate for 2 min. and centrifuging the mixtures for 10 min. as before. Pipet off and add each top aqueous layer to its corresponding previously removed aqueous layer.

17. Extract each combined aqueous layer two more times with TE-saturated phenol and chloroform (3 ml/3 ml). In these second and third extractions, after centrifuging the mixtures, gently shake the mixtures for 2 min. and centrifuge them at room temp. and 4.3 K rpm (2,200 x g) for 10 min. again, before transferring each top aqueous layer of the second extraction to a fresh Kimax tube and each top aqueous layer of the third extraction to a 15 ml Corex tube containing 10 ul 1 mg/ 100 ul tRNA solution and 8.0 ml 100% ethanol (2 shake and centrifugation routines in each extraction).

18. Balance tubes w/ 100% ethanol, cap w/ aluminum foil, and store overnight at -20 °C. The next morning, pellet each tube's precipitated mtDNA by centrifugation at 10 K rpm (12,000 x g), going from less than 4 to 15 °C over 20 min.

19. After centrifugation, the mtDNA should be on each Corex tube's inside, visible as a faint pellet or smear. After carefully pipetting off the ethanol supernatant, remove the mtDNA to a 1.5 ml microcentrifuge tube on ice w/ 1 + 0.4 ml refrigerated 70% ethanol, gently scraping the mtDNA from each tube's inside with the Pasteur pipet.

20. Spin each microcentrifuge tube in an Eppendorf microcentrifuge for 5 min. Pipet off each supernatant, add 1.4 ml cold 70% ethanol to each tube, and again spin each 5 min. in a microcentrifuge. Pipet off supernatant. Repeat last two sentences once.

21. Lyophilize each mtDNA pellet.

22. Solubilize each mtDNA pellet in 10 ul a.ddH20. The tubes may be turned to assist solubilization. Solubilization should be complete in 1 hour.

23. Label each microcentrifuge tube as follows:
A - D) Alfalfa Variety
Date

and store in freezer. Each solution should be sufficient for 1 - 2 restrictions, depending on the yield of mtDNA and the restriction type, e.g., some restrictions could be set up so that a trial lane containing half of the restriction mixture could be run first.

## Analysis of MtDNA

### Stock Solutions

2.0 M NaCl (50 ml)
Dissolve 5.844 g NaCl in 40 ml autoclaved demineralized distilled H20 (a.ddH20). Add a.ddH20 to 50 ml. Sterilize by autoclaving. Store several 1.5 ml aliquots in a freezer.

1.0 M Trizma Base, pH 7.5 (50 ml)
Dissolve 6.055 g Trizma Base in 40 ml a.ddH20. While stirring, adjust the pH to 7.5 by adding concentrated HCl (c. 3 ml) and HCl (aq); add a.ddH20 to 50 ml, maintaining the pH at 7.5 by adding HCl (aq). Sterilize by autoclaving. Store several 1.5 ml aliquots in a freezer.

1.0 M MgCl2 (1.5 ml)
Prepare autoclaved 1.5 ml aliquot from isolation procedure 1.0 M MgCl2 stock solution. Store in a freezer.

10 mg/ml Gelatin (1.5 ml)
Dissolve 15 mg gelatin in less than 1.5 ml a.ddH20. Add a.ddH20 to 1.5 ml. Sterilize by autoclaving. Store in a freezer.

0.1 M DTT (1.5 ml)
Dissolve 23.14 mg DTT in less than 1.5 ml a.ddH20. Add a.ddH20 to 1.5 ml. Do not autoclave; use sterile equipment

in mixing. Store in a freezer.

0.02 M Spermidine (1.5 ml)
Dissolve 7.64 mg Spermidine Trihydrochloride in less than 1.5 ml a.ddH20. Add a.ddH20 to 1.5 ml. Do not autoclave; use sterile equipment in mixing. Store in a freezer.

