A Chemotaxonomic and Serotaxonomic Investigation of Some *Pinus* Species

A thesis presented in partial fulfilment
of the requirements for the degree of

MASTER OF SCIENCE

IN BOTANY

AT MASSEY UNIVERSITY

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1982

ABSTRACT

A relatively simple and rapid method is presented for the extraction of protein from adult tissues of four species of *Pinus*. Protein was extracted using a low pH mixture containing reducing agents, thiols, and polyvinylpolypyrrolidone. The protein interferring components were separated and removed from the protein solution on a Sephadex column.

Protein extracted using this method was found to be useful for separation and analysis by electrophoresis and isoelectric focussing, for enzyme analysis following separation by these techniques, and for antibody production used in serological techniques. These techniques were evaluated for their ability to provide information on the taxonomy of the *Pinus* species examined. The high resolution technique of isozyme analysis by isoelectric focussing, and the serological analysis were found to be most useful.

The relationship between these species suggested by the results support the division of the genus *Pinus* into two subgenera, Haploxylon and Diploxylon, as suggested by Koehne (1893). The results also support the classification suggested by Shaw (1914) but no evidence was found to support the classification suggested by Pilger (1926).


BOGOR
Woodsmans Annual
EXAMINATION
QUESTION 3 : DRAW A PICTURE
OF A TREE AND LABLE ALL
THE DIFFERENT PARTS
(3 MARKS)
the top
the middle
the side
the other side
the back
the front
the bottom

ACKNOWLEDGEMENTS

The production of this thesis would not have been possible without the assistance, advice and encouragement from many people. In particular, I would like to thank Dr. D. Fountain for his friendly advice and encouragement, and the time he has given over the past four years.

I would also like to thank:

- Staff and fellow students in the Botany and Zoology Department for many helpful discussions, and, in particular, Mr B. Campbell and Mr C.L. Kan for their able technical assistance.

- Forest Research Institute in Rotorua for supporting this work, for the loan of equipment, and for supplying samples.

- Dr. D. Smith for his kind help and assistance during my stays in Rotorua, and for the collection of samples.

- Dr. D. Copes for his assistance at the beginning of this work.

- Dr. R. Lill and Mr J. Merton for reading and critically appraising the draft copy.

- Mrs J. Tucker not only for her excellent typing, but also for her encouragement and understanding.

My gratitude is also due to my wife, Irene, who has stood by me through the years involved in this work, and to my parents who have encouraged me during all of my education.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Bis	-	N, N', -methylene-bis-acrylamide
DIECA	-	Sodium diethyldithiocarbamate
EDTA	-	Ethylenediaminetetraacetic acid
MCE	-	2-Mercaptoethanol
PVP	-	polyvinylpyrrolidone
PVPP	-	polyvinylpolypyrrolidone
TEMED	-	N, N, N', N', -tetramethylethylenediamine
Tris	-	Tris (hydroxymethyl) aminomethane

1. INTRODUCTION

The genus *Pinus* is a well recognised taxon of the Gymnospermae, its members being economically important trees easily differentiated from other conifers. Pilger (1926) considered the family Pinaceae to consist of two subfamilies; the Pinoideae, with *Pinus* its only genus; and the Abietinoideae, containing the remaining eight genera (*Abies, Cedrus, Larix, Pseudolarix, Tsuga, Pseudotsuga, Picea and Keteleeria*) (Lawrence 1971). Engelmann (1880) stated "No difficulty exists in the circumscription of the genus *Pinus*; floral characters unite with vegetative to establish it so firmly and so plainly that nobody fails to recognise the species belonging to it". The exact number and classification of species belonging to this genus is not so clear. Engelmann continued "... but when we come to analyse and to group 60 or 70 species of pines, ... we find that they appear so similar that all attempts to arrange them satisfactorily have failed". Even recent attempts at classification have not been entirely successful. Mirov (1967) states "the genus *Pinus* consists of over one hundred species, the exact number being a matter of individual judgement of botanists".

