



National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

ANALYSIS OF THE MITOCHONDRIAL
GENOME OF *LARIX*

BY

LINDA L. DEVERNO

A THESIS SUBMITTED TO
THE FACULTY OF GRADUATE STUDIES AND
RESEARCH

IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF BIOLOGY
UNIVERSITY OF OTTAWA
OTTAWA, ONTARIO, CANADA



National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your No. Votre référence

Our No. Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-80044-5

Canada



UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

The gymnosperms, though composed of fewer genera and species in comparison to the angiosperms, are considered important components of forests worldwide both ecologically and commercially. As descendants of some of the most primitive land plants, conifers have unique physiology and genetics. Coniferous trees, in particular, are known to have had few changes at the chromosomal level during evolution (Pederick, 1970). However, limited information is available on conifer mitochondrial genome complexity, mode of inheritance, and the effect of long term somatic embryogenic cell culture on the organization of this genome. We have isolated both mitochondrial and total genomic DNA from *Larix* species to examine the physical and genetic complexity of mitochondrial DNA and to determine mitochondrial inheritance patterns in *Larix* hybrid crosses, using restriction endonuclease and Southern hybridization analyses with specific wheat mitochondrial gene probes. In addition, these techniques have been used to identify restriction fragment length polymorphisms in somatic embryogenic cell cultures and corresponding regenerated hybrid *Larix* trees.

Molecular hybridizations using specific wheat mitochondrial gene probes demonstrated organizational complexity within the mitochondrial genome of *Larix*. In general, the data suggest that genes found in the mitochondria of other plants are also present in *Larix* mitochondria and that many of these genes are present in more than one copy in the genome, which is in contrast to many other plants where the majority of the genes are in single copy.

Plant cell culture and subsequent regeneration of whole plants may be a process which increases the molecular diversity of the mitochondrial genome. Changes in the mitochondrial genome of *Larix* were detected by observing differences in the hybridization banding patterns of somatic embryogenic cell cultures, trees regenerated from these cell cultures, and trees grown from natural seed. This is the first study to describe molecular

diversity of the mitochondrial genome induced by in vitro somatic embryogenic cell culture of a conifer species. Qualitative and quantitative mitochondrial DNA differences have been demonstrated between a four year old somatic embryogenic cell culture of *Larix X eurolepis*, trees regenerated from this culture, and callus regenerated after cryopreservation of this culture. Changes in the mitochondrial genome occurring during cell culture and regeneration of whole plants may result in undesirable phenotypes of the mature plant such as fertility-related abnormalities, reduced vigor, and poor growth yield.

Evaluation of mitochondrial gene hybridization patterns of two *Larix* species and their reciprocal hybrids demonstrated a maternal mode of inheritance of mitochondrial DNA gene sequences. Conifers appear to have evolved mechanisms for organelle exclusion during fertilization, resulting in a predominantly uniparental mode of inheritance of their organelles (Owens and Morris, 1990). Maternal inheritance of the mitochondrial genome together with paternal inheritance of the chloroplast genome in *Larix* hybrid crosses will be useful in the determination of maternal and paternal contributions in hybrid and introgressed *Larix* populations, and will increase the precision in determining phylogenies of this species.

RÉSUMÉ

Les gymnospermes comprennent moins de genres et d'espèces que les plantes angiospermes. Toutefois, elles sont des composantes importantes des forêts mondiales aux points de vue écologique et commercial. Leurs physiologie et génétique sont uniques lorsqu'elles sont comparées aux angiospermes car elles sont plus anciennes dans l'échelle évolutive. Plus particulièrement, les conifères présentent peu de changements chromosomiques au cours de leur évolution (Pederick, 1970). Cependant, très peu d'information est disponible sur la complexité du génome mitochondrial et de son mode de transmission sexuel, de même que sur son intégrité lors du processus de culture de tissus qu'est l'embryogénèse somatique. Les ADNs mitochondrial et total d'espèces de mélèze ont été analysés pour déterminer la complexité génétique et physique et le mode de transmission du génome mitochondrial. Ceci a été accompli en utilisant les outils de biologie moléculaire que sont les enzymes de restriction, l'hybridation de type Southern et des sondes obtenues à partir du génome mitochondrial du blé. De plus, ces techniques ont permis d'identifier des polymorphismes des sites de restriction spécifiques aux lignées embryogènes cultivées *in vitro* et aux arbres régénérés à partir de ces lignées.

Les hybridations moléculaires avec des sondes génétiques mitochondriales du blé ont permis de caractériser la complexité du génome mitochondrial du mélèze. Généralement, les données suggèrent que les gènes présents chez d'autres espèces sont aussi présents chez le mélèze et que la plupart de ces gènes sont présents en plus d'une copie par génome. Ceci contraste avec la majorité des cas où on les retrouve en copie unique.

La culture de tissus par embryogénèse somatique et la régénération subséquente de plantes entières sembleraient augmenter la diversité moléculaire du génome mitochondrial car différents patrons de restriction ont été observés entre ces tissus et des tissus d'arbres provenant de graines. Ceci est la première étude qui démontre la présence de tels

changements induits par la culture *in vitro* chez le mélèze. Des changements qualitatifs et quantitatifs ont été observés entre une lignée d'embryons somatiques de *Larix X eurolepis* âgée de quatre ans, des arbres régénérés de cette lignée et des cals régénérés après cryoconservation de cette lignée. Ces changements peuvent avoir des conséquences indésirables comme des anomalies de fertilité, une vigueur réduite et un faible taux de croissance.

Les patrons d'hybridation de deux espèces de *Larix* et de leurs hybrides réciproques ont mis en évidence la transmission maternelle du génome mitochondrial. L'évolution des conifères semble avoir résultée en des mécanismes d'exclusion des organelles durant la fertilisation qui donneraient un mode de transmission uniparental des organelles (Owens and Morris, 1990). La transmission maternelle de génome mitochondrial et paternelle du génome chloroplastique des croisements de mélèze et des populations introgressées vont augmenter la précision des phylogénies des espèces du genre *Larix*.

ACKNOWLEDGEMENTS

I would like to thank my thesis supervisor Dr. Linda Bonen of the University of Ottawa, and co-supervisor Dr. Pierre Charest of the Petawawa National Forestry Institute for their continuous support throughout the course of this thesis. I would also like to express my appreciation to Dr. V.N. Iyer of Carleton University and Dr. D. Johnson of the University of Ottawa for their participation on my supervising committee. In addition, I wish to thank Dr. Robert Rutledge for his kind advice and support. I would also like to acknowledge the support and encouragement of Dr. Gordon Murray, Program Director, Forest Genetics and Biotechnology, Petawawa National Forestry Institute, Chalk River, Ontario.

I also acknowledge the technical assistance of Ms. Cathy Jones and Ms. Chris Ward for the growth and maintenance of the cell culture material, and Dr. Krystyna Klimaszewska who initiated the somatic embryogenic cell cultures of *Larix* and produced the regenerated trees. In addition, I would like to thank Mr. Ernie Gilcrest for climbing the *Larix X eurolepis* tree to show that the top of the tree was the same as the bottom, and Mr. Steven Handke for producing the photographic plates. Special thanks are dedicated to the late Mr. Jack Pitel whose commitment and optimistic approach to his work in molecular biology was an example for me to follow.

This work was supported financially by Forestry Canada, Petawawa National Forestry Institute, Chalk River, Ontario, Canada.

DEDICATION

This thesis is dedicated to my son Mark and daughter Michelle, and to my husband Michael. Without their support and encouragement this work could not have been completed.

This thesis is also dedicated to my parents, Laura Dargie and the late Melville Raaflaub, and to my grandmother, Norah Carter, who all encouraged me to seek higher education and strive for the best.

TABLE OF CONTENTS

ABSTRACT.....	ii
RESUME.....	iv
ACKNOWLEDGEMENTS.....	vi
DEDICATION.....	vii
TABLE OF CONTENTS.....	viii
LIST OF TABLES.....	x
LIST OF FIGURES.....	xi
LIST OF ABBREVIATIONS.....	xii
1. INTRODUCTION.....	1
1.1. TAXONOMY OF <i>LARIX</i> (Dallimore and Jackson, 1966).....	3
1.1.1. ISOZYME VARIATIONS IN <i>LARIX</i>	4
1.1.2. MOLECULAR GENETICS OF <i>LARIX</i>	5
1.2. CHARACTERISTICS OF THE CONIFER NUCLEAR GENOME.....	6
1.3. MITOCHONDRIAL GENOME OF HIGHER PLANTS.....	9
1.3.1. Origin and Evolution of Mitochondria.....	9
1.3.2. Size, Organization, Structure, and Conformation of the Genome.....	11
1.3.3. Mitochondrial Genes and Coding Capacity.....	14
1.4. TISSUE CULTURE INDUCED ORGANELLAR GENOME ALTERATIONS.....	16
1.5. INHERITANCE OF MITOCHONDRIA IN HIGHER PLANTS.....	18
1.6. THESIS GOALS.....	21
2. MATERIALS AND METHODS.....	22
2.1. PLANT AND <i>IN VITRO</i> CULTURE MATERIALS.....	22
2.2. PLANT DNA ISOLATION AND ANALYSIS.....	23
2.2.1. Total Genomic DNA Isolation from Tree Needles.....	23
2.2.2. Total Genomic DNA Isolation from Callus Tissue and Cell Cultures of <i>Larix</i>	24
2.2.3. Mitochondrial DNA Isolation from Cell Cultures of <i>Larix</i>	25
2.2.4. Restriction Endonuclease Digestion and Gel Electrophoresis of <i>Larix</i> DNA.....	26
2.2.5. Transfer of DNA to Nylon Membranes.....	26
2.2.6. Preparation of Mitochondrial Gene Probes.....	27
2.2.7. Purification of Wheat Mitochondrial Gene Fragments.....	28
2.2.8. Random Primer Labelling of Mitochondrial Gene Probes.....	29
2.2.9. Southern Hybridization Analysis.....	30
3. RESULTS.....	32
3.1. ISOLATION AND PURIFICATION OF MITOCHONDRIAL DNA FROM <i>LARIX</i> CELL CULTURE.....	32
3.2. PROCEDURES TO MINIMIZE ARTIFACTS.....	35
3.2.1. Restriction Endonuclease Digestion Controls.....	35
3.2.2. Investigation of Hybridization with Vector Sequences.....	36
3.2.3. Hybridization of Wheat Mitochondrial Gene Probes with <i>Larix</i> Total DNA.....	36
3.2.4. Wheat Mitochondrial Gene Clones.....	40
3.3. HYBRIDIZATION OF WHEAT MITOCHONDRIAL GENE PROBES WITH <i>LARIX</i> <i>LEPTOLEPIS</i> TOTAL DNA FROM TREE NEEDLES.....	40
3.4. HYBRIDIZATION PATTERNS OF TREE DNA COMPARED TO <i>IN VITRO</i> MATERIAL AND REGENERATED PLANTS.....	49
3.5. INHERITANCE OF MITOCHONDRIAL DNA SEQUENCES IN <i>LARIX</i> HYBRID CROSSES.....	57
4. DISCUSSION.....	63
4.1. ISOLATION OF MITOCHONDRIAL DNA OF <i>LARIX</i>	63
4.2. GENOME STRUCTURE OF <i>LARIX</i> MITOCHONDRIA.....	65
4.3 TISSUE CULTURE INDUCED VARIATIONS OF THE MITOCHONDRIAL	

GENOME	70
4.4. INHERITANCE OF MITOCHONDRIAL DNA IN <i>LARIX</i>	72
5. CONCLUSION.....	75
REFERENCES.....	79
APPENDIX I.....	100
APPENDIX II.....	101

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
1.1	Species of the genus <i>Larix</i> Mill.....	4
1.1.2	Comparison of gymnosperm and angiosperm gene homologies.....	6
1.2	Gymnosperm nuclear genomes compared to angiosperms.....	8
1.3	Plant mitochondrial genes.....	15
1.5	Organellar inheritance in plants and animals.....	20
2.2	Wheat mitochondrial gene probes used for hybridization experiments.....	27
3.3	Hybridization results for mitochondrial genes of <i>Larix leptolepis</i>	47
3.4	Mitochondrial DNA variation between regenerated trees and cell cultures.....	55
4.2	Comparison of gene copy number in several plant species for genes used in this study.....	70

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE
2.2	Restriction maps of wheat mitochondrial gene probes cloned in pUC vector.....	27b
3.1	Purification and restriction endonuclease digestion of mitochondrial DNA from <i>Larix</i>	33
3.2	Purification of intact mitochondria through a Percoll gradient after DNaseI treatment.....	34
3.3	Effect of <i>Larix</i> total DNA on restriction endonuclease activity.....	37
3.4	Methylation and non-specific hybridization of <i>Larix</i> mitochondrial DNA.....	39
3.5	Hybridization of <i>Larix leptolepis</i> DNA with wheat mitochondrial gene probes <i>atpA</i> and <i>atp9</i>	41
3.6	Hybridization of <i>Larix leptolepis</i> DNA with wheat mitochondrial gene probes <i>nad3/rps12</i> and <i>nad5</i>	43
3.7	Hybridization of <i>Larix leptolepis</i> total DNA and mtDNA with wheat mitochondrial gene probe <i>orf25</i>	45
3.8	Total DNA from <i>Larix</i> species and other gymnosperms hybridized with <i>orf25</i>	46
3.9	Hybridization of <i>Larix leptolepis</i> DNA with wheat mitochondrial gene probes <i>cox1</i> and <i>cob</i>	48
3.10	Comparison of DNA hybridization patterns between <i>Larix</i> trees and cell culture using wheat mitochondrial gene probe <i>atpA/atp9</i>	50
3.11	Comparison of DNA hybridization patterns between <i>Larix</i> trees and cell culture using wheat mitochondrial gene probe <i>nad5</i>	52
3.12	Mitochondrial RFLP analysis of hybrid larch DNA using wheat mitochondrial gene probe <i>atp9</i>	54
3.13	Preliminary investigation of within-tree variation of <i>Larix</i> mitochondrial DNA.....	56
3.14	Identification of RFLPs unique to <i>Larix leptolepis</i> or <i>Larix decidua</i> by hybridization of total genomic DNA with wheat mitochondrial gene probe <i>orf25</i>	58
3.15	Inheritance patterns of hybrid <i>Larix</i> mitochondrial DNA sequences homologous to <i>orf25</i>	59
3.16	Inheritance patterns of hybrid <i>Larix</i> mitochondrial DNA sequences homologous to <i>nad3/rps12</i>	60
3.17	RFLP analysis of <i>Larix</i> cell culture DNA by hybridization with wheat mitochondrial gene probes <i>orf25</i> and <i>nad3/rps12</i>	62

LIST OF ABBREVIATIONS

%	percent
ATP	adenosine triphosphate
ATPase	ATP synthase
cm	centimeter
cpDNA	chloroplast DNA
CsCl	cesium chloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
g	gram
G	centrifugal force
G+C	guanine plus cytosine
HCl	hydrochloric acid
kb	kilobase pair
L	litre
M	molar
m	metre
mg	milligram
mJoules	milliJoules
mM	millimolar
mtDNA	mitochondrial DNA
mw	molecular weight

Na-	sodium-
NaCl	sodium chloride
NaOH	sodium hydroxide
°C	degrees Celsius
OD	optical density
pg	picograms
pH	negative log hydrogen ion concentration
PNFI	Petawawa National Forestry Institute
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
μ-	micro-
V	volts
v	volume
w	weight
X	genetic cross

Some specific abbreviations are explained in the text.

1. INTRODUCTION

Conifers are important forest species all over the world and are descendants of some of the most primitive land plants, with unique physiology and genetics. Thus, only a limited amount of the molecular genetic information gained from the study of agricultural crops can be applied directly to conifers. Until conifer genes and genomes are better understood, it will be difficult to exploit superior trees for the production of new and commercially beneficial genotypes. There is an increasing interest in using the conifer tree species *Larix* in reforestation programs as well as in the forest products industry (Fowler, 1986). Larches (*Larix* species) are known for their rapid juvenile height growth and adaptation to marginal sites, especially those that are excessively moist. These traits make larch a potentially valuable reforestation species (Park and Fowler, 1983). Certain larch hybrids exhibit superior growth rates compared to their parental species over a wide range of sites. The most promising hybrids are those between Japanese larch (*Larix leptolepis*) and European larch (*L. decidua*) [Fowler, 1986]. Research has been conducted on *Larix* species at the Petawawa National Forestry Institute (PNFI), Chalk River, Ontario, Canada since 1926. Experimental results show that the larches have excellent potential as short rotation crops on fertile sites. Under such conditions, in eastern Canada, the yield often exceeds that of the native spruces (Boyle *et al.*, 1989). In addition, somatic embryogenic cultures of *Larix* species and their hybrids have been initiated and maintained at PNFI since 1987 (Klimaszewska, 1989). Development of a method of *in vitro* embryogenesis for conifers was a significant breakthrough because no other practical method is available for the regeneration of conifer plantlets from protoplast or cell culture.

In higher plants there is a sophisticated coordination of gene expression from three different organellar compartments: nucleus, chloroplast, and mitochondrion. Inheritance of organelle genomes in conifers is different from that in angiosperms. Certain conifers appear

to have evolved mechanisms to exclude the maternal transmission of plastids and the paternal transmission of mitochondria leading to uniparental inheritance of organelles (Neale and Sederoff, 1989). The maternal inheritance of one organelle genome and the paternal inheritance of the other genome presents a unique opportunity for evolutionary and population evaluations. This genetic system makes it possible to determine and compare maternal and paternal lineages in the same tree. Independent maternal and paternal phylogenies in conifers can be constructed which could be more informative than either one alone. However, more data are needed on the inheritance and variability of mitochondrial DNA (mtDNA) in conifers before mtDNA polymorphisms can be used for phylogenetic analysis.

Genetic information contained in mitochondrial DNA and its organization has been studied in a wide range of plant species (Birky, 1988; Levings and Brown, 1989; Lonsdale, 1984; Newton, 1988; for reviews). However, information on the organization of the mitochondrial genome in conifers is limited, in spite of the ecological and commercial importance of these tree species. Until recently (Hakman *et al.*, 1985), conifer trees were considered to be more difficult to regenerate from *in vitro* cell culture than other forest trees. Now however, somatic embryogenesis, plantlet regeneration from cells, and genetic transformation techniques are available for genetic manipulation of conifers. Studies of the mtDNA of conifers should include an examination of both the natural tree and the *in vitro* cell culture material since alterations of conifer genomes in the tissue culture system are important to the understanding of the processes of induction, embryogenesis, maturation, somaclonal variation, and regeneration of *in vitro* propagated conifer trees.

Now there is available a rapidly growing conifer culture system that can be manipulated to facilitate research into conifer genomes using molecular genetics. Although some data are available at the molecular level for the chloroplast genome of *Larix* (Szmídt *et al.*, 1987), including limited sequence data (Bousquet *et al.*, 1990; Hutchison *et al.*, 1990b), little is available yet for the mitochondrial genome of *Larix*.

1.1. TAXONOMY OF LARIX (Dallimore and Jackson, 1966)

DIVISION: Spermatophyta

CLASS: Gymnospermae

ORDER: Coniferales

FAMILY: Pinaceae (Pine Family)

GENUS: *Larix*

The Spermatophytes, or seed plants, differ from Pteridophytes by the absence of easily observed alternation of generations. More obviously, Spermatophytes produce flowers and seeds. The Spermatophyta consists of two subdivisions, the Gymnospermae and the Angiospermae. The gymnosperms differ from angiosperms by having ovules that are not enclosed in an ovary but are borne naked on the scale of a cone or carpel. Their flowers are extremely simple, consisting of just ovules and pollen sacs - no sepals, petals, stamens or stigmas. In addition, resin canals are evident in the stems and there are no vessels in the secondary wood.

Gymnosperms are the remains of a widely varied group of plants that dominated world flora in Mesozoic times. They are an important part of today's flora and are abundant in forests in temperate regions both north and south of the equator. The living Gymnosperms, numbering about 675 species, are divided into four orders - Ginkgoales, Cycadales, Taxales, and Coniferales.

Within Coniferales (Family Pinaceae) the genus *Larix* Mill. has ten recognized species (Dallimore and Jackson, 1966) distributed throughout the northern hemisphere. As shown in Table 1.1, four of these originate from southeast Asia, three from North America, two from the Commonwealth of Independent States (CIS; formerly USSR) and one from Europe (Rauter and Graham, 1983). These deciduous larches are notable for their superior strength, rapid growth and resistance to decay (Schlich, 1904). All of the larches vary considerably at the individual tree level, which exceeds the variation between populations. This between tree variation occurs in traits of economic importance, such as wood properties and form characteristics, that are under strong genetic control (Rauter and Graham, 1983).

TABLE 1.1. Species of the genus *Larix* Mill.

SPECIES	ORIGIN
<i>Larix mastersiana</i> Rehder and Wilson <i>L. griffithii</i> Hook <i>L. potaninii</i> Batal. <i>L. leptolepis</i> (Sieb. and Zucc.) Gord.	Southeast Asia
<i>L. gmelinii</i> (Rupr.) Kuzeneva <i>L. sibirica</i> Ledeb.	CIS
<i>L. decidua</i> Mill.	Europe
<i>L. laricina</i> (Du Roi) K. Koch <i>L. occidentalis</i> Nutt. <i>L. lyallii</i> Parl.	North America

1.1.1. ISOZYME VARIATIONS IN *LARIX*

Isozymes are multiple forms of an enzyme having similar or identical catalytic activities, which differ by the charge, size and shape of their protein and by their specific properties in metabolic regulation (Feret and Bergmann, 1976).

Variation in allozyme patterns may result from different evolutionary forces than those influencing morphological and physiological traits (Wilson *et al.*, 1977). Although studies of forest tree species have shown similar patterns of diversity in comparisons of allozyme and morphological data (Copes, 1981; Millar, 1983; Steinhoff *et al.*, 1983), other studies have shown different patterns of genetic structure (Wheeler and Guries, 1982). Dissimilar patterns of variation suggest that neither type of study alone is sufficient to describe the genetic structure of a species.

Isozyme data are available for only two larch species, *L. occidentalis* and *L. laricina*. For all measures of variation, *L. occidentalis* scored lower than most species, but within the range observed for other western conifers. Most variation was found within, rather than between, populations grouped by geographic origin (Fins and Seeb, 1986). The consistently

low values for genetic variation suggest that genetic drift has played a major role in the genetic history of the species, both through its glacial history of refugia and fire history in recent times, resulting in genetic bottlenecks. In addition, the results of a study of *L. laricina* indicated that the species has genetic variation comparable with that of other woody perennials with extensive transcontinental ranges (Cheliak *et al.* 1988). On average, populations of this species were more differentiated and genetically divergent from one another compared with randomly mated tree species. All these data suggest that populations of *L. laricina*, which are highly variable genetically when considered individually, could be more differentiated than populations of other species of more continuously distributed conifers.

1.1.2 MOLECULAR GENETICS OF *LARIX*

The coniferous species represent some of our major crop plants, yet little is known about the molecular genetic mechanisms governing the regulation of gene expression and growth. The molecular data available for the larches is limited. Restriction enzyme analysis of chloroplast DNA (cpDNA) isolated from European and Japanese larch and their reciprocal hybrids was used to determine the inheritance of cpDNA in *Larix* (Szmidt *et al.*, 1987). The cpDNA fragment patterns generated by *Bam*HI and *Bcl*II were species specific. Paternal inheritance of cpDNA patterns was observed in most *Larix* crosses. However, one hybrid showed maternal cpDNA patterns. In addition, two other hybrids had mixed *Bam*HI patterns. These results are in contrast to the maternal inheritance of cpDNA observed in many angiosperms.

The complete nucleotide sequence of the chloroplast gene coding for the large subunit of ribulose 1,5-bisphosphate carboxylase (*rbc*L) was determined for 9 species in the genera *Abies*, *Larix*, *Picea*, *Pinus* and *Pseudotsuga* using the polymerase chain reaction (PCR) and direct sequencing (Bousquet *et al.*, 1990). On average, DNA sequences were 97% identical in these species, with transitions being almost three times as numerous as

transversions. In addition, the nucleotide sequence of the small subunit of ribulose 1,5-bisphosphate carboxylase (*rbcS*) was determined for *Larix laricina* (Hutchison *et al.*: 1990b). This gene of larch is highly conserved relative to other plants, with the greatest similarity with the *Pinus tunbergii* protein and least similarity to the proteins from monocotyledonous plants. Comparisons were made to gene sequences from pine, pea, tobacco, sunflower, soybean, *Flaveria*, maize and wheat (see Table 1.1.2).

An analysis of the chlorophyll a/b binding protein (*cab*) nuclear gene family during maturation in *Larix laricina* indicates that this group of genes is differentially expressed between juvenile and mature plants (Hutchison *et al.*; 1990a). There appear to be at least five different members of the *cab* gene family expressed in larch, and all members are differentially expressed between juvenile and mature trees. Several conifer genes have recently been sequenced and these sequences have been compared to the corresponding genes of angiosperms. A comparison of gene homologies is presented in Table 1.1.2.

Table 1.1.2: Comparison of gymnosperm and angiosperm gene homologies.

Gymnosperm	Gene	Encoded By	Percent Homology to Gene in Angiosperms	Reference
<i>Larix laricina</i>	<i>rbcS</i>	nucleus	57-62%	Hutchison <i>et al.</i> , 1990b
<i>Pinus contorta</i>	actin	nucleus	85%	Kenny <i>et al.</i> , 1988
<i>Pinus contorta</i>	<i>psbA</i>	chloroplast	87%	Lidholm <i>et al.</i> , 1988
<i>Pinus contorta</i>	<i>gidA</i>	chloroplast	77%	Lidholm and Gustafsson, 1991
<i>Pinus radiata</i>	5S rRNA	nucleus	83-91%	Cullis <i>et al.</i> , 1988
<i>Pinus sylvestris</i>	<i>cab</i>	nucleus	75-87%	Jansson and Gustafsson, 1990
<i>Pseudotsuga menziesii</i>	<i>rbcL</i>	chloroplast	86%	Hipkins <i>et al.</i> , 1990
<i>Thuja plicata</i>	<i>coxI</i>	mitochondrial	88%	Glaubitz and Carlson, 1990

1.2. CHARACTERISTICS OF THE CONIFER NUCLEAR GENOME

The DNA content per cell is much higher in gymnosperms than in angiosperms (Miksche, 1967). The DNA content per haploid genome can vary from about 6pg to 40pg,

but the %G+C is relatively constant at 37-38% (Table 1.2). However, the gymnosperms are cytogenetically extremely stable in comparison to angiosperms. Such stability is also found in their habitat preferences, morphology, and pollination and reproductive systems (Khoshoo, 1960). Polyploidy is totally absent in four Orders of gymnosperms - including Cycadales and Ginkgoales. Almost all conifers are diploid, with the exception of coast redwood (*Sequoia sempervirens* (D. Don) Endl.) which is hexaploid. There are two solitary cases of polyploid conifer individuals: one *Larix decidua* and one *Juniperus virginiana*. Both of these trees survived because they occupied protected habitats where selective forces were absent (Khoshoo, 1960). In gymnosperms it appears that the change in the equilibrium by polyploidy results in weak and slow-growing individuals which have no selective advantage, because in nature fast-growing individuals are favoured.

Karyotypic changes in the evolution and differentiation of gymnosperms, and in conifers in particular, involve various processes such as translocations, fragmentation-fusion processes, segmental interchanges, peri- and paracentric inversions and cryptic structural hybridity (Khoshoo, 1960). These have resulted in alterations in number and morphology, and repatterning of chromosomes without significant changes in the karyotype. In general, however, cytological data follow the taxonomic groupings and in the various families (except Cycadaceae and Podocarpaceae) a single basic number can be recognized.

The decrease in chromosome number and absolute size of chromosomes of a complement seems to be generally correlated with the phylogenetic advancement in conifers. Similarly, the increase in asymmetry can also be correlated in some cases with specialization in habitat, and morphology (Khoshoo, 1960). Since conifers have had few changes in chromosome number and structure during evolution this may suggest that gene mutation played a major role in speciation (Pederick, 1970). Further evidence for genetically conservative conifer chromosome evolution is the similarity among conifer karyotypes (Pederick, 1970; Saylor, 1972). The karyotype of all *Pinus* species studied comprises twelve long chromosomes, eleven of which are metacentric and all of similar length. The twelfth

chromosome is shorter with a submedian centromere.

There are at least three possibilities that could explain the large DNA content of conifers. Conifers may have a higher content of repetitive DNA than other plants. However, in general, the repetitive DNA fraction in conifers is on average 75%, and the single and low copy fraction is about 25%, proportions that are similar to plants with much smaller genomes (Dhillon, 1987). Thus, the extra amount of DNA in conifers, relative to other plants, can not be explained entirely by additional highly repetitive DNA. Other possible explanations for the abundance of DNA in conifers, as compared to other plants, are extra single copy DNA, or an increase in the amount of middle or low repetitive DNA, for example certain ribosomal RNA (rRNA) genes (Cullis *et al.*, 1988).

TABLE 1.2: Gymnosperm Nuclear Genomes Compared to Angiosperms.

SPECIES	%G+C	pg DNA/haploid nucleus	Reference
<i>Juniperus virginiana</i>	37	9.7	Dhillon, 1987
<i>Larix laricina</i>	N/A	9.5	Dhillon, 1987
<i>Larix decidua</i>	N/A	9.8	Dhillon, 1987
<i>Picea glauca</i>	37	8.5	Dhillon, 1987
<i>Picea mariana</i>	38	11	Dhillon, 1987
<i>Pinus banksiana</i>	37	15	Dhillon, 1987
<i>Pinus resinosa</i>	37	20.5	Dhillon, 1987
<i>Tsuga canadensis</i>	37	13.5	Dhillon, 1987
<i>Thuja occidentalis</i>	37	6.3	Dhillon, 1987
<i>Sequoia sempervirens</i>	N/A	6.3	Price <i>et al.</i> , 1974
<i>Zamia pseudo-parasitica</i>	N/A	40	Price <i>et al.</i> , 1974
<i>Cycas circinalis</i>	N/A	25	Price <i>et al.</i> , 1974
<i>Ginkgo biloba</i>	N/A	13.5	Price <i>et al.</i> , 1974
<i>Populus deltoides</i>	34	0.55	Dhillon, 1987
Tobacco	39	3.9	Rogers & Bendich, 1985
Wheat	46	18.1	Flavell <i>et al.</i> , 1974

N/A - not available

1.3. MITOCHONDRIAL GENOME OF HIGHER PLANTS

Almost all eucaryotes contain mitochondria, although over a thousand species of protozoa and a few fungi have no mitochondria at all (Cavalier-Smith, 1987). Mitochondria are self-duplicating organelles that increase their numbers by undergoing division in a manner similar to the binary fission of bacteria and have the machinery necessary for replicating their DNA. Mitochondria are the sites of ATP production linked to oxygen consumption and, especially by virtue of their possession of the Krebs cycle enzymes, they play important roles in many metabolic pathways, including the breakdown and synthesis of carbohydrates, fats, and amino acids. Mitochondria contain their own DNA in linear and/or circular form, transcription and translation apparatus and synthesize a relatively small number of polypeptides essential to their role in energy production. Mitochondria are under the control of two sets of genes: those in the mitochondrion and genes in the nucleus.