4X High Buffer (1 ml)
0.4 M NaCl (200 ul 2.0 M NaCl)
0.2 M Trizma Base (200 ul 1.0 M Trizma Base, pH 7.5)
0.04 M MgCl2 (40 ul 1.0 M MgCl2)
0.4 mg/ml gelatin (40 ul 10 mg/ml gelatin)
0.004 M DTT (40 ul 0.1 M DTT)
0.008 M Spermidine (400 ul 0.02 M Spermidine)
Add 80 ul a.ddH20. Do not autoclave; use sterile equipment in mixing. Store in a freezer.

Lambda DNA Solution
Add 100 ul a.ddH20 to 1 A260 unit lambda DNA (Feb. 1986 Sigma Chem. Co. Cat. No. D 0144). Store in a freezer.

Eco RI Digest of Lambda DNA (100 ul)
9.0 ul Eco RI R. E. (c. 90 units) (Feb. 1986 Sigma Chem. Co. Cat. No. R 2627)
25.0 ul 4X High Buffer
2.0 ul Lambda DNA Solution
64.0 ul a.ddH20
Incubate at 37 ± 1 °C for 24 hours. Store in a freezer.

Eco RI Digest w/o Lambda DNA (200 ul)
18.0 ul Eco RI Restriction Endonuclease (see above)
50.0 ul 4X High Buffer
132.0 ul a.ddH20
Store in a freezer.

10X TBE Buffer (1 liter)
0.178 M Trizma Base (108 g Trizma Base)
0.178 M Boric Acid (55 g Boric Acid)
0.004 M EDTA (40 ml 0.5 M EDTA, pH 8.0)
Add distilled H20 (dH20) to a liter. Do not autoclave.

5X Gel Loading Buffer (1 ml)
0.208 % Bromphenol Blue (2.08 mg)
33.3 % (w/v) Sucrose (0.333 g)
Add dH20 to 1 ml. After mixing, autoclave and store in a freezer.

10 mg/ml Ethidium Bromide (50 ml)
To 0.5 g ethidium bromide add dH20 to 50 ml. After mixing, store in a bottle wrapped in aluminum foil in a refrigerator. Ethidium bromide is mutagenic; wear gloves

when handling the dye or solutions containing it. To detoxify ethidium bromide solutions before disposing, mix with an equal volume of chlorox (5.25 % sodium hypochlorite) and let stand overnight.

18 % Sodium Sulfite (1 liter)
Dissolve 18.0 g sodium sulfite in less than 1 liter dH20. Add dH20 to a liter.

Analysis Solutions

EcoRI Digest of MtDNA (c. 24 ul)
1.8 ul Eco RI Restriction Endonuclease (c. 18 units) (see above)
5.0 ul 4X High Buffer
4.6 - 10.5 ul MtDNA Solution
8.6 - 2.7 ul a.ddH20
After mixing (20 ul total), incubate at 37 ± 1 °C for 6 hours. After incubation, spin briefly in a microcentrifuge to recombine solution evaporated to cap of microcentrifuge tube, measure volume by drawing solution into a micropipet, add a quarter of volume (e.g. 4.7 ul for 18.8 ul, or 4.8 ul for 19.2 ul) 5X Gel Loading Buffer, mix, and store in a freezer.

Eco RI Digest of Lambda DNA Marker Solution (23.5, or 24 ul)
3.0 ul Eco RI Digest of Lambda DNA
15.8, or 16.2 ul Eco RI Digest w/o Lambda DNA
4.7, or 4.8 ul 5X Gel Loading Buffer
Incubate at 60 ± 1 °C for 10 min. Store in a freezer.

1X TBE Buffer (800 ml)
80 ml 10 X TBE Buffer
720 ml dH20

Analysis of MtDNA

1. Add 90 ml dH20 to 0.8 g Type V Agarose (Feb. 1986 Sigma Chem. Cat. No. A 3768) in a small beaker containing a magnetic stir bar. Cover with aluminum foil and place on a hot plate. While stirring, heat to boiling. After vigorous boiling, briefly remove aluminum foil and add 10 ml 10X TBE Buffer. Again, cover with aluminum foil and, while stirring, heat to a vigorous boil. Remove from hot plate and let cool for 6 min.