The classification of pines has proved difficult primarily due to the widespread formation of interspecific hybrids. Many recognised species hybridise with other species which are often apparently distantly related. Some of these pairs have been separated and geographically isolated since the Cretaceous or perhaps Jurassic periods (Mirov 1967) which ended 65 and 135 million years ago respectively.

One of the early divisions of the genus was introduced by Koehne (1893) who divided the genus into two subgenera; the Haploxylon, in which the "fibrovascular" bundle (vascular bundle in the needle) is single; and the Diploxylon, in which it is double. Earlier, Engelmann (1880) had decided against using the morphology of the vascular bundle as a taxonomic character as he occasionally found both single and double bundles in the same species. In spite of this, the division has been accepted by most workers and is now supported by evidence gained in paleobotany, chemotaxonomy, and hybridisation experiments (Mirov 1967).

Some attempts at classifying the pines were made by Shaw (1914) and Pilger (1926). Shaw's classification is based on the structure of the wood rays, the shape of the cone scales, position of the resin ducts in the needles, and the form and method of attachment of the seed wing. Pilger based much of his classification on the number of needles of the short shoot and it shows many differences to Shaw's, the chief differences being; the elmination of the group Leioplyllae, the creation of a new group (Khasia), and the rearrangement of the remaining groups using new names (Mirov 1967).

Shaw had classified the Diploxylon pines *Pinus elliottii*, and *P. taeda* together in the group Australes, and placed *P. radiata* in the group Insignis. Pilger, on the other hand, grouped *P. taeda* and *P. radiata* together in the new group 'Taeda'. The classification of the three species is thus quite different with Shaw suggesting a close relationship between *P. elliottii* and *P. taeda*, and Pilger suggesting the closer relationship between *P. taeda* and *P. radiata*.

More recently, Duffield (1952) used information gained from hybridisation experiments to revise Shaw's subsection Pinaster which includes the species mentioned above, but the relationship between them remained as in Shaw's original classification. Mirov (1967) considers that the "Shaw-Duffield classification provides a framework for future taxonomic studies of pines". Further taxonomic investigation, if it is to be useful, should be able to distinguish between pines belonging to the Haploxylon and Diploxylon subsections, and also ascertain which of the implied relationships between *P. radiata*, *P. elliottii*, and *P. taeda* (that is, Pilger's or Shaw/Duffield's) is the more likely. These criteria can therefore be set up as a test of the 'usefulness' of any new taxonomic investigation by referring to two hypotheses (Figure 1 A, B). Hypothesis one (Figure 1A) is taken from the relationship implied in the Shaw/Duffield taxonomy, while hypothesis two (Figure 1B) is taken from Pilger's classification.

As already discussed, difficulty arises when morphological characters are used in *Pinus* taxonomy. Other characters have therefore been sought to differentiate between "true" species and hybrids. For example, the chemistry of "extraneous material" (Mirov 1967) has added much to the understanding of the relationships within the genus *Pinus*. This method too, has its problems. Mirov warns that "the fact - often disregarded - is that there is variability within a species and that often there are no two trees alike in chemical composition of their extraneous substances ... often (the variability) is so considerable as to cause misunderstanding and confusion".