1.3.1. Origin and Evolution of Mitochondria

The endosymbiotic origin of mitochondria is now generally accepted and supported by ribosomal RNA (rRNA) sequence comparisons (Gray, 1988). Plant mitochondrial ribosomal RNA genes diverge in sequence at a slow rate which has enabled the ancestry of the mitochondrion to be traced to the alpha subdivision of the purple bacteria by sequence comparisons of small subunit rRNAs (Yang *et al.*, 1985). The alpha subdivision contains procaryotes such as the rhizobacteria, the agrobacteria, and the rickettsias, all of which have developed intra- or intercellular relationships with eucaryotic cells. Ultrastructural and biochemical analysis of the plant mitochondrial electron transport chain also links the origin of mitochondria to the alpha subdivision of the purple bacteria (John and Whatley, 1975).

Since the basic function of mtDNA is similar in all eucaryotes, a single endosymbiotic event provides the simplest explanation (Attardi and Schatz, 1988). However, the diversity in size, structure, and arrangement of the mitochondrial genome suggests a complex evolutionary history. The plant mitochondrial genome appears to be a

mosaic of genes derived from different sources at different times during evolution, since plant mtDNA can incorporate sequences of chloroplast and nuclear DNA, and the nuclear DNA can incorporate sequences of mitochondria and chloroplast DNA (Gray, 1988).

The migration of genetic information from ancestral procaryotic endosymbionts into eucaryotic nuclei is thought to have an important role in the evolution of mitochondria and chloroplasts. The major direction of movement of genetic information within the cell during evolution has been from the energy-converting organelles, chloroplasts and mitochondria, to the nucleus which is specialized in information storage and retrieval (Schuster and Brennicke, 1986). Sequence transfer during evolution is suggested by the different gene complements encoded in the present day mitochondrial genomes of different species. For example, the alpha-subunit of ATPase is encoded in the mitochondrial genome of plants, but specified by nuclear genes in most other species (Lonsdale, 1989).

Inter-organellar transfer of genetic material from the plastid to the mitochondrion has occurred on a large scale (Schuster and Brennicke, 1988, for review). Plant mitochondrial genomes of many species contain chloroplast DNA sequences that are usually not expressed because they are truncated or incomplete. Transfer of DNA from the mitochondrion to the plastid genome does not appear to occur at a detectable level, but nuclear-encoded information has been observed to be transferred to the mitochondrion and integrated into the mitochondrial genome (Schuster and Brennicke, 1988). Therefore, the plant mitochondrial genome may be considered an evolutionary mosaic having acquired genetic information from various sources during evolution.

The rates of evolution of DNA sequences of higher plants depend in part on whether the sequences are located in the nuclear, chloroplast, or mitochondrial genome. In angiosperms, mitochondrial DNA sequences evolve at least 5 times more slowly than nuclear sequences, with transitions comprising less than 50% of the substitutions in mitochondrial genes (Wolfe *et al.*, 1987). This is in contrast to the mammalian mitochondrial genome where mtDNA sequences evolve at least 5 times faster than nuclear sequences, with

transitions making up about 90% of the differences between closely related mtDNA sequences (Brown *et al.*, 1982). In addition, plant mitochondrial genomes frequently undergo rearrangements, and vary in size and organization. The mitochondrial DNAs of *Brassica* and *Raphanus* species have undergone numerous internal rearrangements, but the point mutation rate is about four times slower than that of chloroplast DNA, and a hundred times slower than animal mtDNA. The high rate of rearrangements of plant mtDNA was attributed to the abundance of short dispersed repeats, large intergenic spacers, and the presence of sublimons (Palmer and Herbon, 1988). In contrast, the chloroplast genome is highly conserved in size and gene arrangement, and cpDNA sequences evolve at about only half the rate of plant nuclear DNA sequences suggesting that the chloroplast genome evolves slowly (Wolfe *et al.*, 1987). Comparison of plant mitochondrial, chloroplast and nuclear DNA sequences shows that the silent substitution rate in mitochondrial DNA is less than one third that in chloroplast DNA and about one sixth that of nuclear DNA; this slower rate in mtDNA is probably due to a lower point mutation rate (Wolfe *et al.*, 1987).

1.3.2. Size, Organization, Structure, and Conformation of the Genome

Higher plant mitochondrial genomes are larger and more complex than those of other organisms. The genome size varies from about 200kb in *Brassica* species to 2500kb in muskmelon, which is in contrast to the smaller genomes of mammals (15-18kb) and fungi (18-78kb) (Eckenrode and Levings, 1986; Newton, 1988). However, mitochondrial DNA from higher plants has a uniform buoyant density in the range of 1.705-1.707gcm³ for widely divergent species. This buoyant density corresponds to approximately 47% G+C content.

Electron microscopic studies of higher plant mitochondrial genomes reveal predominantly large linear molecules and lesser amounts of open circular and covalently closed circular molecules (Dale, 1981). The circular molecules were often heterogeneous in size and relative abundance and have not been correlated to the size of either the proposed

master chromosome or the predicted circular subgenomic molecules of any higher plant species. Molecular heterogeneity appears widespread among plants, since variation in size and relative abundance is reported in maize, potato, *Oenothera*, virginia creeper, tobacco, flax and sugar beets (Levings, 1983). Minicircles and/or miniliners (less than 2.5kb) are prevalent and are commonly visualized by gel electrophoresis in many species, e.g. maize, tobacco, beans, teosinite.

Recently, the structure of the mitochondrial genome in plants has been investigated by using fluorescence microscopy to make moving pictures of ethidium-stained DNA fractionated by pulsed-field gel electrophoresis (PFGE) and emerging from organelles lysed within gelled agarose (Bendich and Smith, 1990; Bendich, 1991). Most of the DNA was in the form of large linear molecules (50-100kb, 1200kb) and no circular molecules were identified. CHEF and Eckhardt gels have also been used to study the physical structure of mitochondrial DNA from tissue cultured cells of maize (Levy *et al.*, 1991). The proposed 500-600kb master chromosome of maize mitochondria was not detected, although three circular supercoiled molecules (70, 115, 120kb) were identified.

The complexity of plant mitochondrial genomes is apparent in the restriction endonuclease patterns of the mtDNA, being characterized by a large number of fragments of varying stoichiometries. This was first shown by Quetier and Vedel (1977) for a variety of plant mtDNAs and with different restriction endonucleases, and has been reproduced consistently with other plant mtDNAs. The isoschizomers *MspI* and *HpaII* can distinguish between the presence or absence of methylation at the internal C residue in the sequence CCGG, and since these enzymes produce identical restriction patterns with *Triticum aestivum* mtDNA, it is unlikely that site-specific partial methylation is the explanation for the observed complexity of restriction patterns (Ward *et al.*, 1981; Bonen *et al.*, 1980). The complex restriction patterns therefore can be interpreted as a reflection of heterogeneity in the mtDNA population.

Heterogeneity was observed in the small mitochondrial genome of *Brassica* (Palmer

and Shields, 1984). A tripartite model of the *Brassica* mitochondrial genome was postulated, in which a master chromosome can recombine via homologous recombination between direct repeats to produce smaller circular molecules. The more complex maize (Lonsdale *et al.*, 1984) and wheat (Quetier *et al.*, 1985) mitochondrial genomes are also proposed to be organized as multiple circular molecules and recombination between repeated sequences could also account for the circular molecules of various sizes.

Lonsdale (1984) has proposed a general model for angiosperm mitochondrial DNA to explain the existence of the multiple sizes of mitochondrial molecules. In the suggested 'homogeneous genome' there is a circular 'master chromosome' that contains inverted and directly repeated sequences; sub-genomic circles are derived by crossing-over and may combine to reconstitute the master circle or yield smaller sub-genomes by subdivision. In the 'heterogeneous genome' there are small circular and linear species which may or may not have sequence homology to the master chromosome. Lonsdale *et al.* (1984) have analyzed the mitochondrial genome of maize and found that the entire sequence complexity can be represented on a master circle of 570kb which has six repeated sequences. All the repeats, except one, are in direct orientation, which predicts a large number of subgenomic circles. Tripartite structures have been reported for spinach and *Brassica* mitochondria (Stern and Palmer, 1986; Palmer and Shields, 1984)). Genomic organization is more complex in wheat where the mitochondrial genome contains at least ten repeats (Quetier *et al.*, 1985). However, recent physical evidence does not support the proposed 'homogeneous genome' model since no genome sized molecules have been identified, and circular molecules exist which could not be predicted by recombination between repeated sequences (Levy *et al.*, 1991; Bendich, 1991; Bendich and Smith, 1990). *Brassica hirta* has no recombination repeats (Palmer and Herbon, 1987), and likewise the mitochondrial genome of *Marchantia* has been completely sequenced and appears to be a single circular molecule of about 180kb in size, with no recombination repeats, containing 93 possible genes (Ohyama *et al.*, 1991).

In addition to the master chromosome and the subgenomic molecules, linear and

circular DNA plasmids and double-stranded RNAs have been reported in many higher plant mitochondria (reviewed by Pring and Lonsdale, 1985). Linear plasmids have been identified in several higher plant species including *Brassica*, *Sorghum*, and *Zea* (Palmer *et al.*, 1983; Chase and Pring, 1986; Levings and Sederoff, 1983). Small circular DNA species, sometimes referred to as plasmids or minicircular DNAs (Sederoff 1984; Pring and Lonsdale, 1985), are the most abundant extrachromosomal elements associated with plant mitochondria and have been identified in several plant species, including *Zea*, *Beta*, *Phaseolus*, *Vicia*, *Helianthus*, *Sorghum*, *Lupin*, and *Oryza* (Kemble and Bedbrook, 1980; Powling, 1981; Goblet *et al.*, 1983; Leroy *et al.*, 1985; Chase and Pring, 1985; Goraczniak and Augustyniak, 1989; Yamaguchi and Kakiuchi, 1983).

1.3.3. Mitochondrial Genes and Coding Capacity

The large size of plant mitochondrial genomes (200-2500kb) compared to mitochondrial genomes of other organisms (e.g. 16kb for human) could allow coding for extra genes, multiple copies of genes, repetitive DNA, or more introns. However, there appears to be no correlation of mitochondrial genome size with highly repetitive sequences (Ward *et al.*, 1981), mitochondrial volume (Bendich and Gauriloff, 1984), or the number of detectable translation products (Stern and Newton, 1986). Most mitochondrial proteins are encoded by the nucleus, synthesized on cytoplasmic ribosomes and transported post-translationally into the mitochondria (Tzagoloff and Myers, 1986). However, mitochondria have their own protein synthesis apparatus, and the mtDNA codes for a small number of polypeptides as well as for mitochondrial ribosomal RNAs and transfer RNAs (Grivell, 1983). Mitochondrial ribosomes in higher plants differ from those of animals and fungi. They contain a 5S ribosomal RNA and they have 18S and 26S rRNAs, which are larger than those found in human or yeast mitochondria (Newton, 1988). Numerous transfer RNA genes have been identified in higher plant mtDNA (Newton, 1988), and a number of mitochondrial genes encoding small subunit ribosomal proteins have been reported in plants, *rps4*, *rps13*,

and *rps14*, and others (Bland *et al.*, 1986; Schuster *et al.*, 1988; Wahleithner and Wolstenholme, 1988). Almost all mitochondrial genomes examined encode at least subunits I, II, and III of the cytochrome c oxidase complex, subunit 6 of the ATPase complex, and the apocytochrome b of the bc₁ complex (Tzagoloff, 1982). See Table 1.3 for a list of plant mitochondrial genes, and specific information for the genes used in this thesis.

TABLE 1.3: Plant Mitochondrial Genes.

Genes	Larix study	copy #	coding region (base pairs)	% nucleotide homology among plants	introns present	Reference
<i>atp9</i>	+	1 or more	222-240	81-100%	-	a
<i>atpA</i>	+	1 or 2	1521-1530	91-97%	-	b
<i>cob</i>	+	1	1164-1194	98%	-	c
<i>coxI</i>	+	1 or 2	1581-1593	91-99%	-	d
<i>nad3/rps12</i>	+	1		>90%	-	e
<i>nad5</i>	+	1 or 2	3082-4178	95%	+	f
<i>orf25</i>	+	1	576	87-93%	-	g
5S,18S,26S rRNA	-					h
<i>atp6</i>	-					i
<i>rps3,13,14,16,19</i>	-					j
<i>coxII,III</i>	-					k
<i>nad1,2,4</i>	-					l
<i>orfS</i>	-					m
REP	-					n
RT	-					o
tRNA (various)	-					p

a. Grabau *et al.*,1990; Wahleithner & Wolstenholme,1988; Young *et al.*,1986; Kalcikau *et al.*,1990b; Morikami & Nakamura,1987b; Bonhomme *et al.*,1989; Dewey *et al.*,1985; Schulte *et al.*,1989; Begu *et al.*,1989a; Xie & Wu,1990; Kazama *et al.*,1990; Recipon,1990.

b. Schuster & Brennicke, 1986; Begu *et al.*, 1989b; Schulte *et al.*, 1989; Morikami & Nakamura, 1987a; Handa & Nakajima, 1991; Kohler *et al.*, 1990; Kadowaki *et al.*, 1990; Chaumont *et al.*, 1988.; Braun & Levings, 1985; Isaac *et al.*, 1985. c. Dawson *et al.*, 1984; Schuster & Brennicke, 1985; Boer *et al.*, 1985; Kalcikau *et al.*, 1990. d. Grabau,1986; Kemmerer *et al.*,1989; Isaac *et al.*,1985; Hiesel *et al.*,1987; Makaroff *et al.*,1991; Bonen *et al.*,1987; Kadowaki *et al.*,1989. e. Gualberto *et al.*, 1988; Suzuki *et al.*, 1991. f. Wissinger *et al.*, 1988; Ecke *et al.*, 1990. g. Dewey *et al.*, 1986; Bonen *et al.*, 1990; Stamper *et al.*, 1987. h. Spencer *et al.*,1984; Falconet *et al.*, 1985,1986; Gywnn, 1987; Chao *et al.*, 1984. l. Bonen & Bird,1988. j. Bonen, 1987; Schuster *et al.*, 1990; Hunt & Newton, 1991; Conklin & Hanson, 1991. k. Bonen *et al.*, 1984; Gualberto *et al.*, 1990. i. Chapdelaine & Bonen, 1991.Wintz *et al.*, 1984. p. Joyce *et al.*, 1988; Bird *et al.*,1989.

1.4. TISSUE CULTURE INDUCED ORGANELLAR GENOME ALTERATIONS

The process of higher plant tissue culture and subsequent regeneration of plantlets should result in the production of clones that are phenotypically and genetically identical to the original material. However, the passage of cells through *in vitro* culture induces a wide range of variation in many plant species, a phenomenon termed somaclonal variation (Larkin and Scowcroft, 1981). Changes in morphology, biochemical alterations and chromosomal variation arising from tissue culture have been documented (Schaeffer, 1982; Armstrong and Phillips, 1988). As well, changes in methylation and chromosome number and structure have been detected (Edallo *et al.*, 1981; Prat, 1983; Evans and Sharp, 1983; Muller *et al.*, 1990; Grisvard *et al.*, 1990; Lewis-Smith *et al.*, 1990). However, as differentiation and organogenesis occur, the level of DNA variation appears to decrease, and most of the regenerated plants show genetic similarity to the controls (Brown *et al.*, 1991). *In vitro* culture conditions appear to affect the stability of plant genomes, with plant species and genotypes responding differently. Somaclonal variation may provide a valuable source of genetic variants for plant breeding purposes, but is undesirable when genetic stability for production of cloned plantlets is required.

Although the majority of these genetic changes occur in the nuclear genome, analyses of plant mitochondrial DNA (and a few examples of chloroplast DNA) indicate that *in vitro* cell culture induces structural changes (i.e. inversions, deletions/insertions, and transpositions) in the organization of the organellar genomes. Mitochondrial recombination in somatic hybrids of higher plants was first suggested by Belliard *et al.* (1979), where a study of two varieties of tobacco with different cytoplasms displayed distinctly different mitochondrial DNA restriction patterns, with new bands which could not be attributed to either parent. Altered mitochondrial restriction patterns, probably due to mitochondrial recombination or amplification of subgenomic molecules during cell culture, have been found in other somatic hybrids or cybrids including tobacco (Nagy *et al.*, 1981; Galun *et al.*, 1982), *Petunia* (Boeshore *et al.*, 1983), *Daucus* (Matthew and Widholm, 1985) and

Pennisetum X Panicum (Ozias-Akins *et al.*, 1987). Appearance of novel restriction fragments, disappearance of subgenomic DNA molecules, and quantitative variation in some restriction fragments encompassing a given recombinatory repeat have also been reported in studies of the mitochondrial genome of *Triticum* (Hartman *et al.*, 1987; Rode *et al.*, 1987). Furthermore, differences in the organization of the mitochondrial genome between *Triticum* embryogenic and non-embryogenic callus cultures have been detected, suggesting that a particular mitochondrial genome organization may be correlated with the ability of cultured wheat cells to regenerate whole plants (Rode *et al.*, 1988). Alterations of mtDNA due to rearrangements have been reported for *Brassica* (Shirzadegan *et al.*, 1989) and sugar beet (Brears *et al.*, 1989). Variations in relative abundance of certain mtDNA sequences, i.e. variations in stoichiometry of fragments, have been reported for rice (Saleh *et al.*, 1990) and *Chenopodium* (Dorfel *et al.*, 1989).

Unique mitochondrial genomic organizations were determined to be associated with long-term *in vitro* culture in *Oryza sativa* (Chowdhury *et al.*, 1990), *Triticum aestivum* (Hartman *et al.*, 1989), and *Brassica campestris* (Shirzadegan *et al.*, 1991). The rearranged forms of these mitochondrial genomes were found to increase and decrease in varying proportions with continued culturing. The longer the duration of the *in vitro* step prior to regeneration, the higher the probability of obtaining mitochondrial DNA variability in regenerated plants. It has been proposed that the rapid structural alterations *in vitro* result from preferential amplification and reassortment of minor pre-existing forms of the genome, caused by recombination between short dispersed repeated elements, rather than *de novo* rearrangement. There are examples where comparisons of whole plants and cultured cells have not revealed differences in the mitochondrial DNA of carrot, petunia, and *Brassica* (DeBonte and Matthews, 1984; Matthews and DeBonte, 1985; Kool *et al.*, 1985; Kemble *et al.*, 1988).

Generally, the chloroplast genome is more stable than the mitochondrial genome, but has also been shown to be altered during *in vitro* culture. Day and Ellis (1984, 1985) have

reported deletions in the plastid DNA of albino wheat plantlets regenerated from anther culture as well as barley anther culture. Large-scale deletions of the plastid genome has been reported for albino rice plants regenerated from anther culture (Harada *et al.*, 1991).

1.5. INHERITANCE OF MITOCHONDRIA IN HIGHER PLANTS

Genetic information contained within mitochondria and chloroplasts of land plants is distinct from that of the nuclear genome. In higher plants, the mode of inheritance of these organelles is non-Mendelian and predominantly uniparental. Mitochondria of angiosperms are primarily maternally inherited, while their chloroplasts can be inherited maternally (Kirk and Tilney-Basset, 1978; Gillham, 1978; Sears, 1980; Mejnartowicz, 1991), paternally (Boblenz *et al.*, 1990; Schumann and Hancock 1989), or biparentally (Metzlaff *et al.*, 1981; Chiu *et al.*, 1988). In contrast to angiosperms, transmission of chloroplast DNA (cpDNA) in gymnosperms is primarily paternal (Ohba *et al.*, 1971; Neale *et al.*, 1986; Wagner *et al.*, 1987; Stine *et al.*, 1989; Neale *et al.*, 1989; Neale and Sederoff, 1989; Stine and Keathley, 1990; Szmidt *et al.*, 1988; Sutton *et al.*, 1991), with the possible exception of biparental transmission in *Larix* Mill. hybrids (Szmidt *et al.*, 1987). In addition, there is evidence that mitochondrial DNA (mtDNA) is maternally inherited in some conifers (Neale and Sederoff, 1989; Sutton *et al.*, 1991) while in others mitochondrial DNA appears to be paternal in origin (Neale *et al.*, 1989). A summary of the various modes of organellar inheritance in plants and animals appears in Table 1.5. Uniparental inheritance of organelle genomes from opposite parents within the same plant appears to be unique to certain conifers. This indicates that these tree species must have special mechanisms for organelle exclusion and/or degradation. Ultrastructural observations of *Pseudotsuga*, *Pinus* and *Larix* (Camefort, 1968; Chesnoy and Thomas, 1971; Owens and Morris, 1990) provide physical explanations for the phenomena of paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in conifers. Egg cell plastids are transformed into inclusions followed by disruption of the original plastid structure and subsequent destruction of maternal

chloroplast DNA. However, the egg cell mitochondria aggregate, migrate to the perinuclear zone and may become altered prior to fertilization. These maternal mitochondria become incorporated into the cytoplasm of the new embryo (Owens and Morris, 1990).

The significance of this ultrastructural evidence has been demonstrated for various gymnosperms using the technique of restriction fragment length polymorphism (RFLP) analysis. By this method cpDNA has been shown to be predominantly paternally transmitted in *Pseudotsuga menziesii* (Mirb.) Franco (Neale *et al.*, 1986); *Larix* Mill. hybrids (Szmidt *et al.*, 1987); *Pinus* L. hybrids (Wagner *et al.*, 1987); *Picea* A. Dietr. hybrids (Szmidt, 1988; Stine *et al.*, 1989; Stine and Keathley, 1990); *Sequoia sempervirens* D Don Endl. (Neale *et al.*, 1989); and *Pinus taeda* L. (Neale and Sederoff, 1989), *P. banksiana* Lamb. (Wagner *et al.*, 1989) and *Calocedrus decurrens* [Torr.] Florin (Neale *et al.*, 1991). RFLP analysis has been used to follow mtDNA inheritance in only a few gymnosperms. As expected, mitochondrial DNA is maternally inherited in *Pinus taeda* L. and hybrids of *Picea strobus* X *P. griffithii* McClelland as demonstrated by RFLP analysis of intraspecific crosses (Neale and Sederoff, 1989; Neale and Sederoff, 1988; Sutton *et al.*, 1991). However, recent analyses of mtDNA inheritance in *Sequoia sempervirens* D Don Endl. (Family Taxodiaceae) and *Calocedrus decurrens* (Family Cupressaceae) suggests the possibility of paternal inheritance in these conifers (Neale *et al.*, 1989; Neale *et al.*, 1991). Since members of all the Orders of gymnosperms appear to have maternal inheritance of mtDNA, the question arises as to whether paternal inheritance of mtDNA may be limited to members of the Families Cupressaceae and Taxodiaceae in the Order Coniferales, or if members of other Families exhibit this phenomenon.

A predominance of maternal transmission of mitochondria has been demonstrated for many species of animals and plants. Animal species include mammals (Giles *et al.*, 1980; Hutchison *et al.*, 1974; Buzzo *et al.*, 1978), amphibians (Dawid and Blackler, 1972), fish (Awise and Vrijerhoek, 1987), and insects (Lansman *et al.*, 1983; Reilly and Thomas, 1980). Angiosperm plant species exhibiting maternal mitochondrial DNA inheritance include

Equilobium (Schmitz, 1988), *Hordeum* (Soliman *et al.*, 1987), *Maize* (Pring *et al.*, 1977), *Populus* (Radetzky, 1990), *Triticum* (Vedel *et al.*, 1981) and various other plants (Edwardson, 1970; Lonsdale, 1987a). The gymnosperm *Pinus taeda* (Family Pinaceae) also transmits mitochondria through the maternal parent (Neale and Sederoff, 1989). Biparental inheritance of mtDNA has been demonstrated in the angiosperms alfalfa (Fairbanks *et al.*, 1988) and the *Hordeum X Secale* hybrid (Soliman *et al.*, 1987). Although maternal transmission of mitochondrial DNA is predominant, it is not universal. The presence of mitochondrial DNA of paternal origin has recently been demonstrated in mice using the PCR method (Gyllensten *et al.*, 1991). Erickson and Kemble (1990) reported paternal inheritance of mitochondria in the angiosperm *Brassica napus*. In addition, paternal inheritance has been reported in the green algae *Chlamydomonas reinhardtii* (Boynton *et al.*, 1987). Neale *et al.* (1989, 1991) demonstrated paternal transmission in *Sequoia sempervirens* and *Calocedrus decurrens*.

TABLE 1.5: Organellar inheritance in plants and animals.

SPECIES	Mitochondria Maternal	Mitochondria Paternal	Chloroplasts Maternal	Chloroplasts Paternal	References
mammals	+				Hutchison <i>et al.</i> , 1974
amphibians	+				Dawid & Blackler, 1972
fish	+				Avise & Vrijenhoek, 1987
insects	+				Lansman <i>et al.</i> , 1983
many angiosperms	+		+		Kirk & Tilney-Basset, 1978
<i>Brassica napus</i>	+	+	+		Erickson & Kemble, 1990
<i>Hordeum X Secale</i>	+	+	+		Soliman <i>et al.</i> , 1987
alfalfa	+	+	+	+	Fairbanks <i>et al.</i> , 1988
<i>Populus</i>	+		+		Radetzky, 1990
<i>Ginkgo</i>	+		+		Whatley, 1982
Cycads	+		+		Whatley, 1982
<i>Picea</i>	+			+	Szmidt, 1988; Sunc & Keathley, 1990
<i>Pinus</i>	+			+	Wagner <i>et al.</i> , 1987, 1989
<i>Pseudotsuga</i>	+			+	Neale <i>et al.</i> , 1986
<i>Larix</i>	+		(+)?	+	Szmidt <i>et al.</i> , 1988; this study
<i>Sequoia</i>		+		+	Neale <i>et al.</i> , 1989
<i>Calocedrus</i>		+		+	Neale <i>et al.</i> , 1991

1.6. THESIS GOALS

This thesis will examine the mitochondrial genome of the conifer, *Larix* to develop suitable techniques for the isolation of *Larix* mitochondrial DNA from somatic embryogenic cell cultures that could be used in restriction and Southern hybridization analyses. Heterologous mitochondrial gene probes from wheat will be used to establish the presence and copy number of certain protein coding genes.

The mitochondrial genome of some higher plants has been shown to undergo rearrangements during *in vitro* cell culture and subsequent plantlet regeneration. Restriction fragment polymorphism analysis of somatic embryogenic cell cultures and regenerated trees of hybrid *Larix* will be used to examine the effect of cell culture on mitochondrial stability.

Hybridization analysis will also be used to identify probes and restriction endonucleases appropriate for investigating the mode of mitochondrial inheritance in *Larix* hybrid crosses. All members of the Family Pinaceae that have been evaluated for inheritance of organelles appear to inherit chloroplasts from the paternal parent and mitochondria from the maternal parent. However, other conifers inherit both organelles from the paternal parent. Analysis of *Larix* hybrid crosses will be conducted to determine if inheritance of mitochondria is maternal as in other members of the Family Pinaceae, and as also suggested by ultrastructural evidence.

2. MATERIALS AND METHODS

2.1. PLANT AND *in vitro* CULTURE MATERIALS

The larch trees used in this study are part of Experiment # 252-C: Demonstration of larch species and hybrids, located at the Petawawa National Forestry Institute, Chalk River, Ontario. The trees are planted on loamy sand with small gravel in the PNFI Pine Graft Arboretum PA 145, Cottage Rd. - Headquarters Rd. The trees were sown in 1977, transplanted in 1978 and planted at the current location in 1980 on a spacing of 5m X 5m. Nine trees of each of five seedlots were planted: *Larix leptolepis* Lot #J.8951-79050; *L. decidua* Lot # J.7462-748524; Hybrid *L. decidua* X *L. leptolepis* Lot #J.9982-748526; Hybrid *L. leptolepis* X *L. decidua* Lot # J.9981-748525. The seedlot of *L. decidua* originated from Jagesborg, Denmark (Improved Sudeten larch); *L. leptolepis* from Central Honshu, Japan, Nagaro Provenance; and both hybrids from Germany, open pollinated. In addition, 132 seedlings of hybrid *L. X eurolepis* (improved Japanese X improved Alps) were grown from seedlot #7285630, Holbaek, Denmark and used for DNA isolation. Other seedlings used include *L. gmelini* Lot #8380678 from China, *L. laricina* Lot #7930280 from Ontario, *L. olgensis* Lot #8480985 from China, *L. siberica* Lot #8580240 from Russia, *Pinus contorta* Lot #7060480, *Picea glauca* Lot #6730800, *Picea mariana* Lot #8630180 and *Ginkgo biloba* leaves from a tree on the Central Experimental Farm, Agriculture Canada, Ottawa, Ontario.

The *in vitro* cultures used in this study include somatic embryogenic cell cultures of *L. leptolepis* L1, *L. decidua* L7G4, *L. leptolepis* X *L. decidua* L6G4, and *L. decidua* X *L. leptolepis*. Suspensions were maintained on modified Murashige and Skoog (1962) medium containing 1460mg/L glutamine, 2.0mg/L 2,4-D and 0.5mg/L BAP with weekly subculturing. Callus cultures used in this study were labelled as hybrid *L. leptolepis* X *L. decidua*: LB89, L287, L187, L2F7, L2 protoplast. These calli cultures were maintained on the same medium as the cell cultures, solidified with 4g/L gellan gum (K9A40 Kelco, San Diego, CA).

2.2. PLANT DNA ISOLATION AND ANALYSIS

2.2.1. Total Genomic DNA Isolation from Tree Needles

A modification of the procedure of Murray and Thompson (1980) was used successfully to isolate pure high molecular weight DNA from mature needles and seedling tissue of *Larix*. Needle tissue, either fresh or frozen, was frozen in liquid nitrogen and ground to a fine powder in a mortar with a pestle. The powder was mixed with 200mL cold DNA extraction buffer (50mM Tris.HCl, pH8.0; 5mM disodium ethylenediaminetetraacetic acid (EDTA); 0.35M sorbitol; 0.1%w/v bovine serum albumin (BSA); 10%w/v polyethylene glycol (PEG)4000; 0.1%w/v spermine; 0.1%w/v spermidine; 0.1%v/v mercaptoethanol) and incubated on ice for 15 minutes with occasional stirring. The mixture was then filtered through several layers of cheesecloth and one layer of Miracloth (Calbiochem, Frankfurt/Main, FRG). The cellular organelles were pelleted at 15 000G for 15 minutes at 4°C. The pelleted organelles were resuspended in 10mL wash buffer (50mM Tris.HCl, pH8.0; 25mM EDTA; 0.35M sorbitol; 0.1%v/v mercaptoethanol) and allowed to equilibrate to room temperature. One seventh volume of 5M NaCl was added, followed by the addition of one fifth volume of 5% sarcosyl to lyse the organelles. After gentle mixing, this solution was incubated at room temperature for 10 minutes to allow complete lysis of the organelles. Then one tenth volume of 8.6%w/v hexadecylcetyltrimethylammonium bromide (CTAB) in 0.7M NaCl was added, followed by incubation at 60°C for 15 minutes. Organic matter was removed by repeated extractions with chloroform:isoamyl alcohol (24:1 v/v) and centrifuged to remove the organic material. The aqueous supernatant containing the DNA was mixed with three volumes of ice cold absolute ethanol and the precipitated DNA was spooled out of the solution with a bent Pasteur pipette. The DNA was dissolved in TE (10mM Tris.HCl; 1mM EDTA) and mixed with 0.96g/mL CsCl and ethidium bromide to a concentration of 300µg/mL. Centrifugation at 50 000G in a vertical rotor for 16 hours resulted in banding of the DNA and pelleting of organic material. The DNA band was removed from the tube with

an 16 gauge needle and extracted with water-saturated 2-butanol to remove the ethidium bromide. The DNA was then dialyzed against 3 changes of 4L each of TE buffer at 4°C. DNA concentration was determined using a fluorometer, and typical yield was about 500 μ g DNA/100g needle tissue.