2. Pour warm agarose mixture into the 11 x 14 cm gel bed (test tape across both ends) of a Bethesda Research Laboratories (BRL) Model H5 Horizontal Gel Electrophoresis Apparatus. Add 14 well (each 4.8 x 1 mm) comb and cover gel

bed with aluminum foil. Let agarose cool and solidify. After 45 min., remove aluminum foil and test tape, place gel bed in transparent tray, and with a micropipet load thawed samples on agarose gel in desired lane arrangement (e.g., Eco RI Digest of Lambda DNA Marker Solution in lane 3 and Eco RI Digests of MtDNA in lanes 4, 5, etc.).

3. Add 1X TBE Buffer to transparent tray till buffer is almost level with gel surface, but gel remains unsubmerged. Cover tray with safety cover, connect power cords, and begin electrophoresis at 52 volts (and c. 21 milliamps) (i.e. 4 volts/ cm). After 30 min., stop electrophoresis, shallowly submerge the gel by adding the remainder of 1X TBE Buffer, and begin electrophoresis again. Continue electrophoresis at 52 volts (and c. 25, decreasing to c. 22, milliamps) for 7 hours & 30 min. more.

4. After 8 hours of electrophoresis (after the bromphenol blue tracking dye of the 5X Gel Loading Buffer has run off the end of the gel), gently remove the gel to a square glass baking pan containing 500 ml 0.5 ug/ml ethidium bromide (25 ul 10 mg/ml Ethidium Bromide + 500 ml dH20). Stain the gel in 0.5 ug/ml ethidium bromide for 45 min. After 45 min., pour off the ethidium bromide solution and add 500 ml dH20. Destain the gel in dH20 for 45 min. then pour off the dH20, taking care not to let the gel slip and break.

5. Place the gel on a UV transilluminator. Put transparent ruler in place and allign the gel in the field. Photograph the gel with a 2 min. exposure through Kodak No. 23A (red) and No. 9 (yellow) Wratten gelatin filters with Polaroid Type 55 P/N 4 x 5 Land Film. 20 sec. after removing film, seperate the positive from the negative. Place the negative in 100 ml 18 % Sodium Sulfite, and wipe the positive with print coater. After the coating has been removed from the negative, hang both positive and negative to dry overnight, then place both in labeled sleeves.

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APPENDIX 2

## Early Studies

### Early Studies with Clear Fragment Patterns for Results

#### Alfalfa Cultivars and Lines

The alfalfa cultivars Apex, Lahontan, and CUF 101, and the USDA alfalfa lines MN 1292-CMS and MN 1292-MA, were grown for three months or more at about 21 °C under 12-hour, about 600 foot-candle photoperiods, after having been established and grown for about a year at a temperature greater than 21 °C under 18-hour, full light intensity (in growth chamber #36 of the KSU Agronomy Dept.) photoperiods. The plants were cut back and spayed with various insecticides (O-Mite, Cygon, and Orthene) many times, too.

#### Isolation of MtDNA

MtDNA was isolated from these older alfalfa plants using procedures that differed slightly from the one listed in Appendix 1. For example, generally only one shake and centrifugation routine (versus two in the later critical study--Step 17 of the Isolation of MtDNA, Appendix 1) was done in the second and third phenol-chloroform extractions (submitting the bulk of each lysis solution generally to a total of only three--versus five--shake and centrifugation routines). Also, mtDNA was isolated from leaf tissue samples of 2.4 to 6-g. MtDNA from Apex alfalfa was isolated from a 5-g leaf tissue sample, Lahontan--a 2.5-g sample, CUF 101--a 2.4-g sample, and MN 1292-CMS and MN 1292-MA--6-g samples. The smaller samples may have been harvested from but one plant.