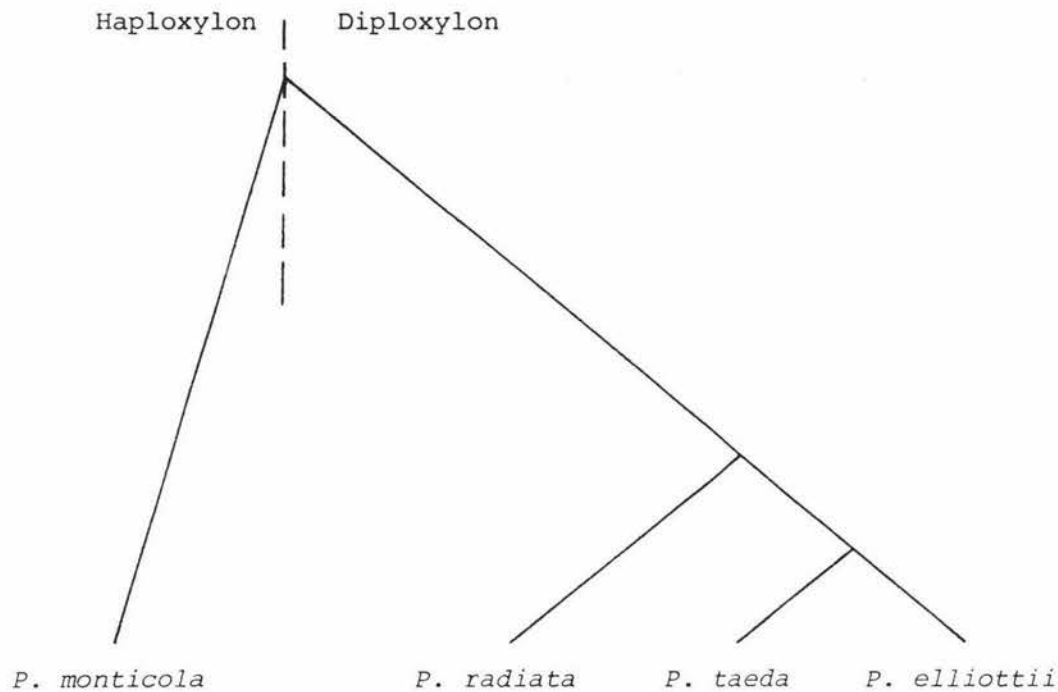

Fig 1A Dendrogram of four *Pinus* species set out to show hypothesis one (see text for details).

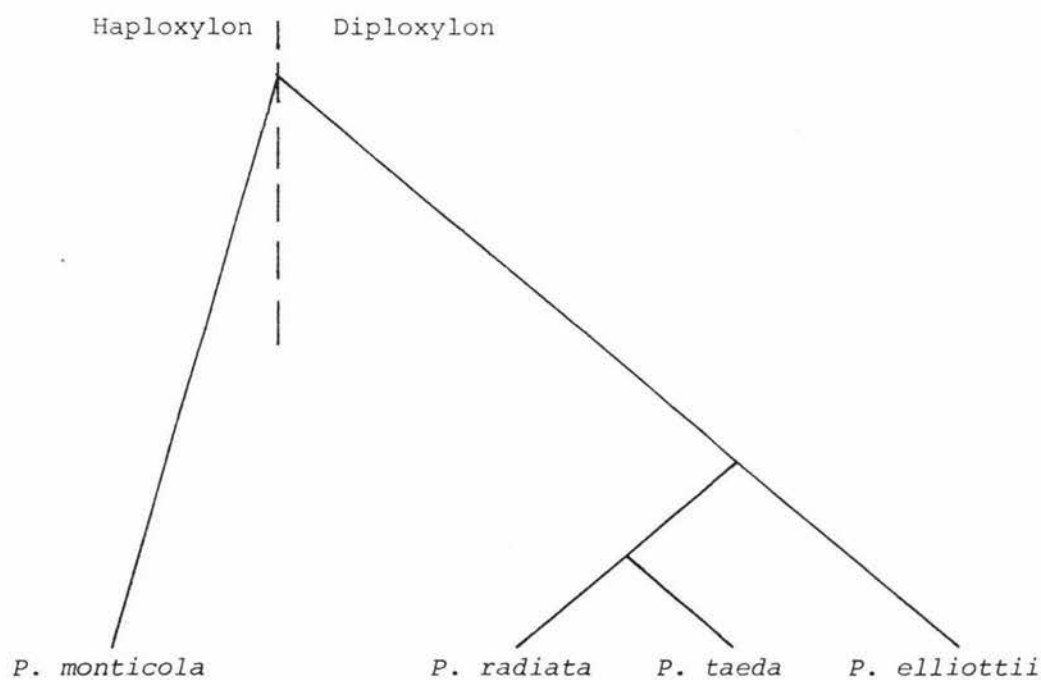

Fig 1B Dendrogram of four *Pinus* species set out to show hypothesis two (see text for details).