2.2.2. Total Genomic DNA Isolation from Callus Tissue and Cell Cultures of *Larix*

This method is a modification of that described by Sanders *et al.* (1987). High molecular weight DNA was extracted from *Larix* callus tissue by grinding frozen tissue in extraction buffer (50mM Tris.HCl pH 8.0, 50mM EDTA, 50mM NaCl, 2% w/v sarcosyl). Organic debris was pelleted by low speed centrifugation (1 000G) and the supernatant was collected and adjusted to 0.96g/mL CsCl and 300 μ g/mL ethidium bromide. The CsCl gradients were centrifuged at 50 000G in a vertical rotor for 16 hours. Ethidium bromide was extracted with water-saturated 2-butanol, the DNA was dialyzed as described previously, and stored at 4°C.

Total genomic DNA was isolated from cell culture using a method adapted from Mettler, (1987). About ten grams of cell culture material was filtered to remove growth medium and transferred to a mortar. Two volumes of homogenization buffer (1%w/v sarcosyl; 0.25M sucrose; 50mM NaCl; 20mM EDTA; 50mM Tris.HCl, pH8.0) were added and the mixture was ground thoroughly to homogeneity. The homogenate was transferred to a centrifuge tube and incubated at room temperature for 30 minutes, with occasional grinding to break any unlysed cells. After incubation an equal volume of phenol (equilibrated with 0.5M NaCl and 100mM Tris.HCl, pH 8.0) was added and mixed until an emulsion formed. The mixture was centrifuged to separate the layers and the upper aqueous phase was removed and extracted twice with chloroform:isoamyl alcohol (24:1). The DNA in the aqueous phase was precipitated by the addition of one tenth volume 3M sodium acetate and 3 volumes absolute ethanol and spooled out with a Pasteur pipette. The DNA was then dissolved in TE, banded on a CsCl gradient and collected as described previously.

The yield was about 1mg DNA/gm cells.

2.2.3. Mitochondrial DNA Isolation from Cell Cultures of *Larix*

Mitochondrial DNA was isolated by a modification of the methods described by Wilson and Chourey (1984) and Radetzky (1990). Cell cultures at 4 days post subculture were ground at 4°C in a mortar and pestle with sand and five volumes/gram fresh weight extraction buffer (0.4M mannitol, 1mM ethylene glycol-bis[aminoethyl ether] N',N',N',N'-tetraacetic acid (EGTA), 20mM N-[2-hydroxyethyl]piperazine-N'-[ethanesulfonic acid] (HEPES), pH 7.5, 0.1% BSA, 0.05% cysteine, 10mM diethyldithiocarbamic acid (DIECA), 0.5% Polyclar AT (BDH Chemicals Ltd., England). After filtration through two layers of Miracloth the homogenate was centrifuged at 150G for 10 minutes. The supernatant was centrifuged three times at 3 000G for 10 minutes to separate cellular debris, nuclei and proplastids from the mitochondria. Mitochondria were pelleted at 10 000G for 20 minutes, resuspended in DNase buffer (0.4M mannitol, 20mM HEPES, pH 7.5, 10mM MgCl₂) and treated with DNase I (0.1mg/mL) at 4°C for one hour. After washing the mitochondria with DNase inhibiting buffer (100mM EGTA, 0.4M mannitol, 20mM HEPES, pH 7.5) mitochondria were further purified by centrifugation in a discontinuous Percoll gradient (45%, 21%, 14% Percoll) at 15 000G for 15 minutes. The mitochondria that banded were pooled and diluted with resuspension buffer (0.4M mannitol, 20mM HEPES, pH 7.5, 10mM EGTA, 10mM DIECA) and centrifuged several times at 10 000G for 10 minutes to remove Percoll. The washed and pelleted mitochondria were resuspended in lysis buffer (0.1M Tris.HCl, pH 8.0, 0.05M EDTA, 0.1M NaCl, 1%w/v sodium dodecyl sulfate [SDS], 1%w/v sarcosyl) and incubated at 65°C for 30 minutes. Organic material was removed by addition of 5M potassium acetate with incubation on ice for 20 minutes. After centrifugation the supernatant was mixed with an equal volume of isopropanol, DNA was precipitated and dissolved in TE. DNA was further purified by extraction with phenol (buffered with TE), followed by two extractions with chloroform:isoamyl alcohol (24:1v/v), reprecipitated and

dissolved in TE buffer. RNA was removed by addition of RNase during the restriction endonuclease digestion of the mtDNA.

2.2.4. Restriction Endonuclease Digestion and Gel Electrophoresis of *Larix* DNA

Mitochondrial and total genomic DNAs were digested with various restriction endonucleases according to the manufacturer's instructions (BRL, Berlin, FRG) with slight modifications. The volume of 5 μ g of DNA in TE buffer was kept at or below one quarter of the final reaction volume, BSA was omitted, and enzyme volume did not exceed one tenth final reaction volume. Water for the reactions was HPLC (high performance liquid chromatography) purified as purchased from Fisher Scientific (Ottawa, ONT). Enzyme was added at 10units/ μ g and was added in 4 equivalent aliquots at hourly intervals, with a total digestion time of 5 hours. Preliminary digestion trials had indicated that the mitochondrial DNA fraction was totally digested in under one hour, and 5 hours was sufficient to digest the nuclear DNA fraction to near completion. If nuclear DNA was not sufficiently digested the hybridizing mitochondrial bands were distorted. Digested DNAs were electrophoresed through horizontal gels (0.7% SeaKem agarose, FMC BioProducts, Rockland, ME) at 0.5V/cm overnight in TAE buffer (40mM Tris-acetate; 2mM EDTA, pH 8.0). DNA fragments were stained with ethidium bromide and gels were photographed with an MP-4 Polaroid camera under ultraviolet light using Polaroid 55 or 57 land film. Lambda DNA, digested with *HindIII*, was used as a standard molecular weight marker to determine the sizes of individual fragments.

2.2.5. Transfer of DNA to Nylon Membranes

DNA fragments separated by agarose gel electrophoresis were transferred to Biotrans nylon membrane (ICN, Irvine, CA) using the LKB 2016 VacuGene Vacuum Blotting System which uses a low pressure vacuum to transfer nucleic acids from a gel to a membrane. The gel to be transferred was placed on the nylon membrane which was held within a window in

the waterproof plastic frame. The frame was supported on the porous screen in the base of the VacuGene unit. After locking the upper frame to the base, a vacuum of 40 cm.H₂O was applied and the gel was flooded sequentially with depurination (0.25N HCl), denaturation (1.5M NaCl, 0.5M NaOH), neutralization (3M NaAcetate, pH 5.5) and transfer solutions (3M NaCl, 0.3M Trisodium citrate, pH 7.6 [20XSSC]). After transfer was complete the membrane was air dried and DNA was cross-linked to the nylon membrane with ultraviolet irradiation at 120mJoules/cm² with the FB-UVXL-1000 Cross Linker (Fisher Scientific).

2.2.6. Preparation of Mitochondrial Gene Probes

Wheat mitochondrial gene probes were obtained from L. Bonen, University of Ottawa. These genes had been cloned into pUC vectors within the multiple cloning site and fragments used as probes were excised by appropriate restriction endonuclease digestion. The following mitochondrial gene probes were chosen because they have been well characterized from several higher plant mitochondrial genomes. Maps of these probes appear in Figure 2.2, and the specific fragments used as probes are indicated. Table 2.2 describes the probes used for the hybridization experiments.

TABLE 2.2: Wheat mitochondrial gene probes used for hybridization experiments.

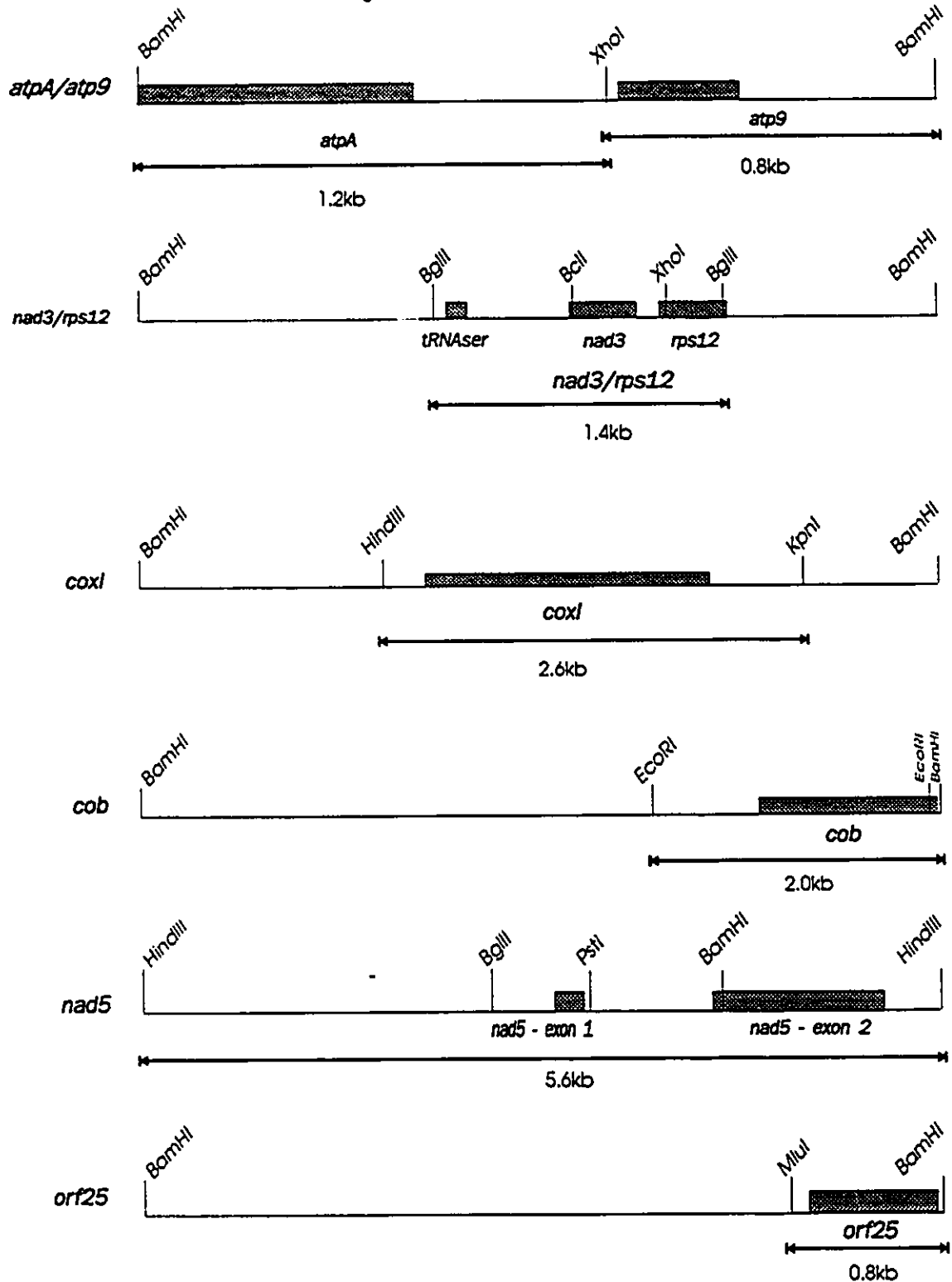
Gene Probe	Plasmid Clone	Enzymatic Fragment	Probe Length (kb)	% of Probe as gene coding region
<i>atpA</i>	B30	<i>XhoI/BamHI</i>	1.2	83.3%
<i>atp9</i>	B30	<i>XhoI/BamHI</i>	0.8	25%
<i>nad3/rps12</i>	B303	<i>BglII</i>	1.4	57%
<i>coxI</i>	B342	<i>HindIII/KpnI</i>	2.4	70.8%
<i>cob</i>	B376	<i>BamHI/EcoRI</i>	2.0	60%
<i>nad5</i>	H4	<i>HindIII</i>	5.5	25.5%
<i>orf25</i>	B314	<i>BamHI/MluI</i>	0.8	71.3%

Transformation of *E. coli* strain JM101 with plasmid clones containing wheat mitochondrial gene fragments was performed according to the rubidium chloride/calcium chloride method as outlined in the manual of Maniatis *et al.*, 1982. Plasmids were isolated

FIGURE 2.2

Restriction maps of wheat mitochondrial gene probes cloned in pUC vector.

Probe segments are indicated by arrows below the map.



and purified by a method adapted from Dale and Greenaway (1984). All buffers and other reagents that were to be stored were autoclaved, used aseptically and stored at 4°C. L-broth containing the selection antibiotic carbenicillin was inoculated with the bacterial colony containing the transformed plasmid and incubated overnight at 37°C with vigorous shaking. Cells were pelleted from 1mL of this overnight culture and resuspended in 100µL of lysis solution (25mM Tris.HCl, pH 8.0; 10mM EDTA, pH8.0; 50mM glucose; 2mg/mL lysozyme) and stored on ice for 30 minutes. Cells were lysed by the addition of 200µL of alkaline SDS (1%w/v SDS in 0.2M NaOH) and incubated on ice for 5 minutes. Proteins and other organic material were precipitated by the addition of 150µL of 3M sodium acetate followed by incubation on ice for 30 minutes. The precipitate was pelleted by centrifugation at 14 000g for 5 minutes and 400µL of the supernatant containing the plasmid DNA was transferred to another tube. The addition of 1mL absolute ethanol and incubation at -20°C facilitated the precipitation of plasmid DNA. The precipitate was centrifuged for 20 minutes at 14 000g to pellet the DNA and this pellet was resuspended in TE buffer. Residual organic material was extracted once with phenol (buffered with TE) and twice with chloroform:isoamyl alcohol (24:1v/v) and DNA was reprecipitated with one tenth volume 3M sodium acetate and three volumes absolute ethanol. Plasmid DNA was recovered by centrifugation for 20 minutes at 14 000g and dissolved in 50µL of TE buffer and then stored at -20°C prior to analysis and gene fragment isolation.

2.2.7. Purification of Wheat Mitochondrial Gene Fragments

After restriction endonuclease digestion of the plasmid clones containing the wheat mitochondrial genes, fragments were purified by agarose gel electrophoresis, bands excised and DNA purified using the GeneClean II Kit (BIO 101 Inc., La Jolla, CA). Purified DNA fragments were then electrophoresed again, excised and the DNA repurified using the GeneClean II Kit according to the manufacturer's instructions as follows: The appropriate DNA band was excised from the agarose gel, weighed and three volumes of 6M NaI stock

solution was added. After incubation for 5 minutes at 50°C to melt the agarose, glassmilk (silica matrix in water) was added at a concentration of 2 μ L/ μ g DNA fragment. After thorough mixing the solution was incubated at room temperature for 15 minutes with occasional mixing. The glassmilk/DNA complex was then pelleted at 1 000g for 5 minutes and the supernatant discarded. The pellet was washed three times with NEW wash (NaCl/ethanol/water) and then resuspended in TE buffer. The mixture was incubated at 50°C for 3 minutes and then centrifuged to pellet the glassmilk. The supernatant containing the DNA was carefully removed to a new tube and the DNA was stored at -20°C.

2.2.8. Random Primer Labelling of Mitochondrial Gene Probes

Mitochondrial gene fragments were labelled with the radioisotope phosphorous-32 using the BRL Random Primers DNA Labelling System (BRL, Gaithersburg, MD) according to the manufacturer's instructions with minor modifications. A boiling water bath was used to denature 25ng of the wheat mitochondrial gene fragment. After denaturation the DNA was transferred immediately to ice and the following additions were made: 2 μ L dATP (0.5M dATP in 3mM Tris.HCl pH7.0, 0.2mM EDTA); 2 μ L dGTP (0.5M dGTP in 3mM Tris.HCl pH7.0, 0.2mM EDTA); 2 μ L dTTP (0.5M dTTP in 3mM Tris.HCl pH7.0, 0.2mM EDTA); 15 μ L Random Primers Buffer Mixture (0.67M HEPES, 0.17M Tris.HCl, 17mM magnesium chloride, 33mM beta-2-mercaptoethanol, 1.33mg/mL BSA, 18 O.D.₂₆₀units/mL oligodeoxyribonucleotide primers [hexamer fraction], pH 6.8); 5 μ L[alpha-³²P] dCTP, 3000 Ci/mmol, 10 μ Ci/ μ L (Amersham Canada Ltd., Oakville,Ontario), distilled water to a total volume of 49 μ L, and 1 μ L Klenow Fragment Polymerase. The reaction was incubated at room temperature overnight. Incorporation was evaluated by spotting 1 μ L of the reaction mix onto a glass fiber filter disk (Whatman GF/C), measuring the initial counts per minute using the Bioscan Quick-Count (Bioscan, Inc., Washington, D.C.), washing the filter disk three times with ice cold 5% trichloroacetic acid containing 1%w/v sodium pyrophosphate, and washing once with ethanol, followed by re-measurement of the counts per minute.

Incorporation was calculated as a percentage of the original counts per minute. Labelled probes were denatured prior to hybridization by the addition of one fifth volume 1N NaOH for two minutes followed by neutralization with one fifth volume 1N HCl. The average specific activity of the probes used in these experiments was 10^9 cpm/ μ g.

2.2.9. Southern Hybridization Analysis

Filters to be hybridized were prehybridized overnight at 60°C in a hybridization solution containing 5X Denhardt's buffer (0.01%w/v Ficoll [400,000 mw], 0.01%w/v polyvinylpyrrolidone [360,000 mw], 0.01%w/v BSA); 5X SSPE (0.8M NaCl, 0.05M sodium phosphate, pH 8.3, 5mM EDTA); and 0.2% SDS. This extended prehybridization was used to obtain maximum blocking of the membrane to prevent high background signals on the autoradiograph. Filters were transferred to hybridization tubes containing 5-10mL fresh hybridization solution. Larger volumes of hybridization solution increased the time required for efficient hybridization of probe to filter, and often resulted in a higher background signal. The hybridization solution used in these experiments contained a total of 0.85M sodium ion concentration as described above. If *Larix* mitochondrial DNA has a G+C content of 46% as in other higher plants, this salt concentration, with hybridization at 60°C, would require theoretically about 61% homology between the probe sequence and *Larix* mtDNA sequences for hybridization to occur. Due to the expected high sequence conservation between mitochondrial genes of angiosperms and gymnosperms, these are conditions of relatively low stringency. The probe was denatured by addition of 1/5 volume NaOH, followed by incubation at room temperature for two minutes prior to neutralization. This method of denaturation was fast, effective, and safer than boiling the radioactive solution. The denatured probe was added to the hybridization buffer and filters were hybridized overnight at 60°C in a hybridization oven (Model 310 Hybridization Incubator, Robbins Scientific Corp., Sunnyvale, CA). Hybridizations performed in plastic containers placed in Lab-line Orbital Environ-Shaker (Lab-line Instruments, Melrose Park, ILL) required larger volumes

of hybridization solution, thereby increasing the hybridization time. Formamide was not used in the hybridization solution because of its toxicity and disposal complications.

Following hybridization, the labelled probe was removed and the filters were washed three times in 2X SSC (equivalent sodium ion concentration as 2X SSPE) with 0.5% sarcosyl (pH 9.0) for 20 minutes each at 55°C. The pH of the washing solution was increased to pH 9.0 to assist in the removal of background signal. This washing solution had a sodium ion concentration of 0.33M, and washing at a temperature of 55°C which would theoretically allow sequences to remain hybridized if they were at least 63% homologous. After washing, the membranes were removed from the glass hybridization tubes, sealed in Kapak plastic bags (Kapak Corporation, Minneapolis, MN) and exposed overnight to Kodak XOMAT XAR film at -70°C in cassettes containing Cronex Lightening Plus intensifying screens. These filters were not used for rehybridization because the second hybridization signal was found to be too weak.

3. RESULTS

3.1. ISOLATION OF MITOCHONDRIAL DNA FROM *Larix* CELL CULTURE

The procedure of Wilson and Chourey (1984), with modifications, was a simple and rapid method for the isolation of crude mitochondrial DNA from somatic embryogenic cell cultures of *Larix leptolepis*. A mortar and pestle and a blender were compared for their efficiency to disrupt cell walls and release cell contents. Microscopic examination of the resulting homogenate indicated that the mortar and pestle was superior to the blender for releasing cell contents. Modifications to the homogenizing buffer were based on a review of literature available on the efficacy of specific buffering compounds. The osmolarity of the homogenizing buffer was adjusted to match that required for isolation of protoplasts from *Larix* somatic embryogenic cell cultures to preserve the integrity of the mitochondrial membrane. Insoluble Polyclar AT was added to the homogenization buffer because of its ability to remove compounds that might degrade the intact mitochondria and interfere with further purification steps (Raderzky, 1990). Repeated (3-5 times) low speed centrifugation was used to attempt to remove most of the intact nuclei, chloroplasts and cell wall debris. However, it was also necessary to increase the DNaseI concentration to 0.1mg/mL during the isolation of purified mitochondria to reduce the nuclear DNA background (Figure 3.1). In a separate experiment, intact mitochondria were isolated and further purified by centrifugation through a Percoll gradient. The mtDNA resulting from this isolation was divided into five aliquots and digested with each of five restriction endonucleases. Gel electrophoresis revealed that this mitochondrial DNA was not free from contaminating nuclear DNA (Figure 3.2) and as a result the gel lanes in Figure 3.2 were overloaded. The Percoll gradient procedure used in these experiments resulted in numerous (about 15) bands throughout the gradient which were all collected and pooled as mitochondria, but nuclear and chloroplast contamination resulted. Some of the bands on the Percoll gradient may not have been mitochondria. However, this DNA was digested with several restriction endonucleases

FIGURE 3.1**Purification and Restriction Endonuclease Digestion of Mitochondrial DNA from
Larix leptolepis.**

Effect of increasing DNaseI concentration prior to lysis of mitochondria to reduce nuclear DNA contamination. Mitochondrial DNA digested with *Hind*III. Lane 1: Lambda *Hind*III digest molecular weight marker. Lane 2: 10 000 units DNaseI. Lane 3: 5 000 units DNaseI. Lane 4: 1 000 units DNaseI. Lane 5: No DNaseI treatment.

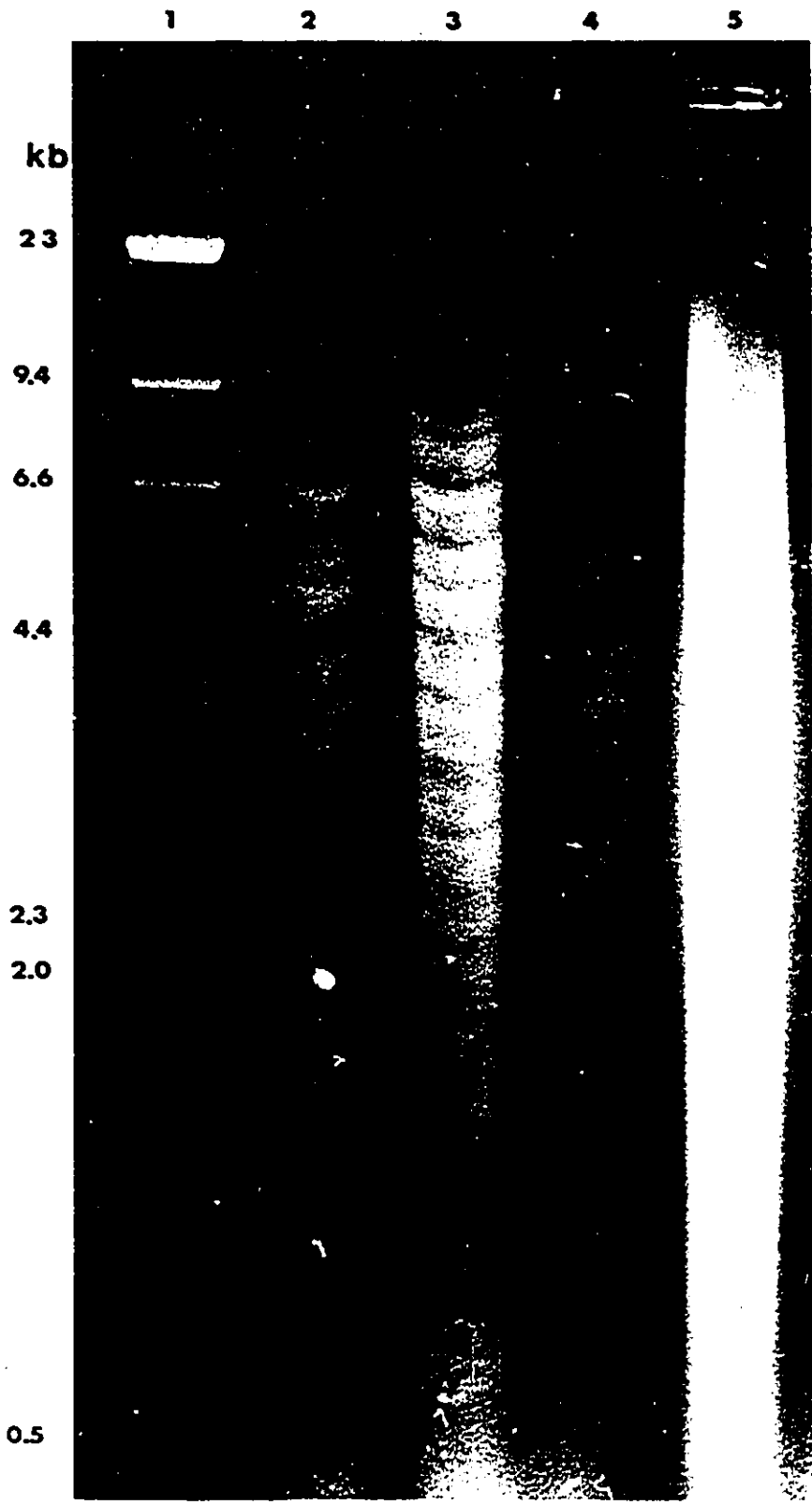
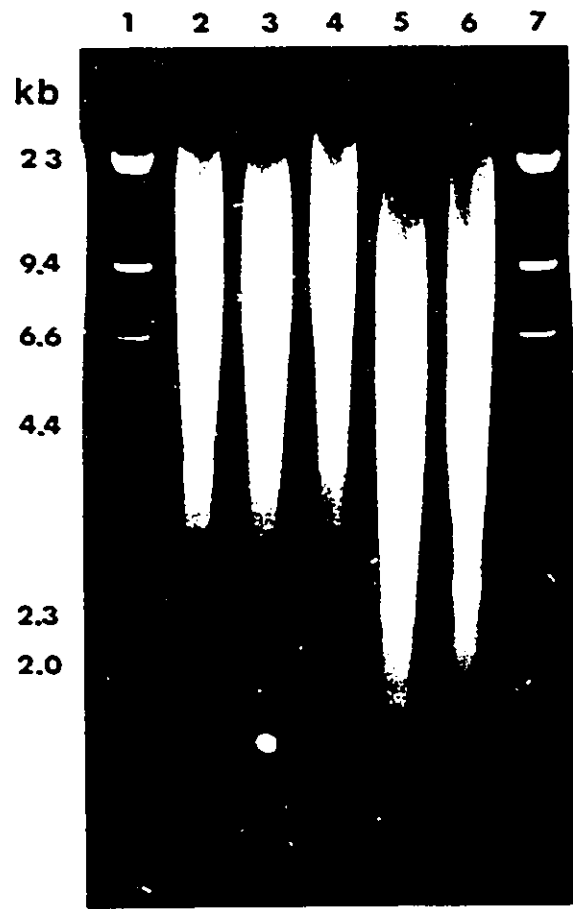


FIGURE 3.2

Purification of Intact Mitochondria Through a Percoll Gradient after DNaseI Treatment.

Lane 1, 7: Lambda *Hind*III digest molecular weight marker. *Larix leptolepis* mitochondrial DNA digested with *Kpn*I (Lane 2), *Hind*III (Lane 3), *Eco*RI (Lane 4), *Dra*I (Lane 5), and *Bam*HI (Lane 6).



and electrophoresed on agarose gels (Figure 3.2) followed by Southern transfer to nylon membranes, and subsequently hybridized with a wheat mitochondrial gene probe (see Figure 3.7 B). Strong hybridization signals indicated that this DNA was enriched for mitochondrial DNA sequences. Therefore, the procedure outlined in Materials and Methods (Section 2.2.3) can be used to enrich for mitochondrial DNA from *Larix* somatic embryogenic cell cultures.

3.2. USE OF TOTAL GENOMIC DNA FROM LARIX FOR MITOCHONDRIAL GENOME ANALYSIS

The experiments in this project required relatively large amounts of DNA, but the yields of purified mitochondria from cell cultures were low, therefore hybridization experiments were performed using total genomic DNA. The feasibility of using total genomic DNA from a gymnosperm, as an alternative to purified mitochondrial DNA, for hybridization with heterologous probes from mitochondria of the angiosperm wheat has to be addressed. Furthermore, the nuclear component of conifer total genomic DNA is composed of highly methylated sequences that can inhibit the action of many restriction endonucleases. Although the mitochondrial DNA component is readily digested, the undigested nuclear DNA can distort the mitochondrial DNA banding pattern. Therefore, several control experiments were performed to ensure that total DNA from *Larix* would hybridize with wheat mitochondrial gene probes and that the resulting mitochondrial hybridization signals were not altered or distorted by the presence of the nuclear and chloroplast DNA components, and the hybridizing signals were the result of wheat mitochondrial gene fragments hybridizing to *Larix* DNA.

3.2.1. Restriction Endonuclease Digestion Controls

Various controls were done to ensure that the regime for digestion of total genomic DNA of *Larix* was free from inhibiting substances that might prevent complete digestion of the mitochondrial DNA. Initially, bacteriophage lambda DNA (1 μ g) was mixed with 5 μ g

total genomic DNA of *Larix* and this mixture was digested with each of six enzymes: *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, and *Kpn*I; according to the protocol outlined in Materials and Methods (Section 2.2.4). Restriction profiles for bacteriophage lambda digested with total genomic DNA of *Larix* matched those of bacteriophage lambda digested alone (Figure 3.3 A), suggesting that the *Larix* DNA preparations did not contain any substances that would inhibit or alter the activity of these restriction endonucleases.

3.2.2. Investigation of Hybridization with Vector Sequences

To reduce the concentration of vector sequence contamination of the radiolabelled gene probes, wheat mitochondrial restriction fragments, free from the pUC vector, were gel purified twice (see Section 2.2.7, page 28). This reduced the probability that hybridizing bands were the result of hybridization of *Larix* DNA to vector sequence DNA rather than to mitochondrial gene probe DNA. In addition, the double gel purification would minimize cross contamination from any closely linked genes or flanking regions, as in the case of the *atpA* and *atp9* genes. To further ensure that vector sequences were not capable of hybridizing to specific *Larix* DNA sequences the pUC vector was radiolabelled, as described in the Materials and Methods (Section 2.2.8), followed by hybridization with restriction endonuclease digested total genomic DNA from each of the four *Larix* species (Figure 3.3 B). No hybridizing bands were observed, indicating that pUC vector sequences do not hybridize to specific *Larix* DNA fragments under the conditions used in these experiments.