#### Analysis of MtDNA

The analysis procedures followed also differed slightly from the one listed in Appendix 1. For example, digests of mtDNA from Apex, Lahontan, and CUF 101 alfalfa were electrophoresed through 0.7 % agarose gels unsubmerged at 50 volts (and about 20 milliamps) for more than eight hours, e.g., ten hours for the Eco RI digest of mtDNA from Lahontan. Hind III digests of phage lambda DNA (prepared by Steven Lee) were often used as markers in electrophoresis of digests of mtDNA. Xho I and Eco RI digests of mtDNA from

MN 1292-CMS and MN 1292-MA alfalfa were electophoresed through a 0.75 % agarose mini-gel unsubmerged at 50 volts (and about 15 mA) for five hours in a mini-sub cell (Bio-Rad 170-4307). Digestion in high buffer of mtDNA from CUF 101 with the restriction endonuclease Xba I (Feb. 1986 Sigma Chem Co. Cat. No. R 8379) also was attempted.

## Results

The Eco RI fragment pattern of mtDNA from Apex differed markedly from the Eco RI fragment pattern of mtDNA from Lahontan alfalfa (Fig. 2) Unlike the patterns of mtDNA from Apex and Lahontan in the later study, higher molecular weight (greater than 7.4 kb) bands were generated. A difference in the two patterns is found amoung these bands; a band about 8 kb in size in the pattern of mtDNA from Apex is not found in the pattern of mtDNA from Lahontan alfalfa (Fig. 2).

Clearer Eco RI than Xho I fragment patterns resulted after electrophoresis in a mini-sub cell of Eco RI and Xho I digests of mtDNA from MN 1292-CMS and MN 1292-MA. The Xho I fragment patterns, though, were much clearer than those generated in the later study. For both restriction endonucleases, the fragment pattern of mtDNA from MN 1292-CMS differed from that of mtDNA from MN 1292-MA alfalfa (data not shown).

After electrophoresis of the Xba I digest of mtDNA from CUF 101 alfalfa, no bands, other than a band of the high molecular weight fraction, seemingly undigested, appeared on the photograph of the gel (data not shown). Dr. Ray J. Rose independently obtained similar results with mtDNA from Regen S alfalfa (Lowell B. Johnson, personal communication).

## Discussion

The Eco RI fragment pattern of mtDNA from Apex alfalfa (Fig. 2, lane A), a Flemish germplasm alfalfa, differed markedly from the Eco RI pattern of mtDNA from Regen S alfalfa documented by Rose et al. (1986). The Eco RI fragment pattern of mtDNA from Lahontan alfalfa (Fig. 2, lane B), a Turkistan germplasm alfalfa, however, appeared nearly identical to the Eco RI fragment pattern documented by Rose et al. (1986) for mtDNA from Regen S alfalfa. Since the germplasm source of Regen S alfalfa is primarily Flemish (see below), these preliminary results indicated that Eco RI restriction endonuclease fragment analysis of mtDNA, despite enabling Apex and Lahontan alfalfa to be distinguished, may not alone be useful for distinguishing or classifying alfalfa according to germplasm source.

Regen S was developed from the cultivars Saranac and

Dupuits (Bingham et al., 1975). The parentage of Saranac can be traced to five of the nine basic alfalfa germplasm sources in approximately the following percentages: Ladak 2, M. varia 7, Turkistan 2, Flemish 87, and Chilean 2; the germplasm source of Dupuits is Flemish (Barnes et al., 1977). The maternal, i.e., cytoplasmic, parentage of Regen S cytoplasm is then likely predominately Flemish, too.

For both Xho I and Eco RI, the fragment pattern of mtDNA from MN 1292-CMS differed from the pattern of mtDNA from MN 1292-MA alfalfa (data not shown). These preliminary results indicated that Xho I and Eco RI restriction endonuclease fragment analysis of mtDNA could be used to distinguish cms from male fertile maintainer alfalfa.