Increasingly, workers have used the large molecules such as proteins as an aid in the taxonomy of many organisms. Smith (1976) states "... it is probable that comparisons between the proteins of different taxa will eventually comprise the bulk of all work involving chemical assay for systematic purposes". Various different protein separation and recognition techniques now available have been used in the taxonomic studies. Each of the techniques utilises one or more of the properties conferred on the proteins by their amino acid composition and/or sequence, for example; net charge, size, or enzymatic function.

In electrophoresis, proteins are separated in an electric field because differences in their net charge (at a particular pH) cause the proteins to migrate at different rates. When electrophoresis is performed in polyacrylamide gels, the gel can act as a molecular sieve so that the proteins are separated by a combination of size and migration rate.

Isoelectric focussing separates proteins according to their isoelectric point. A pH gradient is formed in an electric field by amphoteric substances so that proteins will migrate electrophoretically until they reach a pH at which their net charge is zero (that is, their isoelectric point). Very high resolution is achieved using this technique because of the focussing effect of the electric field counteracting diffusion.

Both isoelectric focussing and electrophoresis can be used to analyse the general protein components from extracts of different species, for example; whitefish (Djupsund 1976), fungi and algae (Shechter 1973), conifers (McMullan and Ebell 1970) or to analyse isozyme (isoenzyme) variation in populations, for example; molluscs (Wright and Rollinson 1979), conifers (Conkle 1971a).

The advantage of using isozyme analysis lies in the ability of these techniques to recognise particular proteins by using specific enzyme stains. Thus the properties of proteins in different individuals or species with the same enzymatic function can be compared.

The serological techniques such as double-diffusion and immunoelectrophoresis also utilise the added dimension of protein recognition, and these techniques have been widely used in taxonomic studies (for example; primates (Goodman and Moore 1971), acacias (El-Tinay *et al.* 1979), and conifers (Prager *et al.* 1976)). The advantage of these techniques is that they are not limited to investigating enzymes but will recognise all proteins which are antigenically similar to those recognised by the animals immune response system.

All these techniques require the extraction of proteins from plant tissues but this presents many problems not usually encountered by workers using animal tissues. Cellular structures, the presence of inhibitory chemicals, and a lower metabolic rate are some of the problems which make plant material less amenable to electrophoresis (O'Malley *et al.* 1979).

The inhibitory chemicals include various organic acids, phenolic compounds, and tannins (Walker 1980). Diphenoloxidase enzymes, which convert diphenols to quinones are also a major problem. Conifers, in particular, appear to be especially difficult due to the relatively large amounts of these interfering substances and the low concentrations of protein especially in the needle material.

The oxidised products of phenols (quinones) are highly reactive and combine with amino acids and proteins rendering them biologically inactive. When plant tissues are disrupted, phenols and quinones will bond with any protein they may come into contact with. "All phenols, unless sterically hindered, take part in hydrogen bonding, and the bond formed between phenols and N-substituted amides is one of the strongest types of hydrogen bonds" (Loomis and Battaile 1966). The amount of bound phenolic material may be up to one third the dry weight of the protein concerned (Loomis 1969). Quinones polymerize with themselves or co-polymerize with amino acides or proteins, inactivating enzymes (Walker 1980). The phenolic compounds are often absent or present in low concentration in juvenile tissue (McMullan and Ebell 1970; Rhoades and Cates 1976) and hence many workers use seed or seedling material for analysis, particularly when working with conifers, for example Prager *et al.* (1976).