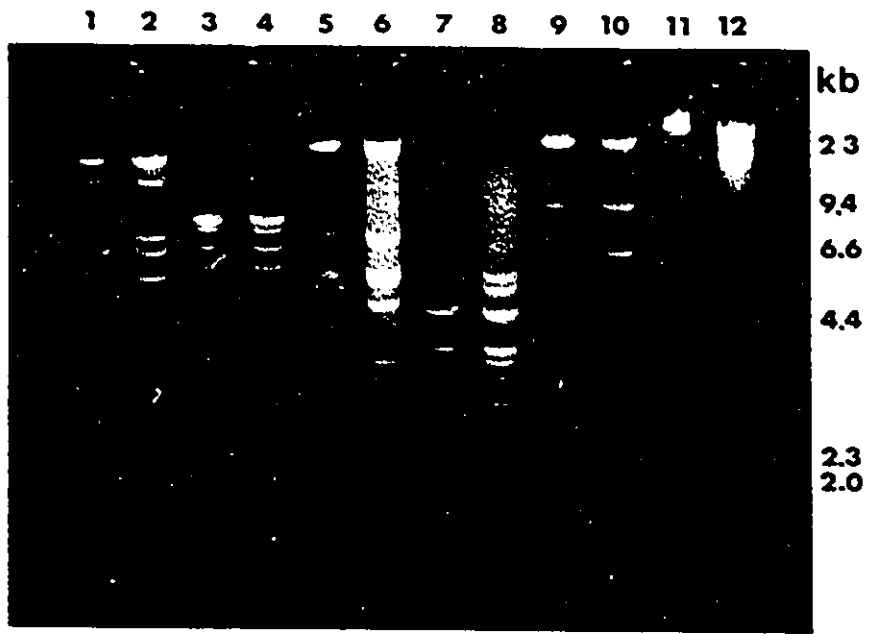
3.2.3. Hybridization of Wheat Mitochondrial Gene Probes with *Larix* Total DNA

To determine if the concentration of restriction endonuclease used in these experiments was sufficient to digest *Larix* mitochondrial DNA to completion, total genomic DNA was digested with various concentrations of restriction endonuclease in a manner similar to that outlined in Materials and Methods (Section 2.2.4), followed by

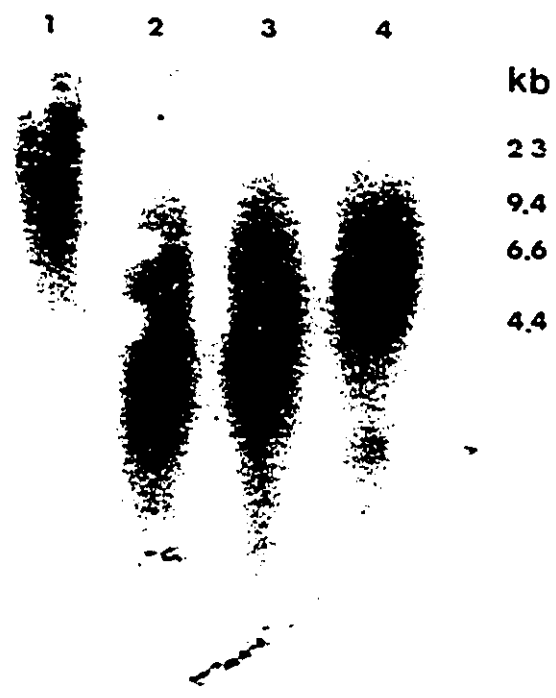
FIGURE 3.3**Effect of *Larix leptolepis* Total DNA on Restriction Endonuclease Activity.**

- A. Lambda DNA fragments generated by restriction endonuclease digestion in the presence of *Larix leptolepis* total genomic DNA compared to Lambda DNA digested alone. Lane 1: Lambda *Bam*HI digest. Lane 2: Lambda and *Larix leptolepis* *Bam*HI digest. Lane 3: Lambda *Dra*I digest. Lane 4: Lambda and *Larix leptolepis* *Dra*I digest. Lane 5: Lambda *Eco*RI digest. Lane 6: Lambda and *Larix leptolepis* *Eco*RI digest. Lane 7: Lambda *Eco*RV digest. Lane 8: Lambda and *Larix leptolepis* *Eco*RV digest. Lane 9: Lambda *Hind*III digest. Lane 10: Lambda and *Larix leptolepis* *Hind*III digest. Lane 11: Lambda *Kpn*I digest. Lane 12: Lambda and *Larix leptolepis* *Kpn*I digest.
- B. pUC vector DNA sequences hybridized with *Hind*III digested *Larix* species total genomic DNA. Lane 1: *Larix leptolepis*. Lane 2: *L. leptolepis* X *L. decidua*. Lane 3: *L. decidua* X *L. leptolepis*. Lane 4: *L. decidua*

A



B

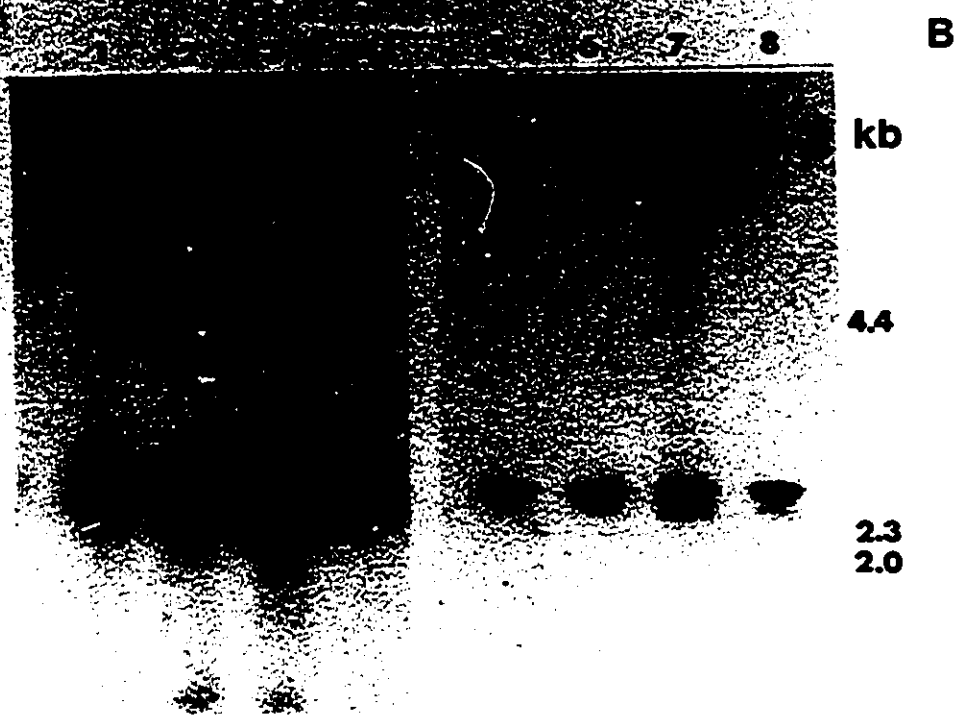
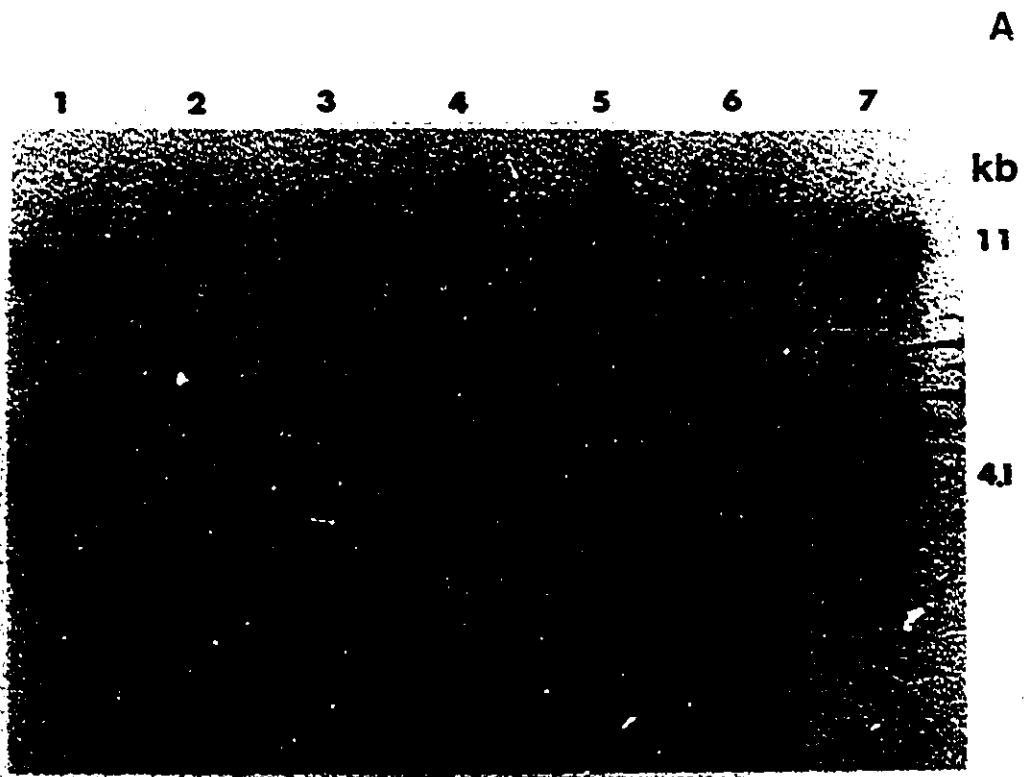


hybridization with a mitochondrial gene probe. Since the hybridization banding pattern was the same for all enzyme concentrations tested the results demonstrated that the mitochondrial DNA was completely digested, even at the lowest concentration of enzyme added (Figure 3.4 A). Therefore, the digestion regime outlined in Materials and Methods (Section 2.2.4) was appropriate for the complete digestion of *Larix* mitochondrial DNA, and total genomic DNA could be used for hybridization with heterologous wheat mitochondrial gene probes. Strong hybridization to lambda DNA marker was due to non-specific hybridization to high copy number fragments, since 350ng of DNA was loaded on the gel.

Complex restriction or hybridization profiles can also result from incomplete digestion caused by methylation of the cytosine, or adenosine residues. To determine if *Larix* mitochondrial DNA was methylated at certain cytosine residues total DNA for each of the four *Larix* species cell cultures was digested with the isoschizomers *MspI* or *HpaII*, followed by hybridization with wheat mitochondrial gene probe *atpA/atp9*. These two enzymes, *MspI* and *HpaII*, can be used to distinguish between DNA that is methylated or non-methylated at cytosine residues since *HpaII* is inhibited by any form of methylation at either C residue in the target sequence C-C-G-G, whereas *MspI* is inhibited by methylation only at the external C residue. Therefore, digestion of DNA containing 5 methylcytosine residues would result in a different restriction pattern with each of these enzymes, and subsequently different hybridization banding patterns. When wheat mitochondrial gene probe *atpA/atp9* was used as a probe in a Southern hybridization experiment the resulting hybridization patterns were identical for both enzymes with each of the four *Larix* species (Figure 3.4 B). Although the higher molecular weight band is the same for all four species of *Larix*, the lower molecular weight band shows different relative intensities between the species. This indicates that 5-methylcytosine is probably not present in *Larix* mtDNA, and therefore does not inhibit complete digestion of *Larix* mitochondrial DNA by enzymes that are sensitive to this type of methylation. Methylation at adenosine residues (N⁶-methyldeoxyadenosine) was not investigated.

FIGURE 3.4**Methylation and non-specific hybridization of *Larix leptolepis* mitochondrial DNA**

- A.** *Larix leptolepis* total DNA digested with various *EcoRI* enzyme concentrations and hybridized with probes *atpA* and *atp9*. Lane 1: 5 units/*ug*. Lane 2: 10 units/*ug*. Lane 3: 20 units/*ug*. Lane 4: 50 units/*ug*. Lane 5: 80 units/*ug*. Lane 6: 120 units/*ug*. Lane 7: 160 units/*ug*.
- B.** Four *Larix* species DNAs digested with restriction endonuclease isoschizomers that have different sensitivities to 5-methylcytosine, hybridized with a wheat mitochondrial gene probe containing sequences for *atpA* and *atp9*. Lane 1: *Larix leptolepis* digested with *HpaII*. Lane 2: *L. leptolepis* X *L. decidua* digested with *HpaII*. Lane 3: *L. decidua* X *L. leptolepis* digested with *HpaII*. Lane 4: *L. decidua* digested with *HpaII*. Lane 5: *Larix leptolepis* digested with *MspI*. Lane 6: *L. leptolepis* X *L. decidua* digested with *MspI*. Lane 7: *L. decidua* X *L. leptolepis* digested with *MspI*. Lane 8: *L. decidua* digested with *MspI*.



3.2.4. Wheat Mitochondrial Gene Clones

To ensure that the fragments generated for each clone matched the corresponding restriction map, which would indicate that the probe to be used in hybridization experiments was in fact the expected gene fragment, the wheat mitochondrial genes cloned in pUC vectors were digested with various enzymes and enzyme combinations, and fragments generated were sized by agarose gel electrophoresis against a molecular weight standard. The fragment sizes generated from digestion of the wheat mitochondrial clones are tabulated in Appendix I. Resulting fragment sizes for each mitochondrial gene clone matched those predicted, therefore it was concluded that the probes used for further hybridization experiments were the mitochondrial gene fragments indicated.

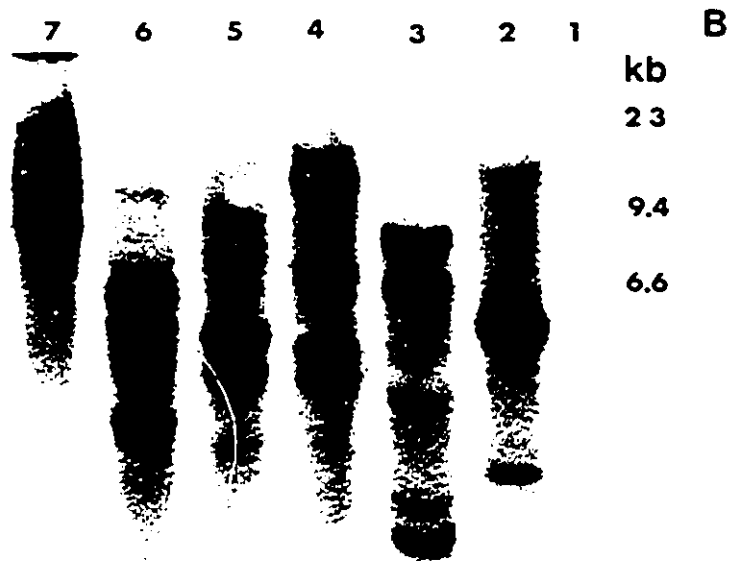
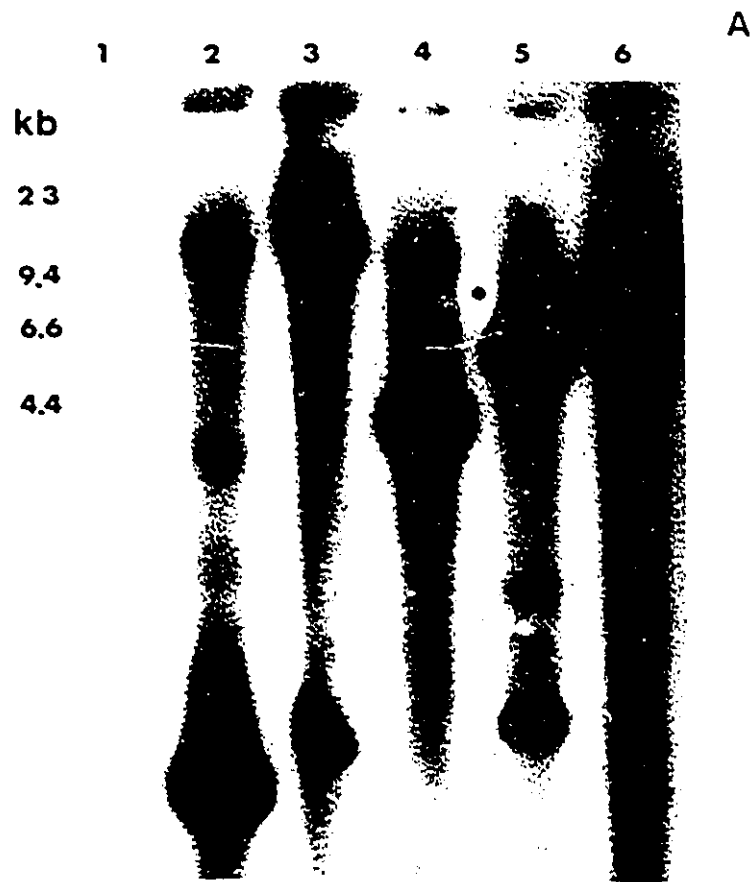
3.3. HYBRIDIZATION OF WHEAT MITOCHONDRIAL GENE PROBES WITH *Larix leptolepis* TOTAL DNA FROM TREE NEEDLES

To determine if sequences homologous to several well characterized genes encoded by the mitochondria in various higher plant species were present in the *Larix* mitochondrial genome, total genomic DNA of *Larix leptolepis* was digested with various enzymes and hybridized with a number of wheat mitochondrial gene probes. The hybridization patterns were also used to examine the organization and complexity of the mitochondrial genes through analysis of the number and intensities of the hybridizing bands. All hybridizations were performed a minimum of two times, with two different DNA isolations, and consistent results were recorded. One exception was the preliminary hybridization data presented on the investigation of within-tree variation which was performed once, with a DNA sample from a single isolation (Figure 3.13). Further hybridization studies will be necessary to verify the results of that experiment. The sizes of the major and minor hybridizing bands (chosen by strength of signal) for each probe are summarized in Table 3.3 (page 47).

Hybridization with probes *atpA* or *atp9* with restriction endonuclease digested *Larix*

FIGURE 3.5**Hybridization of *Larix leptolepis* DNA with Wheat Mitochondrial Gene Probes
atpA and *atp9***

- A.** *Larix leptolepis* total genomic DNA hybridized with *atpA*. Lane 1: Lambda *Hind*III digest molecular weight marker. *Larix leptolepis* DNA digested with *Bam*HI (Lane 2), *Cfo*I (Lane 3), *Eco*RI (Lane 4), *Hind*III (Lane 5), *Kpn*I (Lane 6). Fragment sizes are summarized in Table 3.3.
- B.** *Larix leptolepis* total genomic DNA hybridized with *atp9*. Lane 1: Lambda *Hind*III digest molecular weight marker. *Larix leptolepis* DNA digested with *Bam*HI (Lane 2), *Dra*I (Lane 3), *Eco*RI (Lane 4), *Eco*RV (Lane 5), *Hind*III (Lane 6), *Kpn*I (Lane 7). Fragment sizes are summarized in Table 3.3.

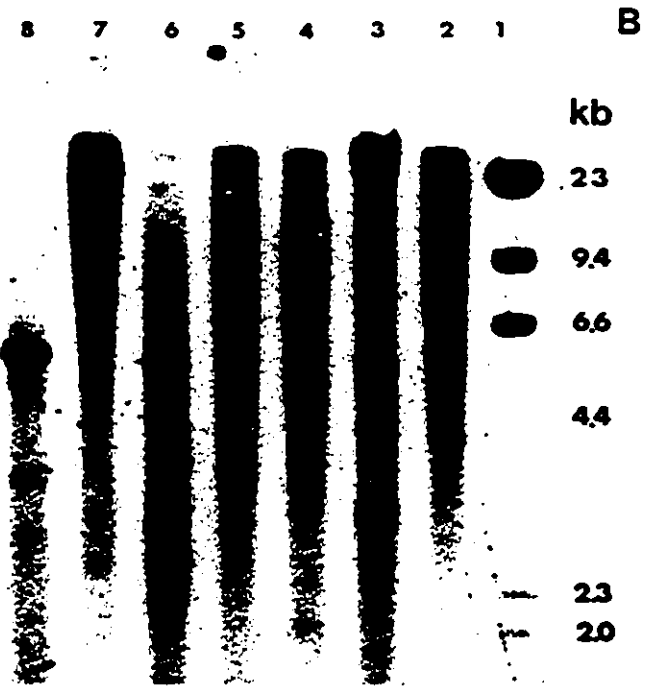


DNA usually resulted in at least two hybridizing bands of unequal intensity (Figure 3.5 A and B). Hybridization with the probe *atpA* resulted in two hybridizing fragments, one major and one minor band, and comparison of the hybridizing fragment sizes produced when *atp9* was used as a probe indicated that the less intense band was probably not due to cross-hybridization with *atp9* sequences. Therefore, it would appear that the *atpA* sequence is present in two copies in the *Larix leptolepis* mitochondrial genome. Hybridization with the *atp9* probe resulted in a complex banding pattern with one or more less intensely hybridizing bands with enzymes *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III and *Kpn*I, that were unique and could not be explained by cross-hybridization with the *atpA* probe. This would suggest that *atp9* is present on at least two different physical forms of the mitochondrial genome. Some data suggest that one copy of *atp9* could be closely linked to *atpA*, because of the similarities in the hybridizing band sizes with these two probes when *Larix* total DNA was digested with *Eco*RI and *Kpn*I (see Table 3.3). However, digestion with *Bam*HI, *Dra*I, *Eco*RV, or *Hind*III resulted in complex hybridization patterns with these two probes that can not be adequately explained if these genes are linked.

Hybridization with probe *nad3/rps12* usually resulted in a single hybridizing band with each of the five restriction endonucleases (Figure 3.6 A). It is likely that sequences homologous to at least one of the three genes present in the probe are hybridizing. Because the tRNA^{ser} gene comprises only a small fraction of the probe it is more likely that the signal is due to hybridization of the *nad3/rps12* sequences. These gene sequences could be present as a linked set in the mitochondrial genome of *Larix* as they are in wheat, because only a single fragment hybridized. If the genes were located at different genomic locations on *Larix* mtDNA, more than one hybridizing fragment would have been expected. Alternatively, only one of the three genes may be present in *Larix* mitochondrial genome, which would also explain a single hybridizing fragment. In addition, hybridization to nuclear or chloroplast DNA sequences is also a possibility, since total genomic DNA was used in these experiments. Hybridization of *nad3/rps12* to wheat total genomic DNA

FIGURE 3.6**Hybridization of *Larix leptolepis* DNA with Wheat Mitochondrial Gene Probes
nad3/rps12 and *nad5*.**

- A.** *Larix leptolepis* total genomic DNA hybridized with *nad3/rps12*. *Larix leptolepis* DNA digested with *DraI* (Lane 1), *EcoRI* (Lane 2), *EcoRV* (Lane 3), *HindIII* (Lane 4), *KpnI* (Lane 5). Lane 6: Lambda *HindIII* digest molecular weight marker. Lane 7: Wheat total genomic DNA digested with *BglII*. Fragment sizes are summarized in Table 3.3.
- B.** *Larix leptolepis* total genomic DNA hybridized with *nad5*. Lane 1: Lambda *HindIII* digest molecular weight marker. *Larix leptolepis* DNA digested with *BamHI* (Lane 2), *DraI* (Lane 3), *EcoRI* (Lane 4), *EcoRV* (Lane 5), *HindIII* (Lane 6), *KpnI* (Lane 7). Lane 8: Total genomic DNA of wheat digested with *HindIII*. Fragment sizes are summarized in Table 3.3.



resulted in two hybridizing bands: the expected band at 1.4kb and a higher molecular weight band (Figure 3.6, Lane 7). This unexpected result was investigated further by comparing the hybridization pattern of *nad3/rps12* with purified wheat mitochondrial DNA, which resulted in a single hybridizing band of 1.4kb, indicating that the higher molecular weight band was the result of homologous sequences present in the chloroplast or nuclear genome of wheat that were able to hybridize under the conditions of stringency used in these experiments.

Hybridization of probe *nad5* with restriction endonuclease digested *Larix* DNA usually resulted in two hybridizing bands of unequal intensities (Figure 3.6 B). This could indicate that this DNA sequence is present in the mitochondrial genome of *Larix* in two components of different sizes on different restriction fragments. Hybridization signals with *Larix* DNA are not as intense as the signals produced when this wheat mitochondrial gene is hybridized with wheat DNA (Figure 3.6 B, Lane 8), and the background signal reduces the resolution of the bands and could have obscured other bands that were present in lower stoichiometries. In addition, this probe was 5.5kb in length, with the *nad5* coding region comprising only about 25% of the probe. Flanking sequences and the group II intron present in the probe may have contributed to the increased background and hybridization complexity. It is also possible that this sequence is present on two different physical forms of the mitochondrial genome, one of which is more abundant than the other. Since hybridization was carried out with total genomic DNA of *Larix* it is also not excluded that low intensity signals are to homologous DNA sequences to this probe are present in the nuclear or chloroplast genome.

Hybridization of probe *orf25* with *Larix* DNA resulted in a single hybridizing fragment for almost all restriction endonucleases (Figure 3.7 A). This indicates that this DNA sequence is present in the *Larix* mitochondrial genome as a single copy. However, hybridization to DNA enriched for mitochondrial DNA produced two or more fragments at varying intensities (Figure 3.7 B). Numerous hybridizing fragments could be the result of

FIGURE 3.7**Hybridization of *Larix leptolepis* Total DNA and mtDNA with Wheat Mitochondrial Probe *orf25*.**

- A.** *Larix leptolepis* total genomic DNA hybridized with *orf25*. Lane 1: Lambda *Hind*III digest molecular weight marker. *Larix leptolepis* DNA digested with *Bam*HI (Lane 2), *Dra*I (Lane 3), *Eco*RI (Lane 4), *Eco*RV (Lane 5), *Hind*III (Lane 6). Fragment sizes are summarized in Table 3.3.
- B.** *Larix leptolepis* mitochondrial DNA hybridized with *orf25*. Lane 1: Lambda *Hind*III digest molecular weight marker. *Larix leptolepis* DNA digested with *Bam*HI (Lane 2), *Dra*I (Lane 3), *Eco*RI (Lane 4), *Hind*III (Lane 5), *Kpn*I (Lane 6). Fragment sizes are summarized in Table 3.3.

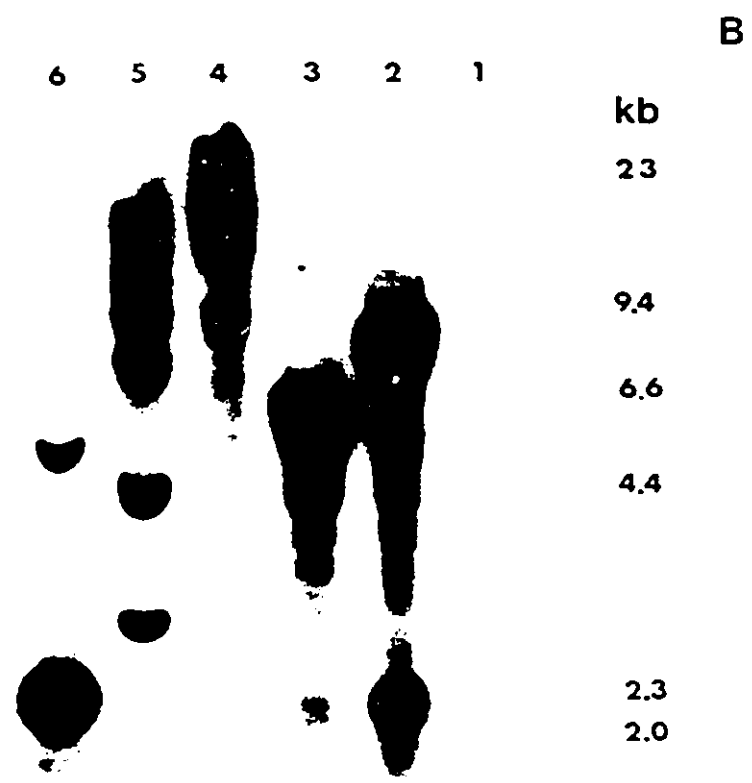
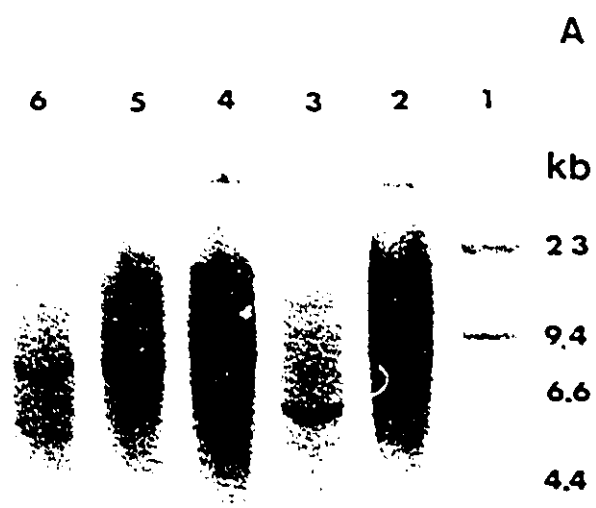
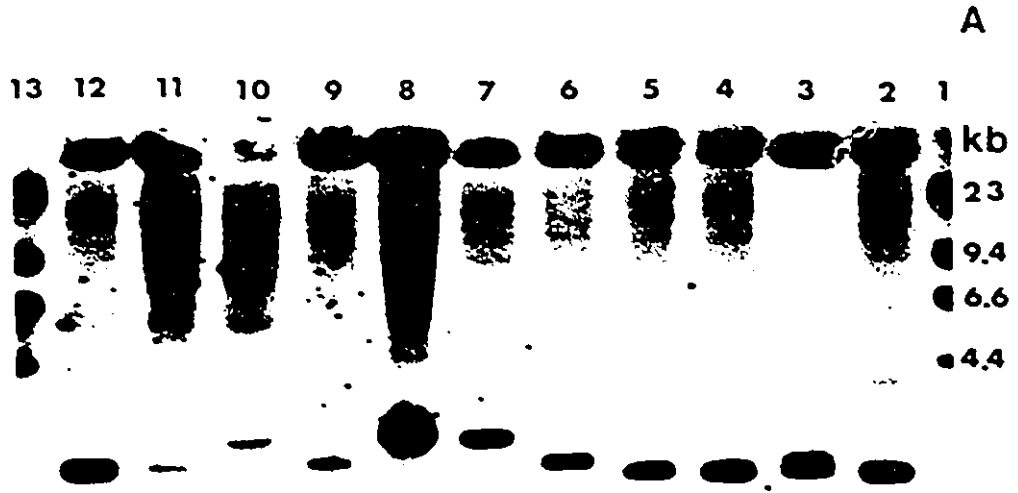


FIGURE 3.8**Total DNA from *Larix* Species and Other Gymnosperms Hybridized with *orf*:5**

- A.** Various *Larix* species total DNA digested with *Kpn*I and hybridized with *orf*:5. Lanes 1 and 13: Lambda DNA *Hind*III digest molecular weight marker. Lane 2: *Larix gmelini*. Lane 3: *L. laricina*. Lane 4 and 5: *L. olgensis*. Lane 6: *L. siberica*. Lanes 7 and 8: *L. decidua*. Lane 9: *L. leptolepis*. Lane 10: *L. decidua* X *L. leptolepis*. Lanes 11 and 12: *L. leptolepis* X *L. decidua*.
- B.** Gymnosperm species total genomic DNA digested with *Dra*I and hybridized with *orf*:5. Lane 1: Lambda DNA *Hind*III digest molecular weight marker. Lane 2: *Picea mariana*. Lane 3: *Ginkgo biloba*. Lane 4: *Pinus contorta*. Lane 5: *Ficea glauca*.



insufficient washing stringency, or there may be multiple copies of short homologous sequences. Sequences homologous to *orf25* were detected in other *Larix* species (Figure 3.8 A) and in other gymnosperms (Figure 3.8 B), as either single copy genes, or as two bands of unequal intensity. The hybridization banding pattern of *Picea mariana* (black spruce) and *Picea glauca* (white spruce) appear to be identical with respect to fragment sizes and relative intensities. This would indicate that *orf25* is present in many divergent plant species.