The Xho I and Eco RI fragment patterns of mtDNA from MN 1292-CMS and MN 1292-MA alfalfa resulted from digestions of about a third of the mtDNA isolated from 6-g leaf tissue samples. Though possible, it is unlikely that a 6-g sample was harvested from a single plant. The pattern generated from a sample then was more likely characteristic of the line, and not of but one plant. The Eco RI fragment pattern of mtDNA from Apex alfalfa (Fig. 2, lane A) resulted from electrophoresis of a digest of about half of the mtDNA isolated from a 5-g leaf tissue sample; this sample may have been, but probably was not, harvested from but one plant. The pattern of mtDNA from Apex alfalfa was then more probably characteristic of Apex alfalfa in general, and not of but one Apex plant. The Eco RI fragment pattern of mtDNA from Lahontan alfalfa resulted from electrophoresis of about 3/5ths of a digest of mtDNA isolated from a 2.5-g leaf tissue sample; the sample may have come from one plant, and the pattern then may be characteristic of only one plant, rather than of Lahontan alfalfa in general.

For restriction endonuclease fragment analysis of mtDNA to be used more definitively to classify, if possible, alfalfas according to background and distinguish cms from male fertile maintainer alfalfa, it was thought best to harvest leaf tissue samples from more than one alfalfa plant. In the later study, the 5-g leaf tissue samples were harvested from five or more plants (Appendix 1, Step 1 of Isolation of MtDNA) to help ensure that the patterns obtained were characteristic of the cultivar or line under study, and not simply of one plant.

Omission of the extra shake and centrifugation routine in the second and third phenol-chloroform extractions in most isolation procedures followed in the early studies may have reduced fragmentation of the mtDNA and helped in the isolation intact of mtDNA sections giving rise to the higher molecular weight (greater than 7.4 kb) Eco RI bands (Fig. 2). Bands greater in size than 7.4 kb, similar in size to the bands in Fig. 2, were present, however, after analysis of mtDNA isolated following a procedure with four

(submitting the bulk of each lysis solution to four shake and centrifugation routines) phenol-choroform extractions (data not shown).

It is difficult to account for the lack of fragment bands in the attempted Xba I digest of mtDNA from CUF 101 alfalfa, particularly in that Dr. Ray J. Rose also found Xba I only minimally, if at all, to digest mtDNA from Regen S alfalfa; Dr. Rose found Xba I of the same batch, though, would digest cpDNA from Regen S alfalfa (Lowell B. Johnson, personal communication). Though unlikely given the size of mtDNA from alfalfa, possibly mtDNA from CUF 101 and Regen S alfalfa lacks Xba I restriction sites.

The parentage of CUF 101 can be traced to the nine basic alfalfa germplasm sources in approximately the following percentages: M. falcata 0, Ladak 1, M. varia 2, Turkistan 11, Flemish 1, and Chilean 7, Peruvian 2, Indian 23, and African 53. (Lehman et al., 1983). Given the diversity of background of CUF 101 and Regen S alfalfas--CUF 101 being primarily of African and Indian germplasm, and Regen S primarily of Flemish germplasm--perhaps the apparent inability of Xba I to digest mtDNA from CUF 101 and Regen S alfalfa is applicable to mtDNA from alfalfa in general. It would be helpful to know the maternal parentage of CUF 101 alfalfa before drawing conclusions.

## Other Preliminary Work

### Work to Isolate and Analyze MtDNA from Leaf Tissue Samples

Attempts to isolate and analyze mtDNA from leaf tissue samples of alfalfa grown solely under the earlier establishment conditions, i.e., temperature higher than 21 °C, and 18-hour, full light intensity photoperiods in growth chamber #36 (much greater than 600 footcandles), using procedures similar to the one listed in the Appendix 1 were ostensibly unsuccessful. Clear bands did not result after electrophoresis of digests of the putative mtDNA.

Negative results also were obtained in an attempt to isolate and analyze mtDNA from the first growth of USDA alfalfa lines grown from seeds entirely under the later conditions of the successful early studies. Though white pellets were present in the microcentrifuge tubes after lyophilization, no bands were visible after electrophoresis of Eco RI digests of the mtDNA.

### Work to Isolate MtDNA from Etiolated Seedlings

An attempt to isolate mtDNA from 10-g samples of eight day old etiolated alfalfa seedlings using a procedure

similar to the one listed in Appendix 1 was unsuccessful. After treatment with Proteinase K, the lysis solutions were found to be quite viscous, resembling raw egg white. The aqueous layers of the first phenol-chloroform extraction were made up of only small amounts free flowing aqueous solution.