To overcome the problems associated with protein extraction from mature *Pinus* tissue, some or all of the following appear to be necessary; as much of the phenolic substances should be removed as quickly as possible; diphenyl oxidase enzymes should be inhibited to prevent the enzymatic oxidation of phenols to quinones; any quinones which are formed enzymatically or non-enzymatically should be reduced or removed; and the protein needs to be separated from the interfering substances to form a stable non-toxic product for immunological studies.

These problems have been approached in various ways by many authors. Loomis and Battaile (1966) produced extracts of peppermint leaves showing enzyme activity after phenols had been absorbed onto insoluble polyvinylpyrrolidone ('polyclar AT' or polyvinylpolypyrrolidone). It is thought that a strong hydrogen bond occurs between the oxygen of the pyrrolidone ring and the hydroxyl group of the phenol (Loomis 1969). Low pH's are required to maintain the hydroxyl group for bonding and maximum phenol binding to polyvinylpyrrolidone (PVP) was found to occur at pH 3.5 for phenols of *Nicotiana tobaccum* (Andersen and Sowers 1968). Higher pH's, can be used to reduce the hydrogen bonding of phenols to proteins but these conditions also reduce the binding of phenols to PVP and, furthermore, favour the "auto-oxidation of phenols" (Walker 1980).

Quinones are produced in cell extracts enzymatically and non-enzymatically. The most active of the phenol oxidising enzymes is o-diphenol: O_2 oxidoreductase (E.C. 1.10.3.1), which oxidises o-diphenols to their corresponding quinone (Anderson 1968).

This enzyme has a requirement for copper which forms the reactive centre of the enzyme and copper chelating agents have been shown to be a potent inhibitor of the enzyme. Sodium diethyldithiocarbamate (DIECA) is one such copper chelating agent used in many extraction systems. Slack (1966) found that DIECA prevented the inhibition of sucrose synthetase enzymes from sugar cane extracts by the oxidation products of phenols. DIECA also, at certain concentrations, binds with the quinone products and this appears to be of critical importance in preventing the build-up of diphenoloxidase products, in comparison to the use of other inhibitors such as cyanide (Anderson, 1968). The general reducing ability of thiols appears to be beneficial in enzyme preparations and they have been included almost routinely by many workers. Slack (1966) found that mercaptoethanol was at least partially effective in reversing the inhibition of sucrose synthetase by p-benzoquinone and attributed this to the reduction of sulphydryl groups by the thiol. Mercaptoethanol may also inhibit diphenoloxidase *per se*, in the manner of other thiols (Walker 1980).

Ascorbate has often been used in enzyme extracts as it readily reduces quinones with the regeneration of the phenols. The ascorbate is required in excess as the continued production of quinones eventually exhausts the supply after which time oxidation can continue unhindered (Anderson 1968). Solubilisation agents such as the non-ionic and cationic detergents may be useful in the reactivation of enzymes from protein - tannin complexes (Goldstein and Swain 1965).

Pinus epitomises the problems associated with protein extraction from plant material and for this reason, only a limited application of the chemo-taxonomic and serotaxonomic methods to *Pinus* has been made to date.

The aim of this study was twofold. Firstly, to develop a method for extracting protein from mature *Pinus* tissue, and secondly, to use the protein extract to investigate the usefulness of some chemo-taxonomic and serotaxonomic techniques as an aid to study the relationships between some species of *Pinus*. The requirements of the method were -

a) It should be useful for screening large numbers of individual trees (as in breeding schemes) and hence should be rapid and technically simple.

b) It should produce an extract free of interfering phenolic and tannin substances.

c) The product should be stable on storage.

d) The product should be non-toxic to the animals used in antiserum production.

The usefulness of each technique was judged by analysing the amount and quality of information the technique provided concerning the two hypothesis regarding *Pinus* taxonomy (Figure 1). The techniques were judged useful if the information gained clearly supported one or other of the hypotheses.