Hybridization of either probes *cox1* or *cob* with restriction endonuclease digested *Larix leptolepis* and *L. decidua* DNA usually resulted in two hybridizing bands of the same size for both species and of approximately equal intensity, with each of three restriction endonucleases (Figure 3.9 A and Figure 3.9 B). This could indicate that sequences homologous to *cox1* and *cob* are present in two copies in both these *Larix* species mitochondrial genomes, or that each of these enzymes have a single recognition site within the hybridizing sequence. Since hybridization was carried out with total genomic DNA of these *Larix* species it is also possible that DNA sequences homologous to this probe are present in the nuclear or chloroplast genomes.

TABLE 3.3: Hybridization results for mitochondrial genes of *Larix leptolepis*.

Sizes of hybridizing fragments are recorded in kilobase pairs.

Probe Enzyme	<i>atpA</i>	<i>atp9</i>	<i>nad3/ rps12</i>	<i>cox1</i>	<i>cob</i>	<i>nad5</i>	<i>orf25</i> Fig.3.7A
BamHI A	1.5	5.0	NS	NS	NS	4.0	8.1
B	11/3.5 ¹	14/2.3/0.5 ¹	NS	NS	NS	5.0	---
DraI A	NS	8.0/6.0	13	2.7	5.9/3.6 ¹	3.1	6.5
B	NS	3.3/1.8/1.5 ¹	---	---	---	5.5	---
EcoRI A	4.1	11/4.1/3.6 ¹	4.1	NS	NS	9.4	18
B	11	6.6	---	NS	NS	5.2	---
EcoRV A	NS	4.6/3.7 ¹	1.8	1.9/1.0	6.0/2.9 ¹	3.7	7.3
B	NS	8.8/1.2	---	2.4	---	---	---
HindIII A	6.0	5.4/2.8 ¹	3.0/0.8	NS	NS	5.5/2.6	7.5
B	1.8	4.6/1.0 ¹	---	NS	NS	---	---
KpnI A	9.0	10/8.8 ¹	---	0.5 ²	NS	8.2 ³	2.4*
B	---	---	---	---	NS	---	---

A = major band. B = minor band. --- = no band. 1 = bands of about equal intensity. *see Figure 3.15. NS = not shown. 2 = not clearly visible on Figure 3.9A. 3. not clearly visible on Figure 3.6B

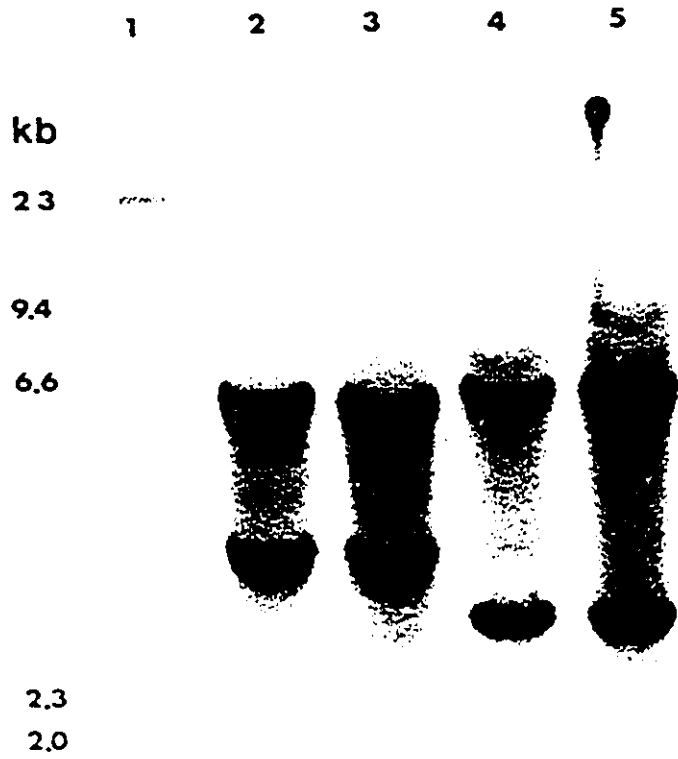
FIGURE 3.9**Hybridization of *Larix leptolepis* and *Larix decidua* DNA with Wheat Mitochondrial Gene Probes *coxI* and *cob***

- A.** *Larix leptolepis* and *Larix decidua* total genomic DNA hybridized with *coxI*. Lane 1: Lambda *Hind*III digest molecular weight marker. Lane 2: *Larix leptolepis* DNA digested with *Kpn*I. Lane 3: *Larix decidua* DNA digested with *Kpn*I. Lane 4: *Larix leptolepis* DNA digested with *Eco*RV. Lane 5: *Larix decidua* DNA digested with *Eco*RV. Lane 6: *Larix leptolepis* DNA digested with *Dra*I. Lane 7: *Larix decidua* DNA digested with *Dra*I. Fragment sizes are summarized in Table 3.3.
- B.** *Larix leptolepis* and *Larix decidua* total genomic DNA hybridized with *cob*. Lane 1: Lambda *Hind*III digest molecular weight marker. Lane 2: *Larix leptolepis* DNA digested with *Dra*I. Lane 3: *Larix decidua* DNA digested with *Dra*I. Lane 4: *Larix leptolepis* DNA digested with *Eco*RV. Lane 5: *Larix decidua* DNA digested with *Eco*RV. Fragment sizes are summarized in Table 3.3.

A



B



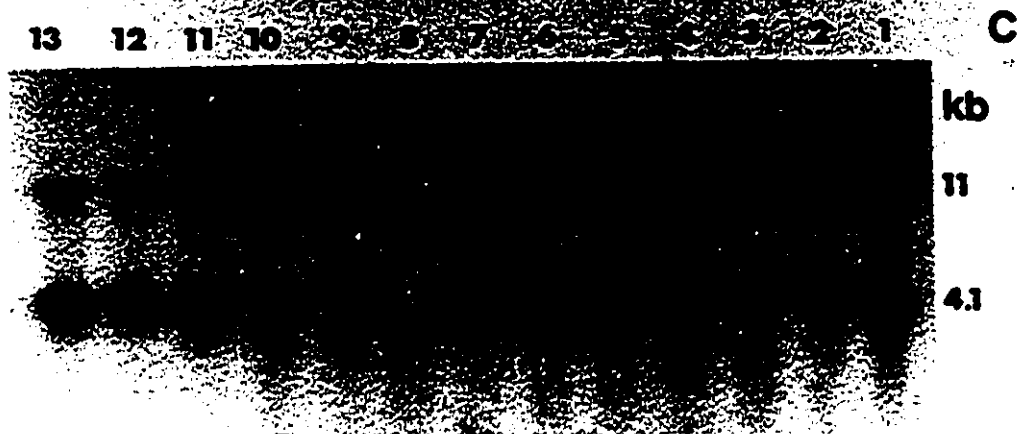
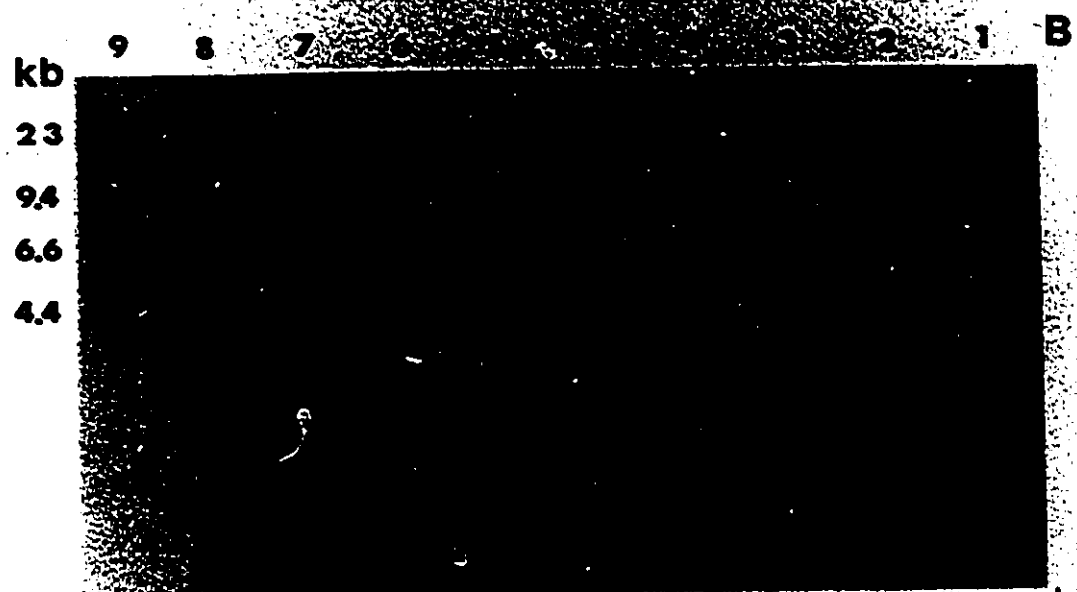
3.4. HYBRIDIZATION PATTERNS OF TREE DNA COMPARED TO *in vitro* MATERIAL AND REGENERATED PLANTS

In vitro cell culture can induce structural changes in the organization of the mitochondrial genome that can be detected by changes in restriction profiles. In addition, *in vitro* cell culture may also change the distribution of size classes of the mitochondrial DNA molecules and this can be detected by variations in restriction fragment stoichiometry. Restriction endonuclease digestion, followed by hybridization with specific wheat mitochondrial gene probes was used to determine if any structural alterations or mitochondrial molecule size class distribution changes had occurred during the *in vitro* somatic embryogenic cell culture of *Larix*.

Hybridization of a 2kb fragment containing sequences of the wheat mitochondrial *atpA* and *atp9* coding regions with DNA of *Larix leptolepis*, *L. decidua*, *L. decidua* X *L. leptolepis*, and *L. leptolepis* X *L. decidua* somatic embryogenic cell cultures and the corresponding tree DNAs digested with *EcoRI*, resulted in two bands of sizes 4.1kb and 11 kb that were of equal intensity in each of the cell cultures. The two bands were of unequal hybridization intensity for all four tree species, with the 4.1kb band hybridizing strongly and a less intense band hybridizing at 11kb (Figure 3.10 A). Similar results were obtained when the same samples were digested with *HindIII* and hybridized with the same probe (Figure 3.10 B). In this case the four cell cultures had two major hybridizing bands of 1.8kb and 6.0kb of equal intensities, whereas the four corresponding tree species had a strongly hybridizing band at 6.0kb and a less intense band at 1.8kb. The quantity of tree needle DNA that was loaded on the gel appears to be less than the amount of cell culture DNA loaded per lane. When DNA from the cell culture of *L. leptolepis* X *L. decidua* (L287) was digested with *EcoRI* and hybridized with sequences of the *atpA* and *atp9* coding regions and compared to the DNA from ten trees that had been regenerated from the corresponding cell culture, the 4.1kb band was of the same intensity as the 11kb band of the cell culture DNA, but was of higher intensity than the 11kb band of the regenerated tree DNA (Figure 3.10 C).

FIGURE 3.10**Comparison of DNA Hybridization Patterns Between *Larix* Trees and Cell Culture
using Wheat Mitochondrial Gene Probe *atpA/atp9*.**

- A. *Eco*RI digested *Larix* cell culture total DNA and *Larix* tree needle total DNA. Lane 1: *Larix decidua* DNA from cell culture. Lane 2: *L. decidua* X *L. leptolepis* DNA from cell culture. Lane 3: *L. leptolepis* X *L. decidua* DNA from cell culture. Lane 4: *L. leptolepis* DNA from cell culture. Lane 5: *Larix decidua* DNA from tree needles. Lane 6: *L. decidua* X *L. leptolepis* DNA from tree needles. Lane 7: *L. leptolepis* X *L. decidua* DNA from tree needles. Lane 8: *L. leptolepis* DNA from tree needles. Lane 9: Lambda *Hind*III digest molecular weight marker.
- B. *Hind*III digested *Larix* cell culture total DNA and *Larix* tree needle total DNA. Lane 1: *Larix decidua* DNA from cell culture. Lane 2: *L. decidua* X *L. leptolepis* DNA from cell culture. Lane 3: *L. leptolepis* X *L. decidua* DNA from cell culture. Lane 4: *L. leptolepis* DNA from cell culture. Lane 5: *Larix decidua* DNA from tree needles. Lane 6: *L. decidua* X *L. leptolepis* DNA from tree needles. Lane 7: *L. leptolepis* X *L. decidua* DNA from tree needles. Lane 8: *L. leptolepis* DNA from tree needles. Lane 9: Lambda *Hind*III digest molecular weight marker.
- C. *Eco*RI digested total DNA from trees regenerated from cell culture compared to total DNA from a tree grown from seed. Lane 1: *L. leptolepis* X *L. decidua* DNA from cell culture (L287). Lane 2: *L. leptolepis* X *L. decidua* DNA from mature tree needles. Lanes 3 to 13: DNA from needles of ten trees regenerated from cell culture (L287).



This would indicate that the hybridizing pattern of the regenerated tree DNA more closely resembles the natural tree than the *in vitro* cell culture from which it was derived.

In a parallel study the same DNA samples were digested with *Bam*HI and hybridized with a 5.5kb fragment containing the *nad5* coding region. DNA from the cell cultures had hybridizing bands of equal intensity at sizes of 5.0kb and 4.0kb. However, DNA from the corresponding tree species had a strongly hybridizing fragment of 4.0kb and a less intense 5.0kb fragment (Figure 3.11 A). In this experiment the quantity of tree needle DNA loaded in the gel lanes appears to be less than the amount of cell culture DNA loaded per lane. When DNA from the cell culture of *L. leptolepis* X *L. decidua* (L287) was digested with *Bam*HI and hybridized with sequences of the *nad5* coding region was compared to the DNA from ten trees that had been regenerated from the corresponding cell culture, the 5.0kb band was of the same intensity as the 4.0kb band of the cell culture DNA, but was of lower intensity than the 4.0kb band of the regenerated tree DNA (Figure 3.11 B). This would also indicate that the hybridizing pattern of the regenerated tree DNA more closely resembles the natural tree than the *in vitro* cell culture from which it was derived .

To further investigate mitochondrial DNA structural changes, DNA was isolated from three lines of hybrid *Larix* callus, one somatic embryogenic cell culture, one mature tree, and three trees regenerated from L287, was digested with *Cfo*I and hybridized with an 800bp fragment containing the coding region of the wheat mitochondrial *atp9* gene (Figure 3.12 A). Although the 3.8kb hybridizing fragment was common for all DNA sources, other fragments are polymorphic (Table 3.4). DNA pooled from 200mg of zygotic embryos of *L. leptolepis* X *L. decidua* open pollinated seeds did not hybridize with sufficient strength, therefore the hybridizing band sizes were not recorded. DNA from the non-embryogenic cell culture (L187, Lane 3) hybridized with *atp9* resulted in hybridization to two fragments of 2.5kb and 1.6kb that did not appear in the embryogenic cell culture (L287, Lane 4).

FIGURE 3.11

Comparison of DNA Hybridization Patterns Between *Larix* Trees and Cell Culture using Wheat Mitochondrial Gene Probe *nad5*.

- A. *Bam*HI digested *Larix* cell culture and tree needle total DNA hybridized with *nad5*.
Lane 1: *Larix decidua* DNA from cell culture. Lane 2: *L. decidua* X *L. leptolepis* DNA from cell culture. Lane 3: *L. leptolepis* X *L. decidua* DNA from cell culture. Lane 4: *L. leptolepis* DNA from cell culture. Lane 5: *Larix decidua* DNA from tree needles. Lane 6: *L. decidua* X *L. leptolepis* DNA from tree needles. Lane 7: *L. leptolepis* X *L. decidua* DNA from tree needles. Lane 8: *L. leptolepis* DNA from tree needles. Lane 9: Lambda *Hind*III digest molecular weight marker.
- B. Probe *nad5* hybridized with *Bam*HI digested DNA from trees regenerated from cell culture compared to a tree grown from seed. Lane 1 and 15: Lambda *Hind*III digest molecular weight marker. Lane 2: *L. leptolepis* X *L. decidua* DNA from cell culture (L287). Lane 3: *L. leptolepis* X *L. decidua* DNA from mature tree needles. Lanes 4 to 14: DNA from needles of ten trees regenerated from cell culture (L287).



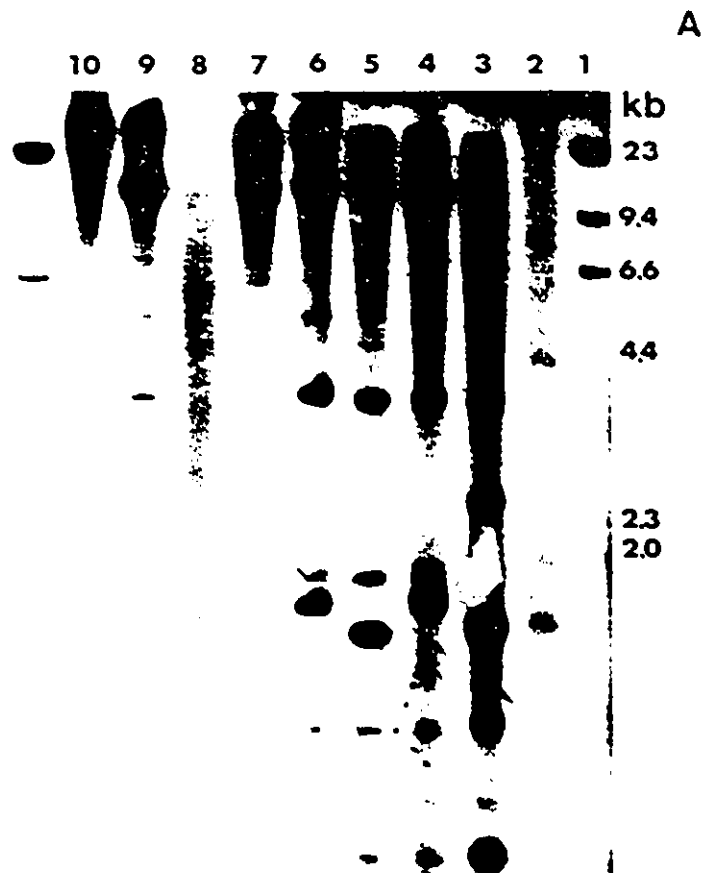
The 1.7kb fragment of L287 was absent in L187. The embryogenic cell culture initiated from an embryo of a *L. leptolepis* X *L. decidua* tree from New Brunswick (Lane 6) produced a banding pattern that differed from both L187 (Lane 3) and L287 (Lane 4) cell cultures, and also differed from callus (Lane 5) used to initiate this cell culture by the presence of a band at 5.4kb. In addition, although the regenerated trees were reported to be from L287(Lane 4), regenerated tree #4 (Lane 7) had a 2.5kb band that corresponds to one found in L187 (Lane 3), whereas regenerated tree #10 (Lane 9) had a 1.7kb fragment that corresponds to DNA from L287. DNA from regenerated tree #7 (Lane 8) appears to be degraded, therefore sizes of the hybridizing bands were not recorded. In addition, DNA from the mature tree did not hybridize strongly enough to produce reliable signals, therefore these data were not recorded.

This variation was investigated further because it has been previously demonstrated that there is genetic variation between individual trees of *Larix*, both at the phenotypic and isozyme levels (Rauter and Graham, 1983; Fins and Seeb, 1986). Since L187 and L287 were initiated from embryos extracted from cones that came from a bulked collection of two *L. leptolepis* X *L. decidua* trees from the plantation experiment 252-C, an additional study was undertaken to evaluate DNA from both source trees, in addition to more regenerated trees. Also included in this study was DNA extracted from L287 cell culture that had been cryopreserved followed by callus regeneration, L287 cell culture that had been regenerated from protoplasts, and somatic embryogenic cell culture LB89 which was initiated from an embryo of a *L. leptolepis* X *L. decidua* tree from the plantation experiment 252-C in 1989. The results of this study are shown in Figure 3.12 B. LB89 (Lane 1) gave a hybridization banding pattern that was similar to L287 (Lane 5), but with one unique and one new band. Cell culture regenerated from protoplasts of L287 (Lane 4) showed no polymorphic bands. However, cell culture regenerated from cryopreserved cells of L287 (Lane 3) had an additional band not present in the original cell culture of L287. This additional band was also evident in four trees regenerated from callus of L287 (Lanes 6, 9, 11,12).

FIGURE 3.12

Mitochondrial RFLP Analysis of Hybrid Larch DNA using Wheat Mitochondrial Gene Probe *atp9*.

- A. *Larix leptolepis* X *L. decidua* total DNA from nine sources digested with *Cfo*I and hybridized with *atp9*. Lane 1: Lambda *Hind*III digest molecular weight marker. Lane 2: DNA from embryos of *L. leptolepis* X *L. decidua*. Lane 3: DNA from non-embryogenic cell culture L187. Lane 4: DNA from embryogenic cell culture L287. Lane 5: DNA from an embryogenic cell callus culture initiated from a zygotic embryo of a *L. leptolepis* X *L. decidua* tree in New Brunswick. Lane 6: DNA from an embryogenic cell suspension culture initiated from the cell callus culture of the zygotic embryo of a *L. leptolepis* X *L. decidua* tree in New Brunswick. Lane 7: DNA from tree #4 regenerated from cell culture L287. Lane 8: DNA from tree #7 regenerated from cell culture L287. Lane 9: DNA from tree #10 regenerated from cell culture L287. Lane 10: DNA from *L. leptolepis* X *L. decidua* tree #9 of plantation experiment 252-C.
- B. *Larix leptolepis* X *L. decidua* total DNA from fourteen sources digested with *Cfo*I and hybridized with *atp9*. Lane 1: DNA from a somatic embryogenic cell culture of *L. leptolepis* X *L. decidua* initiated in 1989 (LB89). Lane 2: DNA from non-embryogenic cell culture L187. Lane 3: DNA from embryogenic cell culture L287, that had been regenerated after cryopreservation. Lane 4: DNA from embryogenic cell culture L287, that had been regenerated after protoplasting. Lane 5: DNA from embryogenic cell culture L287. Lane 6: DNA from tree #2 regenerated from cell culture L287. Lane 7: DNA from tree #4 regenerated from cell culture L287. Lane 8: DNA from tree #5 regenerated from cell culture L287 (degraded). Lane 9: DNA from tree #6 regenerated from cell culture L287. Lane 10: DNA from tree #7 regenerated from cell culture L287 (degraded). Lane 11: DNA from tree #8 regenerated from cell culture L287. Lane 12: DNA from tree #10 regenerated from cell culture L287. Lane 13: DNA from *L. leptolepis* X *L. decidua* tree #3 of plantation experiment 252-C. Lane 14: DNA from *L. leptolepis* X *L. decidua* tree #9 of plantation experiment 252-C.



The hybridization banding pattern of *L. leptolepis* X *L. decidua* tree #8 (Lane 13) matched the patterns of both L187 (Lane 2) and one tree regenerated from L287 (Lane 7). However, *L. leptolepis* X *L. decidua* tree #9 (Lane 14) had a banding pattern that was similar to L287 (Lane 4), but was unique by the absence of one band.

A preliminary investigation was undertaken to determine if variation in the mitochondrial genome might possibly exist within different parts or developmental stages of the tree. DNA was isolated from sexual buds, and needles at the top and bottom of *L. leptolepis* X *L. decidua* tree #1 of the plantation experiment 252-C, and from seedlings of *L. leptolepis* X *L. decidua* seedlot #7285630, Holbaek, Denmark. These seedlings were not genetically related to the hybrid trees. DNA from needles of branches at the top and bottom of the tree appear to have no polymorphic bands, but DNA from the buds had two unique bands (Figure 3.13). In addition, DNA from whole seedlings, including the roots, also had one unique polymorphic band. These preliminary results suggest that there could be mitochondrial DNA structural variation within the tree, but further studies are necessary.

TABLE 3.4: Mitochondrial DNA variation between regenerated trees and cell cultures: DNA digested with *Cfo*I and probed with *atp9*.

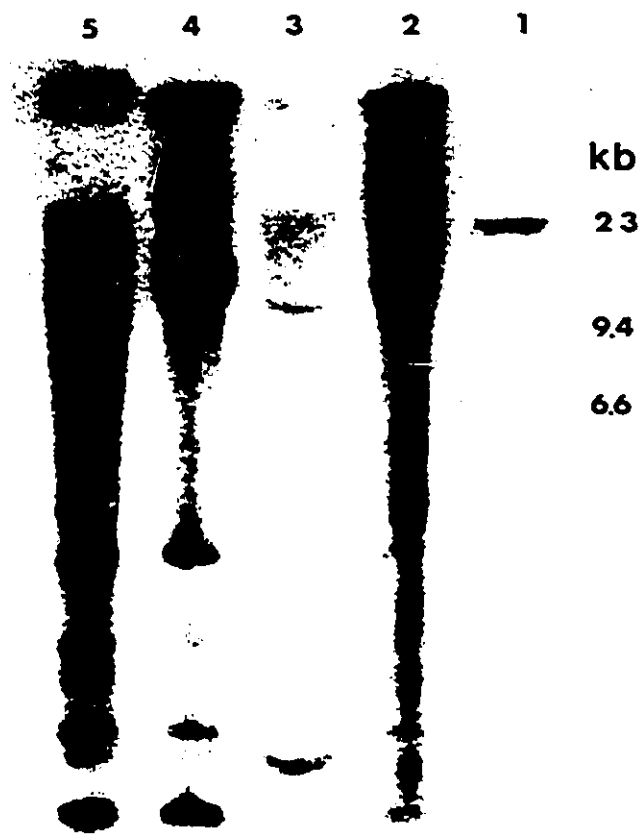
DNA SOURCE	SIZE OF HYBRIDIZING FRAGMENTS (kb)							
	5.4	5.0	3.8	2.5	1.9	1.7	1.6	1.5
Regen #10 ¹	+	-	+	-	-	+	-	-
Regen #4 ¹	-	-	+	+	-	-	-	+
Callus L287 ²	-	+	+	-	+	+	-	-
Callus L187 ³	-	-	+	+	+	-	+	-
Callus 1988 ⁴	-	-	+	-	+	-	-	+
Cell Culture 1988 ⁴	+	-	+	-	+	+	-	-

+presence of a hybridizing fragment of this size. - absence of a hybridizing fragment of this size.

1. Individual tree as numbered that has been regenerated from L287 *in vitro* somatic embryogenic callus culture.
2. Somatic embryogenic callus culture Line 2 initiated in 1987 as somatic embryogenic cells from a single immature embryo of *Larix leptolepis* X *L. decidua* tree #8 or #9 from the plantation experiment 252-C at PNFI.
3. Callus culture Line 1 initiated in 1987 as somatic embryogenic cells from a single immature embryo of *Larix leptolepis* X *L. decidua* tree #8 or #9 from the plantation experiment 252-C at PNFI. This culture has since lost its embryogenic potential.
4. These somatic embryogenic callus and cell cultures were initiated in 1988 from a single immature embryo of *Larix leptolepis* X *L. decidua* of a tree in New Brunswick.

FIGURE 3.13**Preliminary Investigation of Within-Tree Variation of *Larix* Mitochondrial DNA.**

*Cfo*I digested total genomic DNA from different parts and developmental stages of the *L. leptolepis* X *L. decidua* tree hybridized with wheat mitochondrial gene probe *atp9*. Lane 1: Lambda *Hind*III digest molecular weight marker. Lane 2: DNA from needles of a bottom branch of the tree. Lane 3: DNA from sexual buds. Lane 4: DNA from needles of a top branch of the tree. Lane 5: DNA isolated from whole seedlings of a different genotype.



3.5. INHERITANCE OF MITOCHONDRIAL DNA SEQUENCES IN LARIX HYBRID CROSSES

Various restriction digests of total DNA of *L. decidua* and *L. leptolepis* were hybridized to a number of wheat mitochondrial gene probes to identify RFLP's that were specific to each tree species (Figure 3.14). Digestion of total genomic DNA of *L. leptolepis* and *L. decidua* with the restriction endonucleases *Bam*HI, *Cfo*I, *Dra*I, *Eco*RI, *Eco*RV and *Hind*III did not reveal any restriction fragment length polymorphisms (RFLPs) when hybridized with *orf25*. However, digestion with *Kpn*I or *Msp*I produced RFLPs that were specific to *L. decidua* and *L. leptolepis* (Figure 3.14 Lanes 14,15 and 16,17). In addition, total genomic DNA of these two *Larix* species digested with *Dra*I and hybridized with *nad3/rps12* also produced RFLPs that were specific to *L. decidua* and *L. leptolepis* (Figure 3.16). Total DNA digested with *Kpn*I and hybridized with an 800bp *Bam*HI/*Mlu*I fragment of *orf25* derived from wheat produced a 2.4kb band unique to the *L. leptolepis* mitochondrial genome, and a 2.8kb band unique to the *L. decidua* mitochondrial genome (Figure 3.14). When eight trees of each reciprocal cross of these two *Larix* species were analyzed by restriction with *Kpn*I and hybridized with *orf25* all progeny had exclusively the band unique to the maternal parent (Figure 3.15 A). Stringent washing conditions of 0.1X SSC at 65°C removed all but two of the hybridizing bands (Figure 3.15 B). Similarly, total DNA digested with *Dra*I and hybridized with the *nad3/rps12* probe from wheat mitochondria produced a 13kb band unique to the *L. leptolepis* mitochondrial genome and a 10kb band unique to the *L. decidua* mitochondrial genome (Figure 3.16). When these same eight trees were analyzed by restriction with *Dra*I and hybridized with the *nad3/rps12* probe, mitochondrial inheritance is shown to be maternal, except in one tree which has a fragment that was either unique, or was similar to a fragment present at low levels in the maternal species (Figure 3.16). This variation is still under investigation.

FIGURE 3.14

Identification of RFLPs unique to *Larix leptolepis* or *Larix decidua* by Hybridization of Total Genomic DNA with Wheat Mitochondrial Probe *orf25*.