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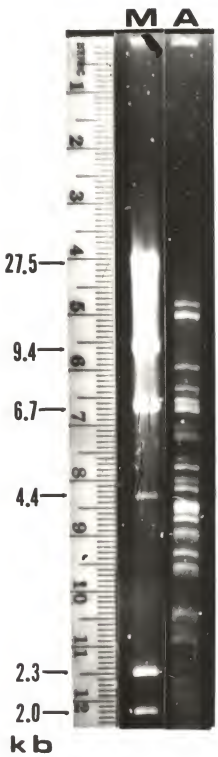
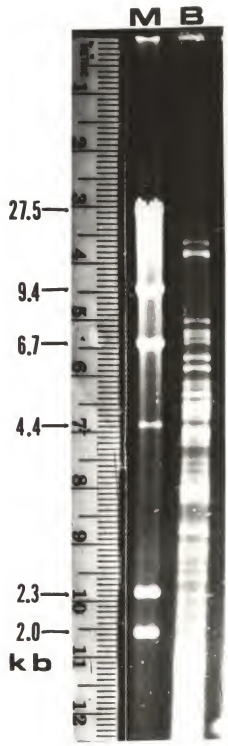
Figure 2. Eco RI fragment patterns of mtDNA from Apex (A) and Lahontan (B) alfalfa of early studies (Appendix 2) following electrophoresis through 0.7% agarose gels. The location and size in kilobase pairs (kb) of Hind III fragments of phage lambda DNA (M) are indicated.


M
A
27.5
9.4
6.7
4.4
2.3
2.0
kb


M
B
27.5
9.4
6.7
4.4
2.3
2.0
kb

# RESTRICTION ENDONUCLEASE FRAGMENT ANALYSIS OF MITOCHONDRIAL DNA FROM MALE FERTILE AND CYTOPLASMIC MALE STERILE ALFALFA (*MEDICAGO SATIVA* L.)

by

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B.A., Saint Louis University, 1981

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AN ABSTACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Genetics

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1986

ABSTRACT

Mitochondrial DNA (mtDNA) was isolated from leaves of male fertile alfalfa (Medicago sativa L.) cultivars and cytoplasmic male sterile (cms) and male fertile maintainer alfalfa lines, of Flemish and Turkistan germplasm sources. Restriction endonuclease fragment analysis was carried out on the isolated mtDNA with the objectives of testing this analysis as a method of:

1) classifying alfalfa according to germplasm source

2) distinguishing cms from male fertile maintainer alfalfa.

Early studies with the restriction endonucleases Xho I and Eco RI yielded clear mtDNA fragment patterns. Gels from a later study, however, did not yield clear patterns with Xho I; only lower molecular weight (generally, less than 7.4 kb) bands were revealed with Eco RI.

In this later study, in contrast to results of the early studies, the Eco RI fragment pattern of mtDNA from the Flemish cultivar (Apex) did not differ from that of the Turkistan cultivar (Lahontan). The Eco RI fragment patterns of mtDNA from the Flemish cms line (MN 1292-CMS) and the Turkistan cms line (MN White-CMS) were indistinguishable. The Eco RI fragment pattern of mtDNA from the Flemish maintainer line (MN 1292-MA), however, did differ slightly from that of the Turkistan maintainer line (MN White-MA); both patterns differed from those of the cms lines.

In the early studies, higher molecular weight bands were generated with Xho I and Eco RI. The Eco RI fragment pattern of mtDNA from the Flemish cultivar (Apex) differed from that of the Turkistan cultivar (Lahontan). For both Xho I and Eco RI, the fragment patterns of mtDNA from the Flemish cms line (MN 1292-CMS) differed from those of the Flemish maintainer line (MN 1292-MA).

The restriction endonuclease Xba I seemingly did not digest mtDNA from the alfalfa cultivar CUF 101.