Lanes 1 and 18: Lambda DNA *Hind*III digest molecular weight marker. Even-numbered lanes contain DNA from *L. decidua*. Odd-numbered lanes contain DNA from *L. leptolepis*. Lanes 2 and 3: DNA digested with *Bam*HI. Lanes 4 and 5: DNA digested with *Cfo*I. Lanes 6 and 7: DNA digested with *Dra*I. Lanes 8 and 9: DNA digested with *Eco*RI. Lanes 10 and 11: DNA digested with *Eco*RV. Lanes 12 and 13: DNA digested with *Hind*III. Lanes 14 and 15: DNA digested with *Kpn*I. Lanes 16 and 17: DNA digested with *Msp*I.

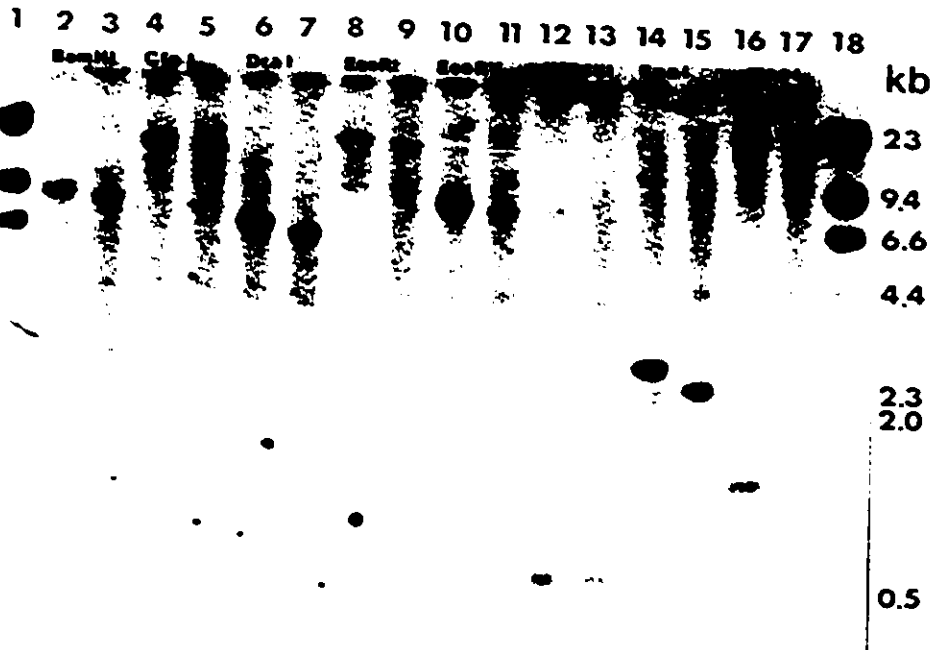


FIGURE 3.15

Inheritance patterns of hybrid *Larix* mitochondrial DNA sequences homologous to *orf25*.

- A.** Low stringency wash (2X SSC at 55°C). Lanes 1 and 20: Lambda DNA *Hind*III digest molecular weight marker. Lane 2: *L. decidua* digested with *Kpn*I. Lanes 3 to 10: *L. decidua* (female) X *L. leptolepis* (male) digested with *Kpn*I. Lanes 11-18: *L. leptolepis* (female) X *L. decidua* (male) digested with *Kpn*I. Lane 19: *L. leptolepis* digested with *Kpn*I.
- B.** High stringency wash (0.1X SSC at 65°C for 4 hours). Arrows indicate the sizes of bands that have faded more than the band at approximately 10kb. Lanes 1 and 20: Lambda DNA *Hind*III digest molecular weight marker. Lane 2: *L. decidua* digested with *Kpn*I. Lanes 3 to 10: *L. decidua* (female) X *L. leptolepis* (male) digested with *Kpn*I. Lanes 11-18: *L. leptolepis* (female) X *L. decidua* (male) digested with *Kpn*I. Lane 19: *L. leptolepis* digested with *Kpn*I.

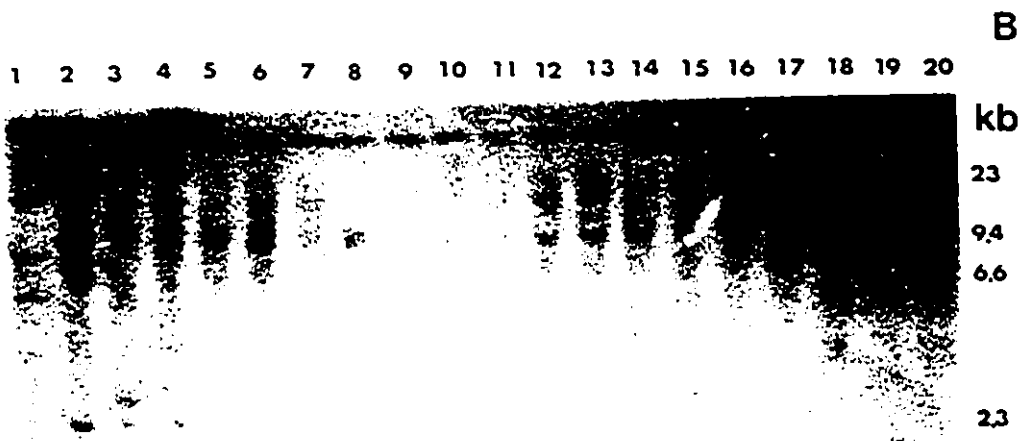
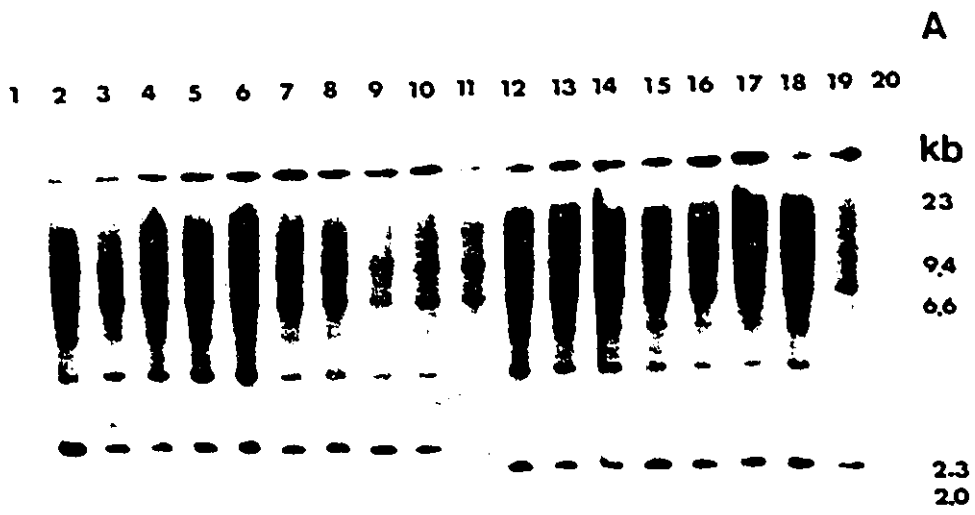


FIGURE 3.16

Inheritance patterns of hybrid *Larix* mitochondrial DNA sequences homologous to *nad3/rps12*.

Lanes 1 and 20: Lambda DNA *Hind*III digest molecular weight marker. Lane 2: *L. decidua* digested with *Dra*I. Lanes 3 to 10: *L. decidua* (female) X *L. leptolepis* (male) digested with *Dra*I. Lanes 11-18: *L. leptolepis* (female) X *L. decidua* (male) digested with *Dra*I Lane 19:*L. leptolepis* digested with *Dra*I.

20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

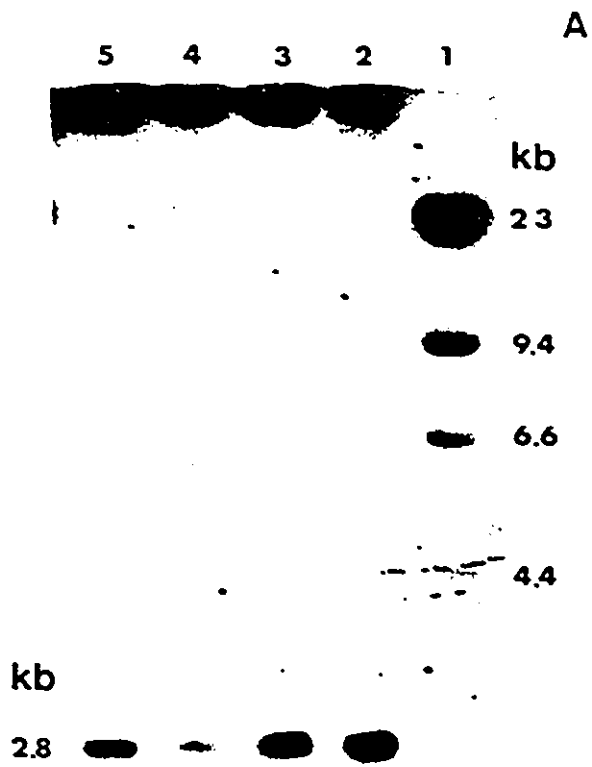


kb
23
9.4
6.6

It should have been feasible to use these same enzyme and mitochondrial gene probe combinations to detect maternal transmission of mitochondria in cell cultures of hybrid *Larix*. However, when total DNA from four cell cultures labelled as *L. decidua*, *L. leptolepis*, *L. decidua X L. leptolepis*, and *L. leptolepis X L. decidua* were digested with *KpnI* and hybridized with the *orf25* probe (Figure 3.17 A); or digested with *DraI* and hybridized with the *nad3/rps12* probe (Figure 3.17 B), all cell cultures had the same hybridizing fragment sizes for each probe. These fragment sizes corresponded to the *L. decidua* 2.8kb unique hybridizing fragment when *orf25* was used as a probe, and to the *L. decidua* 10kb hybridizing fragment when *nad3/rps12* was used as a probe. It is possible that the cell cultures were mislabelled, the original seed embryo used to initiate these cultures was specified incorrectly, or the crosses were not properly controlled.

FIGURE 3.17**RFLP analysis of *Larix* cell culture DNA by Hybridization with Wheat Mitochondrial Gene Probes *orf25* and *nad3/rps12***

- A.** *Larix* species cell culture DNA digested with *KpnI* and hybridized with *orf25*. Lanes 1: Lambda DNA *HindIII* digest molecular weight marker. Lane 2: *L. decidua*. Lane 3: *L. decidua* X *L. leptolepis*. Lane 4: *L. leptolepis* X *L. decidua*. Lane 5: *L. leptolepis*.
- B.** *Larix* species cell culture DNA digested with *DraI* and hybridized with *nad3/rps12*. Lane 1: *L. decidua*. Lane 2: *L. decidua* X *L. leptolepis*. Lane 3: *L. leptolepis* X *L. decidua*. Lane 4: *L. leptolepis*.



4. DISCUSSION

4.1. ISOLATION OF MITOCHONDRIAL DNA of *Larix*

Isolation and purification of mitochondrial DNA has been accomplished from many higher plants, for example wheat (Rode *et al.*, 1985), Brassica (Palmer *et al.*, 1983), and poplar (Radetzky, 1990), but there is no published reliable method to isolate mitochondrial DNA from conifers. The mitochondrial genome size of higher plants varies from 200-2500kb (Newton, 1988) and restriction endonuclease digestion of purified mitochondrial DNA has been used to estimate the genome size for some of the smaller mitochondrial genomes (Palmer *et al.*, 1983; Radetzky, 1990). In this study the isolation of mitochondrial DNA was attempted from cell cultures of *Larix leptolepis*, to develop a reliable isolation method, and to determine if a genome size could be estimated by restriction endonuclease analysis. A fresh weight of about 50g of cells at four days post-subculture were required to give about 15 μ g of DNA enriched for mitochondrial DNA. Tissue culture cells will usually yield 1-5g of purified mitochondria per 500g of cells depending on the status of the cells and the plant species, with an average yield of about 0.1 μ g of mitochondrial DNA per gram of cells (Schuster *et al.*, 1988).

Plant cells are difficult to break because of the rigid cell wall. Some procedures for the isolation of purified mitochondria made use of a blender to disrupt cell walls and release intact mitochondria (Schuster *et al.*, 1988; Hsu and Mullin, 1988). In other procedures a mortar and pestle was used for this purpose (Wilson and Chourey, 1984; Pay and Smith, 1988). Evaluation of the extent of cell breakage and release of cell contents by microscopic examination of the *Larix* cell homogenate showed that grinding the cells in cold homogenizing buffer with sand in a mortar with pestle disrupted more cell walls than homogenizing with a blender. Disruption of cells was done in the cold to minimize degradation of mitochondria (Schuster *et al.*, 1988). Enrichment of mitochondria in the cell lysate was accomplished by differential centrifugation steps, making use of the different

sedimentation rates of broken cells, nuclei, plastids, and mitochondria (Schuster *et al.*, 1988). Several repeats of low speed centrifugation were done to remove as much of the cell wall debris, nuclei and plastids as possible, and pelleting of the mitochondria removed much of the soluble compounds and ribosomal contamination (Schuster *et al.*, 1988). However, at this stage the mitochondrial fraction was still heavily contaminated with nuclear and plastid DNA and RNA due to broken plastids and nuclei, and to co-sedimenting ribosomes (Schuster *et al.*, 1988). To decrease the extent of this contamination a DNaseI digestion of the intact mitochondria was performed. Banding of the DNaseI-treated mitochondria on a density gradient has been used to obtain higher purification of the mitochondria (Jackson *et al.*, 1979; Bergman *et al.*, 1980; Pay and Smith, 1988; Schuster *et al.*, 1988). Mitochondrial density can vary among plant species, and can be a function of homogenization, centrifugation conditions, and physiological state of the plant material (Pay and Smith, 1988), and in the case of *Larix* the mitochondria appeared to have different densities within the cells, if all the bands that were generated in the Percoll gradient were mitochondria. Multiple bands of mitochondria have been observed with other plant species after centrifugation through a sucrose density gradient (Schuster *et al.*, 1988). These purification steps should complement each other because the separation in each step is based on different properties of the material. Centrifugation separates material mainly according to size, and Percoll gradient centrifugation separates according to density. A phase partition step, which separates on the basis of surface properties, could be added before the Percoll gradient to improve purity (Albertsson *et al.*, 1988). In this protocol both SDS and sarcosyl were used in the lysis step. This combination is more effective than either used alone because sarcosyl is more effective at protecting mitochondrial DNA from residual DNase activity, while SDS is more effective at lysing mitochondrial membranes (Hsu and Mullin, 1988). Incorporation of DIECA in the isolation buffer was found to be essential for the isolation of undegraded mitochondrial DNA of tobacco, peanut and sorghum (Wilson and Chourey, 1984) and was added to the *Larix* mitochondrial extraction buffer to protect the mitochondria.

The restriction endonuclease profile of *Larix* mitochondrial DNA consists of a large number of bands of varying intensities. The bands could not be counted because of the complexity of the profile with discrete bands not being clearly resolved. Some bands were more intense than others, indicating variations in stoichiometry of these fragments. The mitochondrial restriction profile of *Larix* appears to be similar in complexity to that of maize mitochondrial DNA digested with *EcoRI* (Wilson and Chourey, 1984) but appears to have more bands, and more band intensity variations, than those of sorghum and peanut (Wilson and Chourey, 1984), tobacco (Pay and Smith, 1988), and cotton mitochondrial DNA digested with *HindIII* (Hsu and Mullin, 1988).

The protocol outlined in Materials and Methods Section 2.2.3 can be used with limited reliability for the isolation of restrictable DNA from *Larix* somatic embryogenic cell cultures enriched specifically for mitochondrial DNA. Further purification of this mtDNA could perhaps be accomplished if the mitochondria were fluorescently tagged using cytochalsin-B and 3,3'-dihexyloxcarbocyanine iodide (Matzke and Matzke, 1989). This tagging could permit the mitochondrial bands on the Percoll gradient to be distinguished from those that were contaminants thereby increasing the purity of the resulting mitochondrial DNA. Restriction endonuclease analysis of *Larix* mitochondrial DNA isolated by the method outlined in Section 2.2.3 could not be used to estimate the size of the genome because of the complexity of the banding pattern. This may indicate that the genome is larger than those mitochondrial genomes for which this technique has been used for size estimation, or that background contamination by nuclear DNA is obscuring the banding pattern.

4.2. GENOME STRUCTURE OF *Larix* MITOCHONDRIA

To identify genes encoded by the mitochondrial genome of *Larix* which are common to most other higher plant mitochondrial genomes, DNA fragments from the coding sequences of well-characterized mitochondrial protein-coding genes (such as those described

in Section 1.1.3) of wheat were used as heterologous hybridization probes under moderate stringency conditions, exploiting the high sequence conservation between homologous genes of distantly related plant species. For example, the mitochondrial gene *cob* has a sequence homology of 98% for maize, wheat and rice (Dawson *et al.*, 1984; Schuster and Brennicke, 1985; Boer *et al.*, 1985; Kaleikau *et al.*, 1990). Several conifer genes have been sequenced and percent homologies to the corresponding angiosperm genes are listed in Table 1.1.2. However, the extent of homology of *Larix* mitochondrial gene sequences to those of a mitochondrial gene probe from wheat has not been determined, therefore the hybridization stringency was reduced to allow stable heteroduplexes to form if the homology was anywhere from about 65% to 100%. These stringency conditions are similar to those used in heterologous hybridization studies using maize mitochondrial gene probes to hybridize with total genomic DNA of *Calocedrus* and *Sequoia* (Neale *et al.*, 1989, 1991).

The stringency used in hybridization experiments is dependent on the melting temperature (T_m) of a DNA heteroduplex, which falls by 1°C for each percent sequence mismatch (Maniatis *et al.*, 1982). T_m is dependent on the G+C content of the DNA and the monovalent cation concentration in the hybridization solution according to the following formula: $T_m = 81.5 + 16.6(\log[Na^+]) + 0.41(\%G+C)$ in a hybridization solution without formamide. In this study hybridizations were carried out in a solution with a monovalent cation concentration of 0.85M at a temperature of 60°C. If the mitochondrial DNA of *Larix* has a G+C of 47%, as is common for higher plants, then this would allow for the formation of a heteroduplex with a maximum 35% sequence mismatch (i.e. sequences that had a homology of 65% or more). These hybridization conditions were satisfactory for the detection of homologous mitochondrial gene sequences in *Larix* for the seven wheat mitochondrial gene probes that were analyzed.

The complexity of the hybridization patterns for each mitochondrial gene was evaluated to compare the organization of *Larix* mitochondrial genes to mitochondrial genes for other higher plants. However, it was first necessary to perform various controls (Section

3.2) to minimize artifacts that would result from incomplete digestion of the mitochondrial DNA, or from hybridization of the wheat gene cloning vector with *Larix* sequences. Restriction endonuclease digestion conditions were shown to result in complete digestion of *Larix* mitochondrial DNA, and it was also confirmed that vector DNA sequences did not hybridize to any specific *Larix* mtDNA restriction fragments. In addition, digestion by a restriction endonuclease sensitive to 5-methylcytosine at the internal cytosine residue (the most common type of methylation found in nuclear DNA) was shown to result in the same hybridization banding pattern as the isoschizomer that is not affected by this type of methylation, indicating that 5-methylcytosine was not contributing to the complexity of the hybridization banding pattern. Therefore, the resulting hybridizing fragment pattern for each mitochondrial gene probe analyzed was determined to be specific hybridization of wheat mitochondrial gene sequences to homologous *Larix* mitochondrial gene sequences.

Wheat mitochondrial gene probes *atpA* and *atp9* hybridized with *Larix* DNA resulted in at least two hybridizing bands of unequal intensity for each gene. When the restriction endonucleases *EcoRI* and *KpnI* were used to digest *Larix* DNA, the hybridizing bands for *atpA* and *atp9* were of the same molecular weight, differing only in the relative intensities of the two bands. This may suggest that these two genes are closely linked in the *Larix* mitochondrial genome. In addition, hybridization data analysis suggests that *atpA* is likely to occur as two copies in the mitochondrial genome, whereas *atp9* appears to occur as more than two different physical forms in the mitochondrial genome of *Larix*. These two genes are also closely linked in wheat (Bonhomme *et al.*, 1989) and pea (Morikami and Nakamura, 1987). The ATPase alpha subunit (*atpA*) is encoded by the nuclear genome in most organisms (Neupert and Schatz, 1981), but it is encoded by the mitochondrial genome in higher plants (Boutry *et al.*, 1983), and the conifer, *Larix*. The *atp9* gene is located in different compartments in different organisms. In yeast and some higher plants, including *Larix*, this subunit is encoded by the mitochondrial genome. However, in mammals *atp9* is encoded by the nucleus, and in *Neurospora crassa* both the nucleus and mitochondrion

encode this gene.

Wheat mitochondrial gene probe *coxI* hybridized with restriction endonuclease digested *Larix* DNA resulting in two hybridizing bands of approximately equal intensities, suggesting that *coxI* is present in the *Larix* mitochondrial genome in two copies with different genomic locations. This gene also exists as two copies in *Oenothera*, but other plants such as soybean, pea, radish, wheat, rice and maize have only a single copy of this gene (Grabau, 1986; Kemmerer, *et al.*, 1989; Isaac *et al.*, 1985; Hiesel *et al.*, 1987; Makaroff *et al.*, 1991; Bonen *et al.*, 1987; Kadowaki *et al.*, 1989).

Hybridization of *Larix* DNA with wheat mitochondrial gene probe *cob* also resulted in two hybridizing bands of approximately equal intensities, suggesting that this gene is also present in the *Larix* mitochondrial genome in two complete copies with different genomic locations. This gene has a high sequence conservation (98%) among higher plants such as maize, wheat and rice (Dawson *et al.*, 1984; Schuster and Brennicke, 1985; Boer *et al.*, 1985; Kaleikau *et al.*, 1990).

Wheat mt gene probe *nad5* hybridized with restriction endonuclease digested *Larix* DNA resulting in two hybridizing bands of unequal intensities, suggesting that this DNA sequence exists in the mitochondrial genome of *Larix* as two copies of different sizes on different restriction fragments. Alternatively, this sequence may be present on two mitochondrial chromosomes of different abundance. In comparison, the *nad5* gene of *Oenothera* mitochondria occurs as one partial and two intact identical copies (Wissinger *et al.*, 1988). However, in sugar beet the *nad5* gene occurs as a single copy (Ecke *et al.*, 1990).

Hybridization of wheat probe *nad3/rps12* with *Larix* DNA resulted in a single hybridizing band. A single hybridizing fragment in *Larix* may suggest that a similar set of linked genes is present in the mitochondrial genome. A set of genes in maize mitochondria is almost identical to the set in wheat (Gualberto *et al.*, 1988). In addition, a similar set of genes is present in rice mitochondria, linked in the same order, but not preceded by tRNA^{ser} (Suzuki *et al.*, 1991).

Sequences homologous to wheat mitochondrial *orf25* are usually present as a single hybridizing fragment in *Larix*, suggesting that this DNA sequence is present in the mitochondrial genome as a single copy. This open reading frame has also been identified as a single copy gene in wheat (Bonen *et al.*, 1990), and tobacco (Stamper *et al.*, 1987). Although *orf25* sequences have not been identified by sequence comparison in either animal or fungal mitochondrial genomes, it is present in diverse angiosperm plant species such as maize, tobacco, bean, wheat, pea and rice (Dewey *et al.*, 1986; Bonen *et al.*, 1990; Stamper *et al.*, 1987). Properties such as transcription and sequence conservation suggest that *orf25* encodes a functional plant mitochondrial gene. This gene also hybridized to the conifers *Picea glauca*, *Picea mariana* and *Pinus contorta*, in addition to *Ginkgo biloba* which is believed to be the common ancestor of all conifers. This result seems to indicate that *orf25* could be ubiquitous amongst higher plants, since it is common to both angiosperms and gymnosperms.

In general, these data suggest that many of the mitochondrial genes of *Larix leptolepis* are present in more than one copy. Other plants, such as wheat, have predominantly single copy genes with only a few multiple copy genes (Table 4.2). A comprehensive characterization of *Larix* mitochondrial protein-coding genes would require gene isolation and sequence determination with a comparative analysis to previously sequenced mitochondrial genes of other organisms. A further comparison of nuclear and purified mitochondrial DNA could be used to verify that the hybridizing bands are in fact of mitochondrial origin. However, the strength of the hybridization signals would indicate that these hybridizing bands are in fact mitochondrial and in high copy number, since hybridization signals from single or low copy nuclear sequences often requires several weeks exposure to X-ray film before bands are visible.

TABLE 4.2. Comparison of gene copy number in several plant species for genes used in this study.

SPECIES	SINGLE COPY GENES	MULTIPLE COPY GENES
Maize	4	1
Wheat	7	0
Rice	3	0
Oenothera*	1	4
Larix**	2	5

*Copy number was determined from cell culture material, rather than plants.

**Predicted from complexity of hybridization profiles, this study.

4.3. TISSUE CULTURE INDUCED VARIATIONS OF THE MITOCHONDRIAL GENOME

Higher plant *in vitro* cell culture has been shown to increase the molecular diversity of the mitochondrial genome by inducing structural changes in its organization. There is evidence in various plant cell culture systems for the appearance of novel restriction fragments, disappearance of previously existing fragments, variation in restriction fragment stoichiometry, and amplification of subgenomic molecules (Nagy *et al.*, 1981; McNay *et al.*, 1984; Hartmann *et al.*, 1987, Rode *et al.*, 1987). This is the first study to describe molecular diversity of the mitochondrial genome induced by *in vitro* somatic embryogenic cell culture of a conifer species. Qualitative and quantitative mtDNA differences have been illustrated between a four year old embryogenic cell culture of *Larix X eurolepis*, ten trees that were regenerated from this embryogenic cell culture, and callus of this embryogenic cell culture that had been regenerated after cryopreservation for seven days.

When compared to the corresponding callus culture (L287), *Larix* regenerated trees can be considered as mitochondrial variants because of changes in the number of restriction fragments or variations in the stoichiometry of the fragments. When compared to each other, *Larix* regenerated trees do not appear to be exact genetic clones, because of the differences observed in the sizes of the hybridizing fragments. Events such as inversions, deletions/insertions, transpositions, and amplifications could result in the detection of loss or

gain of a restriction fragment. Only a small fraction of the mitochondrial genome was monitored since a single probe was used to detect these variations, therefore it is possible that variation exists in other parts of the genome not yet evaluated. Mitochondrial recombination in somatic hybrids of tobacco also displayed distinctly different mitochondrial DNA restriction patterns, with unique bands which were not present in either parent (Belliard *et al.*, 1979). In addition, altered mitochondrial restrictions patterns have been found in other somatic hybrids or cybrids including *Petunia* (Boeshore *et al.*, 1983), *Daucus* (Matthew and Widholm, 1985) and *Pennisetum X Panicum* (Ozias-Akins *et al.*, 1987). Rearrangements have also been detected in T-cytoplasm maize plants regenerated from cell culture, and the mitochondrial restriction patterns varied from one regenerant to another (Gengenbach *et al.*, 1981).

Alterations in the distribution of the size classes of mitochondrial DNA molecules, resulting in variation in restriction fragment stoichiometry, have also been reported in maize (McNay *et al.*, 1984), rice (Saleh *et al.*, 1990) and *Chenopodium* (Dorfel *et al.*, 1989), and quantitative variation in some restriction fragments encompassing a given recombinatory repeat have been reported in studies of the mitochondrial genome of *Triticum* (Hartman *et al.*, 1987; Rode *et al.*, 1987). Loss of mtDNA subgenomic molecules in response to *in vitro* cell culture has been detected in wheat (Rode *et al.*, 1987) leading to reduced genomic complexity. In addition, it has been shown that the region of the maize mitochondrial *atpA* gene varies considerably within the N and S- maize cytoplasms, suggesting that selective amplification of pre-existing subgenomic molecules could be responsible for the variation detected in this region of the genome (Small *et al.*, 1987). In contrast, no mtDNA rearrangements were detected in antibiotic resistant tobacco protoplast and callus culture regenerants (Nagy *et al.*, 1983).

Variations in the organization of the mitochondrial genome between *Larix* embryogenic and non-embryogenic cell cultures were detected by differences in the restriction patterns. Differences in the organization of the mitochondrial genome between

Triticum embryogenic and non-embryogenic callus cultures have also been detected, and it has been suggested that a particular mitochondrial genome organization may be correlated with the ability of cultured wheat cells to regenerate whole plants (Rode *et al.*, 1988).

Variation in the stoichiometry of certain fragments appears to be the predominant effect of prolonged culture. These changes could arise from alterations in the relative abundance of specific subgenomic DNA molecules. Alternatively, variations such as mutation/deletion in restriction sites could also lead to the rearrangements observed in this study. Hartman *et al.* (1988) and Rode *et al.* (1987) concluded that most changes of the mt genome *in vitro* occur during the early process of wheat callogenesis and that these changes are conserved in later subcultures. However, Shirzadegan *et al.* (1991) found no evidence that structural changes of mtDNA were limited to any particular culture period in *Brassica*, nor did they find that all changes were maintained in subsequent generations. The process of mitochondrial genome variation could be dependent on the cell culture species and should probably be examined independently in the *Larix* somatic embryogenic system using a time course study from initiation, through maintenance and regeneration.

4.4. INHERITANCE OF MITOCHONDRIAL DNA IN *LARIX*

Use of mitochondrial DNA restriction fragment length polymorphisms has demonstrated maternal inheritance of mitochondrial DNA sequences in *Larix* hybrids. Although maternal transmission of mitochondrial DNA is predominant, it is not universal. Ericksen and Kemble (1990) reported paternal inheritance of mitochondria in the angiosperm *Brassica napus*. In addition, paternal inheritance has been reported in the green alga *Chlamydomonas reinhardtii* (Boynton *et al.*, 1987). Neale *et al.* (1989) demonstrated paternal transmission in the gymnosperm *Sequoia sempervirens* (Family Taxodiaceae). Paternal inheritance of mitochondria may also occur in a gymnosperm of the Family Cupressaceae, *Calocedrus decurrens* (Neale *et al.*, 1991).

The various modes of mitochondrial inheritance exhibited by different gymnosperms

can be explained by the characteristics of the male gametes and the ultrastructural changes occurring during fertilization. During fertilization in members of the Orders Ginkgoales and Cycadales, the oldest living gymnosperms, a single spermatozoid nucleus enters the egg cell, with the male cytoplasm being discarded just outside the egg cell near the point of entry (Whatley, 1982). This ultrastructural evidence suggests that only the maternal organelles would be transmitted to the embryo.

Members of the order Coniferales are believed to be descendants of these ancient gymnosperms (Dallimore and Jackson, 1966). Male gametes of the Families Cupressaceae and Taxodiaceae are large complete cells and during fertilization the entire male gamete, including its cytoplasm, penetrates the egg cell. With proembryo development the cytoplasm of the fertilized female gamete degenerates, becomes radically altered and finally collapses (Chesnoy and Thomas, 1971). As a consequence the embryo consists of two nuclei and a cytoplasm originating primarily from the deep zone of the male gamete cytoplasm complete with male plastids, mitochondria and cytoplasmic RNA. This results in the paternal transmission of mitochondria and chloroplasts to the offspring. Molecular techniques have identified paternal mitochondria and chloroplast inheritance in the conifers *Sequoia sempervirens* (Neale *et al.*, 1989) and *Calocedrus decurrens* (Neale *et al.*, 1991). These conifers belong to the Families Taxodiaceae and Cupressaceae, respectively, and therefore have fertilization mechanisms and male gamete characteristics consistent with paternal transmission of both organelles.

In contrast, in members of the Families Pinaceae and Cephalotaxaceae the two male gametes are represented simply by two nuclei and during fertilization the entire contents of the pollen tube enters the egg cell cytoplasm (Lawson, 1907). The mitochondria and plastids brought by the pollen tube are morphologically distinct from those of the female gamete. In the egg cell the plastids are hypertrophied and transformed into large inclusion bodies and subsequently degenerate, but those plastids arising from the male pollen tube seem to persist, together with the mitochondria of male origin (Chesnoy and Thomas, 1971, Owens and

Morris, 1990). Therefore, in conifers of the Families Pinaceae and Cephalotaxaceae the plastids of maternal origin are excluded and only the male plastids remain in the embryo. Although male mitochondria are present in the fertilized egg cell these are greatly outnumbered by the abundance of female mitochondria aggregated around the nucleus. Random drift would lead to the predominance of maternal mitochondria in subsequent divisions (Birky, 1983). Furthermore, studies of fertilization at the ultrastructural level in some members of the Families Podocarpaceae and Taxaceae indicate that the mechanism of fertilization is similar to that outlined for Pinaceae and Cephalotaxaceae. Consequently, members of the Families Pinaceae, Cephalotaxaceae, Podocarpaceae and Taxaceae exhibit paternal plastid inheritance and maternal mitochondrial inheritance.

Mitochondrial DNA sequences of hybrid crosses of the conifer *Larix* have been shown in this study to be of maternal origin, whereas chloroplast DNA is predominantly paternal in origin (Szmidt *et al.*, 1987). Since *Larix* is a member of the Pinaceae Family, these results are consistent with earlier ultrastructural evidence that would predict maternal mitochondrial transmission and paternal chloroplast transmission to progeny (Camefort, 1969; Chesnoy and Thomas, 1971). This phenomenon makes it possible to determine both the maternal and paternal contributions in hybrid and introgressed *Larix* populations. In addition, phylogenies based on data of maternal and paternal origin from an individual tree will be more precise than a single lineage analysis.

5. CONCLUSION

This thesis has shown that available molecular technology can be applied to the identification and characterization of genes of the mitochondrial genome of the conifer *Larix*. Seven well-characterized genes known to be encoded by the mitochondria in other higher plants have also been shown to be encoded by the mitochondria of *Larix*. Studies of the coding capacity of this conifer mitochondrial genome are important for evolutionary and phylogenetic purposes because, as gymnosperms, conifers are members of one of the oldest living groups of higher plants. The complexity of the restriction endonuclease banding pattern of the *Larix* mitochondrial genome suggests that this genome has the capacity to code for many more genes than have been evaluated in this thesis. Of special interest, because of the ancient origin of gymnosperms, would be the sequences of *Larix* mitochondrial ribosomal rRNA genes since these sequences diverge at a particularly slow rate, and have proven to support the endosymbiotic origin of mitochondria (Gray, 1988).

This thesis has also shown that *in vitro* cell culture of *Larix* results in changes in the mitochondrial genome. Plant genetic material has been noted to be capable of undergoing change and generating variability (Marx, 1984). This variation could be the basis for an adaptive response and can lead to changes in phenotype over time as well as production of seed from genetically different branches. Within tree variation, involving entire branches, suggests that certain tissue has developed from meristematic cells containing newly arisen somatic mutations. This genetic mosaicism, for example variegation, is known to occur in the conifer *Cryptomeria* (Ohba et al., 1971), and chloroplast DNA variation between needle samples obtained from different parts of the crown of *Pinus* has also been reported (Govindaraju et al., 1988). Furthermore, in Eucalyptus trees differences in volatile oil composition of different branches is directly correlated to resistance to a defoliating insect (Edwards et al., 1990). In this study of *Larix*, preliminary results suggest that there may be

variation in the mitochondrial genome between the developing sexual buds that give rise to the male and female flowers (and subsequently the seed from which somatic embryogenic cell cultures are derived) and needle tissue from the top of the tree. As a comparison, DNA alterations have arisen in flax during mitosis in response to stress, producing mosaic plants - DNA in the bottom quarter of the stem has the original composition, but altered DNA appears in the upper portion and seeds, and is heritable. A similar mosaic has been found in triticale (Marx, 1984).

Mitochondrial DNA reorganization, without any apparent deleterious effects has been documented in eucaryotes including yeast and fungi, plants, *Drosophila* and animals (Degoul *et al.*, 1991). However, deletions of mtDNA have been reported in cases of human myopathies and neuromuscular diseases (Degoul *et al.*, 1991). In plants energy is needed for seed germination, and mitochondrial respiration supplies much of the needed energy. Mitochondria of the embryo are responsible for producing energy used for elongation of the root/shoot axis and subsequent growth. A study of soybean seed deterioration during storage demonstrated that decreased respiration rates and energy-coupling efficiency of mitochondria in the embryo lead to a decline in seed vigor (Ferguson *et al.*, 1990).

Deletions of mtDNA have been reported to cause phenotypic abnormalities in plants (Kofer *et al.*, 1991; Roussel *et al.*, 1991). An examination of cybrids of tobacco regenerated after protoplast fusion of male-sterile cultivars showed a correlation between mitochondrial DNA changes and changes in floral phenotype, and changes in floral phenotype were always predictive of changes in mtDNA (Kofer *et al.*, 1991). Therefore, this provides evidence that mitochondrial genes are involved in several stages of stamen and petal development and tissue culture induced alterations of the mitochondrial genome could have a significant effect on the fertility of regenerated plants.

Cytoplasmic male sterility (CMS) is caused by a mitochondrial mutation resulting in the inability of plants to produce functional pollen, and is one of two well documented plant mitochondrial dysfunctions. This phenotype has been found in many species including

important crops such as maize, rice, sorghum and sunflower (Lonsdale, 1987b review). CMS is often associated with alloplasmic hybrids and it has been suggested that it is an expression of mitochondrial-nuclear incompatibility, or caused by the expression of certain polypeptides encoded by mitochondrial genes.

The other known plant mitochondrial dysfunction is caused by DNA rearrangements in the mitochondrial genome of maize which create the nonchromosomal stripe (NCS) mutants with abnormal growth phenotypes (Roussell *et al.*, 1991). These phenotypes include striped sectors of pale-green or yellow tissue in the leaves, overall reduction in plant height and vigor and sectors of abnormal corn on ears. Mutant mitochondria exert a pleiotropic effect upon the chloroplasts whereby the bundle sheaths do not accumulate starch and mesophyll chloroplasts fix CO₂ less efficiently. In addition, the light reactions of photosynthesis appear to be affected indicating that chloroplasts may have arrested at late stage in development. The chloroplast may not be only cellular compartment affected. Plants with NCS mutant mt appear to survive only when normal mt are present - heteroplasmy. The NCS mutants are caused by rearrangements in either the *coxII* gene or *nad4* gene.

In conclusion, changes in the mitochondrial genome of plants caused by natural mutations, or induced by tissue culture, may result in a heteroplasmic population of normal and mutant mitochondria (homoplasmic mutants are usually lethal), which in turn can lead to abnormal phenotypes and undesirable traits such as reduced vigor and growth yield.

Unlike mtDNA, the chloroplast genome has been found to remain unchanged during extended *in vitro* culture (Shirzadegan *et al.*, 1989), except for the large scale deletions reported in albino plantlets regenerated from anther cultures of wheat, barley and rice (Day and Ellis, 1984, 1985; Harada *et al.*, 1991). The chloroplast genome was not examined in the present study of *Larix*, but information on its stability or variation during tissue culture would be useful in determining the magnitude of DNA changes induced by *in vitro* cell culture.

Restriction fragment length polymorphisms of mitochondrial DNA can be used to

distinguish between closely related tree species in zones of introgression, for example *Picea engelmanni* and *P. sitchensis* (Sutton *et al.*, 1991). This thesis has identified probes and restriction endonucleases appropriate for investigating the mode of mitochondrial inheritance in hybrid crosses of *Larix decidua* and *L. leptolepis*. As a result, it was shown that mtDNA was maternally inherited in hybrids of these two species. In addition, probes and restriction endonucleases were identified that were useful for the demonstration of restriction fragment length polymorphisms between different *Larix* species. This technique will make it possible to obtain information on the maternal origin of hybrids for breeding purposes. Lineage analysis would be more complete however if this information was combined with similar data on the chloroplast genome of *Larix*, which is paternally inherited (Szmidt *et al.*, 1987). Analysis of cpDNA and mtDNA could also assist breeders in the verification of hybrids resulting from controlled pollination.

REFERENCES

- Albertsson, P-A.; Andersson, B.; Larsson, C.; Akerlund, H-E. Phase partition - a method for purification and analysis of cell organelles and membrane vesicles. *Methods Biochem. Anal.* 28: 115-145
- Armstrong, C.L.; Phillips, R.L. 1988. Genetic and cytogenetic variation in plants regenerated from organogenic and friable, embryogenic tissue cultures of maize. *Crop Sci.* 28: 363-369
- Attardi, G.; Schatz, G. 1988. Biogenesis of mitochondria. *Annu. Rev. Cell Biol.* 4: 289-333
- Avisé, J.C.; Vrijenhoek, R.C. 1987. Mode of inheritance and variation of mitochondrial DNA in hybridogenetic fishes of the genus *Poeciliopsis*. *Mol. Biol. Evol.* 4: 514-525
- Begu, D.; Graves, P-V.; Litvak, S.; Araya, A. 1989a. Nucleotide sequences of the F₀-ATPase subunit 9 gene from two lines of wheat. *Nucleic Acids Res.* 17: 9491
- Begu, D.; Graves, P-V.; Litvak, S.; Araya, A. 1989b. Nucleotide sequence of the F₁-ATPase subunit alpha gene from two lines of wheat. *Nucleic Acids Res.* 17: 9492
- Belliard, G; Vedel, F.; Pelletier, G. 1979. Mitochondrial recombination in cytoplasmic hybrids of *Nicotiana tabacum* by protoplast fusion. *Nature* 281: 401-403
- Bendich, A.J. 1991. Moving picture of DNA released upon lysis from bacteria, chloroplasts, and mitochondria. *Protoplasma* 160: 121-130
- Bendich, A.J.; Smith, S.B. 1990. Moving pictures and pulsed-field gel electrophoresis show linear DNA molecules from chloroplasts and mitochondria. *Curr. Genet.* 17: 421-425
- Bendich, A.J.; Gauriloff, L.P. 1984. Morphometric analysis of cucurbit mitochondria: the relationship between chondriome volume and DNA content. *Protoplasma* 119: 1-7
- Bergman, A.; Gardstrom, P.; Ericson, I. 1980. Method to obtain a chlorophyll-free preparation of intact mitochondria from spinach leaves. *Plant Physiol.* 66: 442-445

- Birky, C.W. Jr. 1988. Evolution and variation in plant chloroplast and mitochondrial genomes. In: Plant Evolutionary Biology, Chapman & Hall, London, New York. pp.23-82
- Birky, C.W. 1983. Relaxed cellular controls and organelle heredity. *Science* 222: 468-475
- Bland, M.M.; Levings, C.S.III.; Matzinger, D.F. 1986. The tobacco mitochondrial ATPase subunit 9 gene is closely linked to an open reading frame for a ribosomal protein. *Mol. Gen. Genet.* 204: 8-16
- Boblenz, K.; Nothnagel, T.; Metzloff, M. 1990. Paternal inheritance of plastids in the genus *Daucus*. *Mol. Gen. Genet.* 220: 489-491
- Boer, P.H.; McIntosh, J.E.; Gray, M.W.; Bonen, L. 1985. The wheat mitochondrial gene for apocytochrome b: absence of a ribosome binding site. *Nucleic Acids Res.* 13: 2281-2292
- Boeshore, M.; Lifshitz, I.; Hanson, M.R.; Izhar, S. 1983. Novel arrangements in the mitochondrial genomes of somatic hybrids derived from CMS and fertile *Petunia* lines. *Mol. Gen. Genet.* 190: 459-467
- Bonen, L.; Huh, T.Y.; Gray, M.W. 1980. Can partial methylation explain the complex fragment patterns observed when plant mitochondrial DNA is cleaved with restriction endonucleases? *FEBS Letters* 111: 340-346
- Bonen, L.; Boer, P.H.; McIntosh, J.E.; Gray, M.W. 1987. Nucleotide sequence of the wheat mitochondrial gene for subunit I of cytochrome oxidase. *Nucleic Acids Res.* 15: 6734
- Bonen, L.; Bird, S.; Belanger, L. 1990. Characterization of the wheat mitochondrial *orf25* gene. *Plant Mol. Biol.* 15: 793-795
- Bonhomme, S.; Bird, S.; Bonen, L. 1989. Comparison of the wheat mitochondrial *atp9* gene sequence with mitochondrial and chloroplast homologues from other plants. *Plant Mol. Biol.* 13: 395-397
- Bousquet, J.; Strauss, S.H.; Doerksen, A.H.; Price, R.A.; Hipkins, V.D. 1990. Diversity of *rbcL* sequences in the Pinaceae. In: Abstracts, IUFRO Molecular Genetics Working

- Party. S2.04.06. Lake Tahoe, California, September 30-October 4, 1990
- Boutry, M.; Briquet, M.; Goffeau, A. 1983. The alpha subunit of a plant mitochondrial F₁-ATPase is translated in mitochondria. *J. Biol. Chem.* 258: 8524-8526
- Boyle, T.J.B.; Nieman, T.C.; Magnussen, S.; Veen, J. 1989. Species, provenance, and progeny tests of the genus *Larix* by the Petawawa National Forestry Institute. Inf. Rep. PI-X-94 70pp.
- Boynton, J.E.; Harris, E.H.; Burkhart, B.D.; Lamerson, P.M.; Gillham, N.W. 1987. Transmission of mitochondrial and chloroplast genomes in crosses of *Chlamydomonas*. *Proc. Natl. Acad. Sci. USA* 84: 2391-2395
- Braun, C.J.; Levings, C.S.III. 1985. Nucleotide sequence of the F₁-ATPase alpha subunit gene from maize mitochondria. *Plant Physiol.* 79: 571-577
- Brears, T.; Curtis, G.J.; Lonsdale, D.M. 1989. A specific rearrangement of mitochondrial DNA induced by tissue culture. *Theor. Appl. Genet.* 77: 620-624
- Brown, P.T.H.; Gobel, E.; Lorz, H. 1991. RFLP analysis of *Zea mays* callus cultures and their regenerated plants. *Theor. Appl. Genet.* 81: 227-232
- Brown, W.M.; Prager, E.M.; Wang, A.; Wilson, A.C. 1982. Mitochondrial DNA sequences of primates: tempo and mode of evolution. *J. Mol. Evol.* 18: 225-239
- Buzzo, K.; Fouts, D.L.; Wolstenholme, D.R. 1978. *EcoRI* cleavage site variants of mitochondrial DNA molecules from rats. *Proc. Natl. Acad. Sci. USA* 75: 909-913
- Camefort, H. 1968. Sur l'organisation du neocytoplasme dans les proembryons tetranuclees du *Larix decidua* Mill. (*Larix europea* D.C.) et l'origine des mitochondries et des plastes de l'embryon de cette espece. *Comptes Rend. Acad. Sci. Paris* 266(D): 88-91
- Cavalier-Smith, T. 1987. Eukaryotes with no mitochondria. *Nature* 326: 332-333
- Chase, C.D.; Pring, D.R. 1985. Circular plasmid DNAs from mitochondria of *Sorghum bicolor*. *Plant Mol. Biol.* 5: 303-312
- Chase, C.D.; Pring, D.R. 1986. Properties of the linear N1 and N2 plasmid-like DNAs from mitochondria of cytoplasmic male sterile *Sorghum bicolor*. *Plant Mol. Biol.* 6: 53-64

- Chaumont, F.; Boutry, M.; Briquet, M.; Vassarotti, A. 1988. Sequence of the gene encoding the mitochondrial F1-ATPase alpha subunit from *Nicotiana plumbaginifolia*. *Nucleic Acids Res.* 16: 6247
- Cheliak, W.M.; Wang, J.; Pitel, J.A. 1988. Population structure and genetic diversity in Tamarack, *Larix laricina* (Du Roi) K. Koch. *Can. J. For. Res.* 18: 1318-1324
- Chesnoy, L.; Thomas, M.J. 1971. Electron microscopy studies on gametogenesis and fertilization in gymnosperms. *Phytomorph.* 21: 50-63
- Chiu, W-L.; Stubbe, W.; Sears, B.B. 1988. Plastid inheritance in *Oenothera*: organelle genome modifies the extent of biparental plastid transmission. *Curr. Genet.* 13: 181-189
- Chowdhury, M.K.U.; Schaeffer, G.W.; Smith, R.L.; DeBonte, L.R.; Matthews, B.F. 1990. Mitochondrial DNA variation in long-term tissue cultured rice lines. *Theor. Appl. Genet.* 80: 81-87
- Copes, D. L. 1981. Isozyme uniformity in western red cedar seedlings from Oregon and Washington. *Can. J. For. Res.* 11: 451-453
- Cullis, C.A.; Creissen, G.P.; Gorman, S.W.; Teasdale, R.D. 1988. The 25S, 18S, and 5S ribosomal RNA genes from *Pinus radiata* D. Don. In: *Molecular genetics of forest trees. Proceedings, Second IUFRO Working Party on Molecular Genetics.* Petawawa National Forestry Institute Information Report PI-X-80 pp. 34-40
- Dale, J.W.; Greenaway, P.J. 1984. Small-scale plasmid DNA preparation. In: *Methods in Molecular Biology Vol. 2: Nucleic Acids.* J.M. Walker ed. Humana Press, Clifton, New Jersey. pp 191-196
- Dale, R.M.K. 1981. Sequence homology among different size classes of plant mtDNAs. *Proc. Natl. Acad. Sci. USA* 78: 4453-4457
- Dallimore, W.; Jackson, A.B. 1966. *A Handbook of Coniferae and Ginkgoaceae.* Fourth edition. Revised by S. G. Harrison. Edward Arnold (Publishers) Ltd. London.
- Dawid, I.B.; Blackler, A.W. 1972. Maternal and cytoplasmic inheritance of mitochondrial

- DNA in *Xenopus*. *Devel. Biol.* 29: 152-161
- Dawson, A.J.; Jones, V.P.; Leaver, C.J. 1984. The apocytochrome b gene in maize mitochondria does not contain introns and is preceded by a potential ribosome binding site. *EMBO J.* 3: 2107-2113
- Day, A.; Ellis, T.H.N. 1984. Chloroplast DNA deletions associated with wheat plants regenerated from pollen: possible basis for maternal inheritance of chloroplasts. *Cell* 39: 359-368
- Day, A.; Ellis, T.H.N. 1985. Deleted forms of plastid DNA in albino plants from cereal anther culture. *Curr. Genet.* 9: 671-678
- DeBonte, L.R.; Matthews, B.F. 1984. Rapid isolation and purification of plastid and mitochondrial DNA from carrot cell suspensions. *Plant Mol. Biol. Rep.* 2: 32-36
- Degoul, F.; Nelson, I.; Amselem, S.; Romero, N.; Obermaier-Kusser, B.; Ponsot, G.; Marsac, C.; Lestienne, P. 1991. Different mechanisms inferred from sequences of human mitochondrial DNA deletions in ocular myopathies. *Nucleic Acids Res.* 19: 493-496
- Dewey, R.E.; Schuster, A.M.; Levings, C.S.III; Timothy, D.H. 1985. Nucleotide sequence of F₀-ATPase proteolipid (subunit 9) gene of maize mitochondria. *Proc. Natl. Acad. Sci USA* 82: 1015-1019
- Dewey, R.E.; Levings, C.S.III.; Timothy, D.H. 1986. Novel recombinations in the maize mitochondria! genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. *Cell* 44: 439-449
- Dhillon, S.S. 1987. DNA in trees species. In: *Cell and Tissue Culture in Forestry. Vol. 1. General principles and biotechnology.* Edited by Bonga, J.M. and Durzan, D.J. Martinus Nijhoff Publishers pp298-313.
- Dorfel, P.; Weihe, A.; Knosche, R.; Borner, T. 1989. Mitochondrial DNA of *Chenopodium album* (L.): a comparison of leaves and suspension cultures. *Curr. Genet.* 16: 375-380
- Ecke, W.; Schmitz, U.; Michaelis, G. 1990. The mitochondrial *nad5* gene of sugar beet

- (*Beta vulgaris*) encoding a subunit of the respiratory NADH dehydrogenase. *Curr. Genet.* 18: 133-139
- Eckenrode, V.K.; Levings, C.S. III. 1986. Maize mitochondrial genes. *In Vitro Cell & Dev. Biol.* 22: 169-176
- Edallo, S.; Zucchini, C.; Perenzin, M.; Salamini, F. 1981. Chromosomal variation and frequency of spontaneous mutation associated with *in vitro* culture and plant regeneration in maize. *Maydica* 26: 39-56
- Edwards, P.B.; Wanjura, W.J.; Brown, W.V. 1990. Mosaic resistance in plants. *Nature* 347: 434
- Edwardson, J.R. 1970. Cytoplasmic male sterility. *Bot. Rev.* 36: 341-420
- Erickson, L.; Kemble, R. 1990. Paternal inheritance of mitochondria in rapeseed (*Brassica napus*). *Mol. Gen. Genet.* 222: 135-139
- Evans, D.A.; Sharp, W.R. 1983. Single gene mutations in tomato plants regenerated from tissue culture. *Science* 221: 949-951
- Fairbanks, D.J.; Smith, S.E.; Brown, J.K. 1988. Inheritance of large mitochondrial RNAs in alfalfa. *Theor. Appl. Genet.* 76: 619-622
- Feret, P.P.; Bergmann, F. 1976. Gel electrophoresis of proteins and enzymes. In: Miksche, J.P. (ed.) *Modern methods in forest genetics*. Springer, Berlin Heidelberg New York, pp49-77
- Fins, L. Seeb, L.W. 1986. Genetic variation in allozymes of western larch. *Can. J. For. Res.* 16: 1013-1018
- Fowler, D.P. 1986. Strategy for the genetic improvement of *Larix* species and hybrids in the Maritimes. *Proc 1986 Larch Workshop, New Brunswick Forest Research Advisory Committee, Fredericton, N.B.* pp 73-78
- Galun, E.; Arzee-Gonen, P.; Fluhr, R.; Edelman, M.; Aviv, D. 1982. Cytoplasmic hybridization in *Nicotiana*: Mitochondrial DNA analysis in progenies resulting from fusion between protoplasts having different organelle constitutions. *Mol. Gen Genet.*

186: 50-56

- Gengenbach, B.G.; Connelly, J.A. 1981. Mitochondrial DNA variation in maize plants regenerated during tissue culture selection. *Theor. Appl. Genet.* 59: 161-167
- Giles, R.E.; Blanc, H.; Cann, H.M.; Wallace, D.C. 1980. Maternal inheritance of human mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* 77: 6715-6719
- Gilham, N.W. 1978. *Organelle heredity*. Raven Press, New York. pp. 1-79, 405-415
- Glaubitz, J.C.; Carlson, J.E. 1990. Nucleotide sequence of a portion of the western red cedar mitochondrial gene for subunit I of cytochrome oxidase. Abstract in: 5th Annual Meeting of the Canadian Society for Plant Molecular Biology, Dalhousie University, Halifax, Nova Scotia, June 11-13, 1990
- Goblet, J-P.; Boutry, M.; Duc, G.; Briquet, M. 1983. Mitochondrial plasmid-like molecules in fertile and male sterile *Vicia faba* L. *Plant Mol. Biol.* 2: 305-309
- Goraczniak, R.M.; Augustyniak, H. 1989. Characterization and sequence of lupin mitochondrial plasmid-like DNA. *Curr. Genet.* 16: 469-471
- Govindaraju, D.R.; Wagner, D.B. 1988. Chloroplast DNA variation within individual trees of a *Pinus banksiana*-*Pinus contorta* sympatric region. *Can. J. For. Res.* 18: 1347-1350
- Grabau, E.A.; Asleson, C.M.; Hegenbach, B.G. 1990. Nucleotide sequence and transcription of the soybean mitochondria ATPase subunit 9 gene. *Plant Mol. Biol.* 15: 183-186
- Grabau, E. A. 1986. Nucleotide sequence of the cytochrome oxidase subunit I gene from soybean mitochondria. *Plant Mol. Biol.* 7: 377-384
- Gray, M.W.; Cedergren, R.; Abel, Y.; Sankoff, D. 1989. On the evolutionary origin of the plant mitochondrion and its genome. *Proc. Natl. Acad. Sci. USA* 86: 2267-2271
- Grisvard, J.; Seignac, M.; Chateau, M.; Branchard, M. 1990. Changes in a repetitive DNA sequence during callus culture of *Cucumis melo*. *Plant Sci.* 72: 81-91
- Grivell, L. 1983. Mitochondrial DNA. *Sci. Am.* 248: 78-89

- Gualberto, J.M.; Wintz, H.; Weil, J-H.; Grienenberger, J-M. 1988. The genes coding for subunit 3 of NADH dehydrogenase and for ribosomal protein S12 are present in the wheat and maize mitochondrial genomes and are co-transcribed. *Mol. Gen. Genet.* 215: 118-127
- Gyllensten, U.; Wharton, D.; Josefsson, A.; Wilson, A.C. 1991. Paternal inheritance of mitochondrial DNA in mice. *Nature* 352: 255-257
- Hakman, I.; Fowke, L.C.; von Arnold, S.; Eriksson, T. 1985. The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway Spruce). *Plant Sci.* 38: 53-95
- Handa, H.; Nakajima, K. 1991. Nucleotide sequence and transcription analyses of the rapeseed (*Brassica napus* L.) mitochondrial F1-ATPase alpha subunit gene. *Plant Mol. Biol.* 16: 361-364
- Harada, T.; Sato, T.; Asaka, D.; Matsukawa, I. 1991. Large-scale deletions of rice plastid DNA in anther culture. *Theor. Appl. Genet.* 81: 157-161
- Hartmann, C.; Henry, Y.; De Buyser, J.; Aubry, C. Rode, A. 1989. Identification of new mitochondrial genome organizations in wheat plants regenerated from somatic tissue cultures. *Theor. Appl. Genet.* 77: 169-175
- Hartmann, C.; de Buyser, J.; Henry, Y.; Falconet, D.; Lejeune, B.; Benslimane, A-A.; Quetier, F.; Rode, A. 1987. Time-course of mitochondrial genome variation in wheat embryogenic somatic tissue cultures. *Plant Sci.* 53: 191-198
- Hiesel, R.; Schobel, W.; Schuster, W.; Brennicke, A. 1987. The cytochrome oxidase subunit I and subunit III genes in *Oenothera* mitochondria are transcribed from identical promoter sequences. *EMBO J.* 6: 29-34
- Hipkins, V.D.; Tsai, C.H.; Strauss, S.H. 1990. Sequence of the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase from a gymnosperm, Douglas-fir. *Plant Mol. Biol.* 15: 505-507
- Hsu, C.L.; Mullin, B.C. 1988. A new protocol for isolation of mitochondrial DNA from

- cotton seedlings. *Plant Cell Rep.* 7: 356-360
- Hutchison, K. W.; Singer, P.B.; Greenwood, M.S. 1990a. Structure and expression of the *cab* gene family during maturation in conifers. In: Abstracts, IUFRO Molecular Genetics Working Party. S2.04.06. Lake Tahoe, California, September 30-October 4, 1990
- Hutchison, K. W.; Harvie, P.D.; Singer, P.B.; Brunner, A.F.; Greenwood, M.S. 1990b. Nucleotide sequence of the small subunit of ribulose-1,5-bisphosphate carboxylase from the conifer *Larix laricina*. *Plant Mol. Biol.* 14: 281-284
- Hutchison, C.A. III.; Newbold, J.E.; Potter, S.S.; Edgell, M.H. 1974. Maternal inheritance of mammalian mitochondrial DNA. *Nature* 251: 536-538
- Isaac, P.G.; Brennicke, A.; Sunbar, S.M.; Leaver, C.J. 1985. The mitochondrial genome of fertile maize (*Zea mays* L.) contains 2 copies of the gene encoding the alpha subunit of the F1-ATPase. *Curr. Genet.* 10: 321-328
- Isaac, P.G.; Jones, V.P.; Leaver, C.J. 1985. The maize cytochrome C oxidase subunit I gene: sequence, expression and rearrangement in cytoplasmic male sterile plants. *EMBO J.* 4: 1617-1623
- Jackson, C.; Dench, J.E.; Hall, D.O.; Moore, A.L. 1979. Separation of mitochondria from contaminating subcellular structures utilizing silica sol gradient centrifugation. *Plant Physiol.* 64: 150-153
- Jansson, S.; Gustafsson, P. 1990. Type I and type II genes for the chlorophyll *a/b*-binding protein in the gymnosperm *Pinus sylvestris* (Scots pine): cDNA cloning and sequence analysis. *Plant Mol. Biol.* 14: 287-296
- John, P.; Whatley, F.R. 1975. *Paracoccus denitrificans* and the evolutionary origin of the mitochondrion. *Nature* 254: 495-498
- Kadowaki, K.; Kazama, S.; Suzuki, T. 1990. Nucleotide sequence of the F1-ATPase alpha subunit gene from rice mitochondria. *Nucleic Acids Res.* 18: 1302
- Kadowaki, K.; Suzuki, T.; Kazama, S.; Oh-fuchi, T.; Sakamoto, W. 1989. Nucleotide

- sequence of the cytochrome oxidase subunit I gene from rice mitochondria. *Nucleic Acids Res.* 17: 7519
- Kaleikau, E.K.; Andre, C.P.; Doshi, B.; Walbot, V. 1990a. Sequence of the rice mitochondrial gene for apocytochrome b. *Nucleic Acids Res.* 18: 372
- Kaleikau, E.K.; Andre, C.P.; Walbot, V. 1990b. Sequence of the F₀-ATPase proteolipid (*atp9*) gene from rice mitochondria. *Nucleic Acids Res.* 18: 370
- Kazama, S.; Suzuki, T.; Kadowaki, K.; Akihama, T. 1990. Nucleotide sequence of the F₀-ATPase subunit 9 gene from tomato mitochondria. *Nucleic Acids Res.* 18: 5879
- Kemble, R.J.; Bedbrook, J.R. 1980. Low molecular weight circular and linear DNA molecules in mitochondria from normal and male-sterile cytoplasms of *Zea mays*. *Nature* 284: 565-566
- Kemble, R.J.; Yarrow, S.A.; Wu, S-C.; Barsby, T.L. 1988. Absence of mitochondrial and chloroplast DNA recombinations in *Brassica napus* plants regenerated from protoplasts, protoplast fusions and anther culture. *Theor. Appl. Genet.* 75: 875-881
- Kemmerer, E.C.; Kao, T.; Deng, G.; Wu, R. 1989. Isolation and nucleotide sequence of the pea cytochrome oxidase subunit I gene. *Plant Mol. Biol.* 13: 121-124
- Kenny, J.R.; Dancik, B.P.; Florence, L.Z.; Nargang, F.E. 1988. Nucleotide sequence of the carboxy-terminal portion of a lodgepole pine actin gene. *Can J. For. Res.* 18: 1595-1602
- Khoshoo, T.N. 1960. Chromosome numbers in gymnosperms. *Silv. Genet.* 10: 1-9
- Kirk, J.T.O.; Tilney-Bassett, R.A.E. 1978. The plastids: their chemistry, structure, growth and inheritance. 2nd edition, Elsevier/North-Holland Biomedical Press, Amsterdam. pp. 251-554
- Klimaszewska, K. 1989. Plantlet development from immature zygotic embryos of hybrid larch through somatic embryogenesis. *Plant Sci.* 63: 95-103
- Kofer, W.; Glimelius, K.; Bonnett, H.T. 1991. Modifications of mitochondrial DNA cause changes in floral development in homeotic-like mutants of tobacco. *Plant Cell* 3: 759-

769

- Kohler, R.H.; Lossl, A.; Zetsche, K. 1990. Nucleotide sequence of the F1-ATPase alpha subunit gene of sunflower mitochondria. *Nucleic Acids Res.* 18: 4588
- Kool, A.J.; de Haas, J.M.; Mol, J.N.M.; van Marrewijk, G.A.M. 1985. Isolation and physicochemical characterization of mitochondrial DNA from cultured cells of *Petunia hybrida*. *Theor. Appl. Genet.* 69: 223-233
- Larkin, P.J.; Scowcroft, W.R. 1981. Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* 60: 197-214
- Lawson, A.A. 1907. The gametophytes and embryo of the Cupressineae with special reference to *Libocedrus decurrens*. *Ann. Bot.* 21: 281-301
- Lansman, R.A.; Avise, J.C.; Huettel, M.D. 1983. Critical experimental test of the possibility of "paternal leakage" of mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* 80: 1969-1971
- Leroy, P.; Bazetouz, S.; Quetier, F.; Delbut, J.; Berville, A. 1985. A comparison between mitochondrial DNA of an isogenic male-sterile (S) and male-fertile (F) couple (HA89) of sunflower. *Curr. Genet.* 9: 242-251
- Levings, C.S. III. 1983. The plant mitochondrial genome and its mutants. *Cell* 32: 659-661
- Levings, C.S. III; Sederoff, R.R. 1983. Nucleotide sequence of the S-2 mitochondrial DNA from the S cytoplasm of maize. *Proc. Natl. Acad. Sci. USA* 80: 4055-4059
- Levings, C.S. III.; Brown, G.G. 1989. Molecular biology of plant mitochondria. *Cell* 56: 171-179
- Levy, A.A.; Andre, C.P.; Walbot, V. 1991. Analysis of a 120-kilobase mitochondrial chromosome in maize. *Genetics* 128: 417-424
- Lewis-Smith, A.C.; Chamberlain, M.; Smith, S.M. 1990. Genetic and chromosomal variation in *Petunia hybrida* plants regenerated from protoplast and callus cultures. *Biol. Plant. (Praha)* 32: 247-255
- Lidholm, J.; Gustafsson, P. 1991. Homologues of the green algal *gidA* gene and the

- liverwort *frxC* gene are present on the chloroplast genomes of conifers. *Plant Mol. Biol.* 17: 787-798
- Lidholm, J.; Szmidt, A.; Gustafsson, P. 1988. Lack of one rDNA repeat and duplication of *psbA* in the chloroplast genome of lodgepole pine (*Pinus contorta* Dougl.). In: Cheliak, W.M.; Yapa, A.C., eds. *Molecular genetics of forest trees*. PNFI Inf. Rep. PI-X-80, pp 67-74
- Lonsdale, D.M. 1984. A review of the structure and organization of the mitochondrial genome of higher plants. *Plant Mol. Biol.* 3: 201-206
- Lonsdale, D.M.; Hodge, T.P.; Fauron, C.M-R. 1984. The physical map and organization of the mitochondrial genome from the fertile cytoplasm of maize. *Nucleic Acids Res.* 12: 9249-9261
- Lonsdale, D.M. 1987a. The physical and genetic map of the mitochondrial genome from the WF9-N fertile cytoplasm. *Maize Genet. Coop. Newsl.* 61: 148-149
- Lonsdale, D.M. 1987b. Cytoplasmic male sterility: a molecular perspective. *Plant Physiol. Biochem.* 25: 265-271
- Lonsdale D.M. 1989. The plant mitochondrial genome. In: *The biochemistry of plants: a comprehensive treatise*. Vol. 15, A. Marcus, ed. (New York, Academic Press)
- Makaroff, C.A.; Apel, I.J.; Palmer, J.D. 1991. The role of *coxI*-associated repeated sequences in plant mitochondrial DNA rearrangements and radish cytoplasmic male sterility. *Curr. Genet.* 19: 183-190
- Maniatis, T.; Fritsch, E.F.; Sambrook, J. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Press, Cold Spring Harbor, New York
- Marmur, J.; Doty, P. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.* 5: 109-118
- Marx, J.L. Instability in plants and the ghost of Lamarck. *Science* 224: 1415-1416
- Matthews, B.F.; DeBonte, L.R. 1985. Chloroplast and mitochondrial DNAs of carrot and its wild relatives. *Plant Mol. Biol. Rep.* 3: 12-14

- Matthews, B.F.; Widholm, J.M. 1985. Organelle DNA compositions and isoenzyme expression in an interspecific somatic hybrid of *Daucus*. *Mol. Gen. Genet.* 198: 371-376
- Matzke, .A.; Matzke, A.J.M. 1986. Visualization of mitochondria and nuclei in living plant cells by the use of a potential-sensitive fluorescent dye. *Plant Cell Environ.* 9: 73-78
- McNay, J.W.; Chourey, P.S.; Pring, D.R. 1984. Molecular analysis of genomic stability of mitochondrial DNA in tissue cultured cells of maize. *Theor. Appl. Genet.* 67: 433-437
- Mejnartowicz, M. 1991. Inheritance of chloroplast DNA in *Populus*. *Theor. Appl. Genet.* 82: 477-480
- Mettler, I.J. 1987. A simple and rapid method for minipreparation of DNA from tissue cultured plant cells. *Plant Mol. Biol. Rep.* 5: 346-349
- Metzlaff, M.; Borner, T.; Hagemann, R. 1981. Variations of chloroplast DNAs in the genus *Pelargonium* and their biparental inheritance. *Theor. Appl. Genet.* 60: 37-41
- Miksche, J.P. 1967. Variation in DNA content of several gymnosperms. *Can. J. Genet. Cytol.* 9: 717-722
- Millar, C.I. 1983. A step cline in *Pinus muricata*. *Evolution (Lawrence, Kans.)*, 37: 311-319
- Morikami, A.; Nakamura, K. 1987. Structure and expression of pea mitochondrial F1-ATPase alpha subunit gene and its pseudogene involved in homologous recombination. *J. Biochem.* 101: 967-976
- Morikami, A.; Nakamura, K. 1987b. The pea mitochondrial ATPase subunit 9 gene is located upstream of the ATPase alpha subunit gene. *Nucleic Acids Res.* 15: 4692
- Muller, E.; Brown, P.T.H.; Hartke, S.; Lorz, H. 1990. DNA variation in tissue-culture-derived rice plants. *Theor. Appl. Genet.* 80: 673-679
- Murashige, T.; Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497
- Murray, M.G.; Thompson, W.F. 1980. Rapid isolation of high molecular weight plant

- DNA. *Nucleic Acids Res.* 8: 4321-4325
- Nagy, F.; Torok, I.; Maliga, P. 1981. Extensive rearrangements in the mitochondrial DNA in somatic hybrids of *Nicotiana tabacum* and *Nicotiana knightiana*. *Mol. Gen. Genet.* 183: 437-439
- Nagy, F.; Lazar, G.; Menczel, L.; Maliga, P. 1983. A heteroplasmic state induced by protoplast fusion is a necessary condition for detecting rearrangements in *Nicotiana* mitochondrial DNA. *Theor. Appl. Genet.* 66: 203-207
- Neale, D.B.; Sederoff, R.R. 1989. Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. *Theor. Appl. Genet.* 77: 212-216
- Neale, D.B.; Wheeler, N.C.; Allard, R.W. 1986. Paternal inheritance of chloroplast DNA in Douglas-fir. *Can. J. For. Res.* 16: 1152-1154
- Neale, D.B.; Marshall, K.A.; Sederoff, R.R. 1989. Chloroplast and mitochondrial DNA are paternally inherited in *Sequoia sempervirens* D. Don Endl. *Proc. Natl. Acad. Sci. USA* 86: 9347-9349
- Neale, D.B.; Marshall, K.A.; Harry, D.E. 1991. Inheritance of chloroplast and mitochondrial DNA in incense-cedar (*Calocedrus decurrens*). *Can. J. For. Res.* 21: 717-720
- Neupert, W.; Schatz, G. 1981. How proteins are transported into mitochondria. *Trends Biochem. Sci.* 6: 1-4
- Newton, K.J. 1988. Plant mitochondrial genomes: organization, expression and variation. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 39: 503-532
- Ohba, K.; Iwakawa, M.; Okada, Y.; Murai, M. 1971. Paternal transmission of a plastid anomaly in some reciprocal crosses of Sugi, *Cryptomeria japonica* D. Don. *Silv. Genet.* 20: 101-107
- Ohyama, K.; Oda, K.; Yamato, E.; Ohta, Y.; Nakamura, M.; Takemura, M.; Nozato, N.; Akashi, K.; Kanegae, T. 1991. Gene organization of Liverwort *Marchantia polymorpha* mitochondrial DNA. Program and Abstracts, International Society of

- Plant Molecular Biology. Third International Congress: Molecular Biology of Plant Growth and Development. Tucson, Arizona, October 6-11, 1991. Abstract #214
- Owens, J.N.; Morris, S.J. 1990. Cytological basis for cytoplasmic inheritance in *Pseudotsuga menziesii*. I. Pollen tube and archegonial development. *Am. J. Bot.* 77: 433-445
- Ozias-Akins, P.; Pring, D.R.; Vasil, I.K. 1987. Rearrangements in the mitochondrial genome of somatic hybrid cell lines of *Pennisetum americanum* (L.) K. Schum. + *Panicum maximum* Jacq. *Theor. Appl. Genet.* 74: 15-20
- Palmer, J.D., Herbon, L.A. 1988. Plant mitochondrial DNA evolves rapidly in structure, but slowly in sequence. *J. Mol. Evol.* 28: 87-97
- Palmer, J.D. 1990. Contrasting modes and tempos of genome evolution in land plant organelles. *Trends Genet.* 6: 115-120
- Palmer, J.D.; Shields, C.R. 1984. Tripartite structure of the *Brassica campestris* mitochondrial genome. *Nature* 307: 437-440
- Palmer, J.D.; Shields, C.R.; Cohen, D.B.; Orton, T.J. 1983. An unusual mitochondrial DNA plasmid in the genus *Brassica*. *Nature* 301: 725-728
- Palmer, J.D.; Herbon, L.A. 1987. Unicircular structure of the *Brassica hirta* mitochondrial genome. *Curr. Genet.* 11: 565-570
- Park, Y.S.; Fowler, D.P. 1983. Effects of inbreeding and genetic variances in a natural population of tamarack (*Larix laricina* (Du Roi) K. Koch.) in eastern Canada. *Silv. Genet.* 13: 21-26
- Pay, A.; Smith, M.A. 1988. A rapid method for purification of organelles for DNA isolation: self-generated Percoll gradients. *Plant Cell Rep.* 7: 96-99
- Pederick, L.A. 1970. Chromosome relationships between *Pinus* species. *Silv. Genet.* 19: 171-180
- Powling, A. 1981. Species of small DNA molecules found in mitochondria from sugar beet with normal and male sterile cytoplasms. *Mol. Gen. Genet.* 183: 82-84

- Prat, D. 1983. Genetic variability induced in *Nicotiana sylvestris* by protoplast culture. *Theor. Appl. Genet.* 64: 223-230
- Price, H.J.; Sparrow, A.H.; Nauman, A.F. 1974. Evolutionary and developmental considerations of the variability of nuclear parameters in higher plants. I. Genome volume, interphase chromosome volume, and estimated DNA content of 236 gymnosperms. *Brookhaven Symp. Biol.* 25: 390-421
- Pring, D.R., Lonsdale, D.M. 1985. Molecular biology of higher plant mitochondrial DNA. *Int. Rev. Cytol.* 97: 1-46
- Pring, D.R.; Levings, C.S.III.; Hu, W.W.L., Timothy, D.H. 1977. Unique DNA associated with mitochondria in the "S" type cytoplasm of male-sterile maize. *Proc. Natl. Acad. Sci. USA* 74: 2904-2908
- Quetier, F.; Vedel, F. 1977. Heterogeneous population of mitochondrial DNA molecules in higher plants. *Nature* 268: 365-368
- Quetier, F.; Lejeune, B.; Delorme, S.; Falconet, D. 1985. Molecular organization and expression of the mitochondrial genome of higher plants. In: *Encyclopedia of Plant Physiology* 18 ed. R. Douce, D.A. Day, pp. 25-36. Berlin: Springer-Verlag
- Radetzky, R. 1990. Analysis of mitochondrial DNA and its inheritance in *Populus*. *Curr. Genet.* 18: 429-434
- Rauter, R.M.; Graham, B.J. 1983. The genetics and tree improvement potential of larches in Ontario. *Larch Symposium, Toronto 1982*. Fac. Forestry, Univ. Toronto and Ont. Min. Nat. Res. pp. 11-26
- Recipon, H. 1990. The sequence of the sunflower mitochondrial ATPase subunit 9 gene. *Nucleic Acids Res.* 18: 1644
- Reilly, J.G.; Thomas, C.A.Jr. 1980. Length polymorphisms, restriction site variation, and maternal inheritance of mitochondrial DNA of *Drosophila melanogaster*. *Plasmid* 3: 109-115
- Rode, A.; Hartmann, C.; Falconet, D.; Lejeune, B.; Quetier, F.; Benslimane, A.; Henry, Y.;

- de Buyser, J. 1987. Extensive mitochondrial DNA variation in somatic tissue cultures initiated from wheat immature embryos. *Curr. Genet.* 12: 369-376
- Rode, A.; Hartmann, C.; De Buyser, J.; Henry, Y. 1988. Evidence for a direct relationship between mitochondrial genome organization and regeneration ability in hexaploid wheat somatic tissue cultures. *Curr. Genet.* 14: 387-394
- Rode, A.; Hartmann, C.; Dron, M.; Picard, E.; Ouetier, F. 1985. Organelle genome stability in anther-derived doubled haploids of wheat (*Triticum aestivum* L., cv. 'Moisson'). *Theor. Appl. Genet.* 71: 320-324
- Roussell, D.L.; Thompson, D.L.; Pallardy, S.G.; Miles, D.; Newton, K.J. 1991. Chloroplast structure and function is altered in the NCS2 maize mitochondrial mutant. *Plant Physiol.* 96: 232-238
- Saleh, N.M.; Gupta, H.S.; Finch, R.P.; Cocking, E.C.; Mulligan, B.J. 1990. Stability of mitochondrial DNA in tissue-cultured cells of rice. *Theor. Appl. Genet.* 79: 342-346
- Sanders, P.R.; Winter, J.A.; Barnason, A.R.; Rogers, S.G.; Fraley, R.T. 1987. Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. *Nucleic Acids Res.* 15: 1543-1558
- Saylor, L.C. 1972. Karyotype analysis of the genus *Pinus* - subgenus *Pinus*. *Silv. Genet.* 21: 155-163
- Schaeffer, G.W. 1982. Recovery of heritable variability in anther-derived doubled-haploid rice. *Crop Sci.* 22: 1160-1164
- Schlich, W. 1904. *Manual of Forestry. Vol.III: Silviculture Third Edition.* Bradbury, Agnue, London
- Schmitz, U.K. 1988. Molecular analysis of mitochondrial DNA and its inheritance in *Epilobium*. *Curr. Genet.* 13: 411-415
- Schulte, E.; Staubach, S.; Laser, B.; Kuck, U. 1989. Wheat mitochondrial DNA: organization and sequences of *atpA* and *atp9* genes. *Nucleic Acids Res.* 17: 7531
- Schumann, C.M.; Hancock, J.F. 1989. Paternal inheritance of plastids in *Medicago sativa*.

- Theor. Appl. Genet. 78: 863-866
- Schuster, W.; Hiesel, R.; Wissinger, B.; Schobel, W.; Brennicke, A. 1988. Isolation and analysis of plant mitochondria and their genomes. Shaw, C.H. ed. Practical approach series: Plant molecular biology: a practical approach. pp 79-102
- Schuster, W.; Brennicke, A. 1985. TGA-termination codon in the apocytochrome b gene from *Oenothera* mitochondria. *Curr. Genet.* 9: 157-163
- Schuster, W.; Brennicke, A. 1988. Interorganellar sequence transfer: plant mitochondrial DNA is nuclear, is plastid, is mitochondrial. *Plant Science* 54: 1-10
- Schuster, W.; Brennicke, A. 1986. Pseudocopies of the ATPase alpha subunit gene in *Oenothera* are present on different circular molecules. *Mol. Gen. Genet.* 204: 29-35
- Sears, B.B. 1980. Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. *Plasmid* 4: 233-255
- Sederoff, R.R. 1984. Structural variation in mitochondrial DNA. *Adv. Genet.* 22: 2-108
- Shirzadegan, M.; Palmer, J.D.; Christey, M.; Earle, E.D. 1991. Patterns of mitochondrial DNA instability in *Brassica campestris* cultured cells. *Plant Mol. Biol.* 16: 21-37
- Shirzadegan, M.; Christey, M.; Earle, E.D.; Palmer, J.D. 1989. Rearrangement, amplification, and assortment of mitochondrial DNA molecules in cultured cells of *Brassica campestris*. *Theor. Appl. Genet.* 77: 17-25
- Small, I.D.; Isaac, P.G.; Leaver, C.J. 1987. Stoichiometric differences in DNA molecules containing the *atpA* gene suggest mechanisms for the generation of mitochondrial genome diversity in maize. *EMBO J.* 6: 865-869
- Soliman, K.; Fedak, G.; Allard, R.W. 1987. Inheritance of organelle DNA in barley and *Hordeum X Secale* intergeneric hybrids. *Genome* 29: 867-872
- Stamper, S.E.; Dewey, R.E.; Bland, M.M.; Levings, C.S.III. 1987. Characterization of the gene *urf13-T* and an unidentified reading frame, *orf25*, in maize and tobacco mitochondria. *Curr. Genet.* 12: 457-463
- Steinhoff, R.J.; Joyce, D.G.; Fins, L. 1983. Isozyme variation in *Pinus monticola*. *Can. J.*

- For. Res. 13: 1122-1132
- Stern, D.B.; Newton, K.J. 1986. Isolation of plant mitochondrial RNA. *Methods Enzymol.* 118: 488-496
- Stern, D.B.; Palmer, J.D. 1986. Tripartite mitochondrial genome of spinach: physical structure, mitochondrial gene mapping, and locations of transposed chloroplast DNA sequences. *Nucleic Acids Res.* 14: 5651-5666
- Stine, M.; Sears, B.B.; Keathley, D.E. 1989. Inheritance of plastids in interspecific hybrids of blue spruce and white spruce. *Theor. Appl. Genet.* 78: 768-774
- Stine, M.; Keathley, D.E. 1990. Paternal inheritance of plastids in Engelmann X Blue Spruce hybrids. *J. Hered.* 81: 443-446
- Sutton, B.C.S.; Flanagan, D.J.; Gawley, J.R.; Newton, C.H.; Lester, D.T.; El-Kassaby, Y.A. 1991. Inheritance of chloroplast and mitochondrial DNA in *Picea* and composition of hybrids from introgression zones. *Theor. Appl. Genet.* 82: 242-248
- Suzuki, T.; Kazama, S.; Hirai, A.; Akihama, T.; Kadowaki, K. 1991. The rice mitochondrial *nad3* gene has an external reading frame at its 5' end: nucleotide sequence analysis of rice *trnS*, *nad3* and *rps12* genes. *Curr. Genet.* 20: 331-337
- Szmidt, A. E.; Alden, T.; Hallgren, J-E. 1987. Paternal inheritance of chloroplast DNA in *Larix*. *Plant Mol. Biol.* 9: 59-64
- Szmidt, A.E.; El-Kassaby, Y.A.; Sigurgeirsson, A.; Alden, T.; Lindgren, D.; Hallgren, J-E. 1988. Classifying seedlots of *Picea sitchensis* and *P. glauca* in zones of introgression using restriction analysis of chloroplast DNA. *Theor. Appl. Genet.* 76: 841-845
- Tzagoloff, A.; Myers, A.M. 1986. Genetics of mitochondrial biogenesis. *Ann. Rev. Biochem.* 55: 249-285
- Tzagoloff, A. 1982. *Mitochondria*. New York: Plenum. 342pp.
- Vedel, F.; Quetier, F.; Cauderon, Y.; Dosba, F.; Doussinault, G. 1981. Studies on maternal inheritance in polyploid wheats with cytoplasmic DNAs as genetic markers. *Theor. Appl. Genet.* 59: 239-245

- Wagner, D.B.; Furnier, G.R.; Saghai-Marroof, M.A.; Williams, S.M.; Dancik, B.P.; Allard, R.W. 1987. Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. *Proc. Natl. Acad. Sci. USA* 84: 2097-2100
- Wahleithner, J.A.; Wolstenholme, D.R. 1988. Ribosomal protein S14 genes in broad bean mitochondrial DNA. *Nucleic Acids Res.* 16: 6897-6913
- Ward, B.L.; Anderson, R.S., Bendich, A.J. 1981. The size of the mitochondrial genome is large and variable in a family of plants (Cucurbitaceae). *Cell* 25: 793-803
- Whatley, J.M. 1982. Ultrastructure of plastid inheritance: green algae to angiosperms. *Biol. Rev.* 57: 527-569
- Wheeler, N.C.; Guries, R.P. Population structure, genic diversity, and morphological variation in *Pinus contorta* Dougl. *Can. J. For. Res.* 12: 595-606
- Wilson, A.C.; Carlson, S.S.; White, T.J. 1977. Biochemical evolution. *Annu. Rev. Biochem.* 46: 573-639
- Wilson, A.J.; Chourey, P.S. 1984. A rapid inexpensive method for the isolation of restrictable mitochondrial DNA from various plant sources. *Plant Cell Reports* 3: 237-239
- Wissinger, B.; Hiesel, R.; Schuster, W.; Brennicke, A. 1988. The NADH-dehydrogenase subunit 5 gene in *Oenothera* mitochondria contains 2 introns and is co-transcribed with the 5S rRNA gene. *Mol. Gen. Genet.* 212: 56-65
- Wolfe, K.H.; Li, W-H.; Sharp, P.M. 1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proc. Natl. Acad. Sci. USA* 84: 9054-9058
- Xie, Y.; Wu, R. 1990. Nucleotide sequences of mitochondrial ATPase subunit 9 genes from three lines of rice (*Oryza sativa* L.). *Nucleic Acids Res.* 18: 4268
- Yamaguchi, H.; Kakiuchi, H. 1983. Electrophoretic analysis of mitochondrial DNA from normal and male-sterile cytoplasm in rice. *Jpn. J. Genet.* 58: 607-611
- Yang, D.; Oyaizu, Y.; Oyaizu, H.; Olsen, G.J.; Woese, C.R. 1985. Mitochondrial origins.

Proc. Natl. Acad. Sci. USA. 82: 4443-4447

Young, E.G.; Hanson, M.R.; Dierks, P.M. 1986. Sequence and transcription analysis of the *Petunia* mitochondrial gene for the ATP synthase proteolipid subunit. Nucleic Acids Res. 14: 7995-8006

APPENDIX I

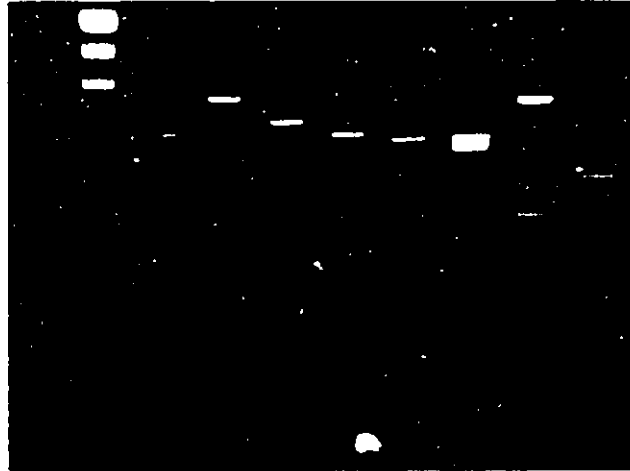
Restriction Analysis of Wheat Mitochondrial Genes Cloned in pUC Vector.

Each wheat mitochondrial gene cloned in pUC vector was digested with single and multiple enzyme combinations to compare resulting fragment sizes to the restriction map for the clone. The fragment sizes for these clones, with the enzymes tested, matched the restriction map, indicating that the correct gene fragments were isolated for use as probes.

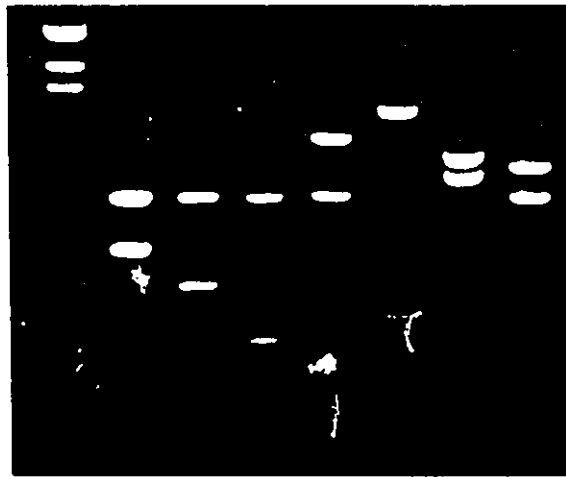
FIGURE LEGEND

LANE	PLASMID	GENE	ENZYME(S)	FRAGMENT SIZES (kb)
1		Lambda	<i>HindIII</i>	23, 9.4, 6.6, 4.4 2.3, 2.0, 0.5
2	B342	<i>coxI</i>	<i>BamHI</i>	4.5, 2.7
3			<i>HindIII</i>	5.8, 0.8, 0.6
4			<i>PstI</i>	4.8, 2.4
5			<i>BamHI/EcoRI</i>	4.5, 2.7
6			<i>BamHI/XbaI</i>	4.4, 2.7
7	H4	<i>nad5</i>	<i>BamHI</i>	4.3, 4.0
8			<i>HindIII</i>	5.5, 2.7
9			<i>HindIII/PstI</i>	3.0, 2.7, 2.1
10		Lambda	<i>HindIII</i>	23, 9.4, 6.6, 4.4 2.3, 2.0, 0.5
11	B30	<i>atpA</i> and <i>atp9</i>	<i>BamHI</i>	2.7, 2.0
12			<i>BamHI/SstI</i>	2.7, 1.7, 0.3
13			<i>BamHI/XhoI</i>	2.7, 1.2, 0.8
14	B303	(rRNA ^{scr} , <i>nad3.rps12</i>)	<i>BamHI</i>	3.8, 2.7
15			<i>BglII</i>	5.1, 1.4
16			<i>HindIII</i>	3.5, 3.0
17			<i>BamHI/SstI</i>	3.1, 2.7, 0.7
18		Lambda	<i>HindIII</i>	23, 9.4, 6.6, 4.4 2.3, 2.0, 0.5
19	B314	<i>orf25</i>	<i>BamHI</i>	4.8, 2.7
20			<i>BamHI/MluI</i>	3.8, 2.7, 0.8
21			<i>BamHI/SstI</i>	3.2, 2.7, 1.6
22	B376	<i>cob</i>	<i>BamHI</i>	5.6, 2.7
23			<i>BamHI/EcoRI</i>	3.6, 2.7, 1.8
24			<i>BamHI/SstI</i>	2.7, 2.6, 2.0, 1.0
25			<i>BamHI/XbaI</i>	3.6, 2.7, 1.8, 0.2
26			<i>BamHI/MluI</i>	5.6, 2.7
27			<i>BamHI/XhoI</i>	5.6, 2.7

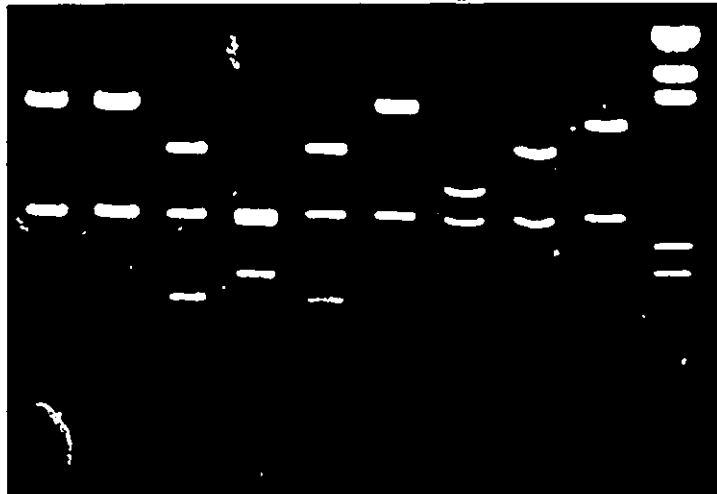
1 2 3 4 5 6 7 8 9



10 11 12 13 14 15 16 17



27 26 25 24 23 22 21 20 19 18



APPENDIX II

This table consists of preliminary restriction fragment length data obtained from single hybridization experiments with wheat mitochondrial gene probes. Sizes are in kilobase pairs.

Probe Enzyme	<i>atp6</i>	<i>atp9</i>	<i>atpA</i>	<i>nad3/ rps12</i>	<i>nad5</i>	<i>orf25</i>	<i>cox1</i>	<i>cob</i>
AvaI	nd	nd	6.0/4.0	nd	nd	nd	nd	nd
BamHI	10	Table 3.3	Table 3.3	Table 3.3	Table 3.3	Table 3.3	9.4	5.6/4.1
CfoI	3.2	14/3.8/2.0/1.9/1.2/ 0.8/0.4	11/3.8	---	6.5/3.5/1	15	16/2.5	0.3
DraI	nd	Table 3.3	6.0	Table 3.3	Table 3.3	Table 3.3	Table 3.3	Table 3.3
EcoRI	17/8.2	Table 3.3	Table 3.3	Table 3.3	Table 3.3	Table 3.3	6/3.5	2.3
EcoRV	nd	Table 3.3	4.6/3.2	Table 3.3	Table 3.3	Table 3.3	Table 3.3	Table 3.3
HincII	nd	nd	3.8/1	nd	nd	nd	nd	nd
HindIII	7.0/4.0	Table 3.3	Table 3.3	Table 3.3	Table 3.3	Table 3.3	1.5	1.7
KpnI	20/11/10	Table 3.3	Table 3.3	Table 3.3	Table 3.3	Table 3.3	Table 3.3	6/3.2/2.8
MspI	nd	nd	2.5	nd	1.2	0.7	0.8	nd
HpaII	nd	nd	2.5	nd	nd	nd	nd	nd
NciI	nd	nd	4.4	nd	nd	nd	nd	nd
NruI	nd	nd	23+	nd	nd	nd	nd	nd
SalI	nd	nd	nd	nd	nd	15	nd	nd
ScaI	nd	nd	14/8.2	nd	nd	nd	nd	nd

nd - not done

--- no band

Table 3.3 - this data is available in Table 3.3, page 47