A Chemotaxonomic and Serotaxonomic Investigation of Some *Pinus* Species

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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

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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".

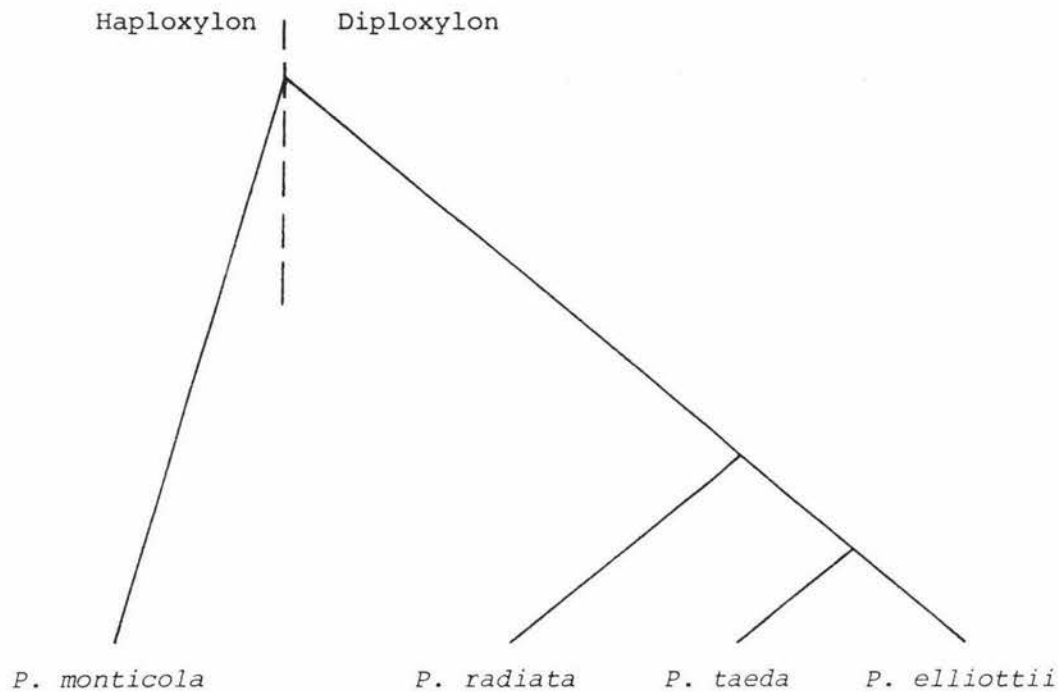

Fig 1A Dendrogram of four *Pinus* species set out to show hypothesis one (see text for details).

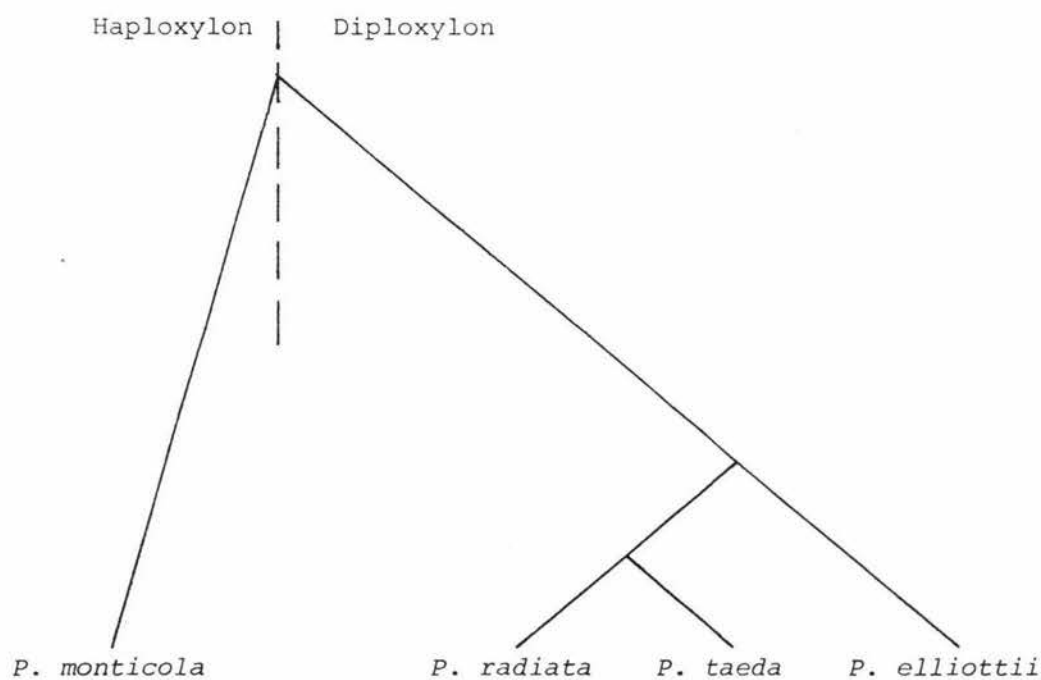

Fig 1B Dendrogram of four *Pinus* species set out to show hypothesis two (see text for details).

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.

2. MATERIALS AND METHODS

2.1 PLANT MATERIAL

Shoot tip samples with varying amounts of needle material visible (Figure 2) were taken from individual trees or clones of *P. radiata* D. Don ("Monterey pine"), *P. elliottii var. elliottii* Engelm ("Slash pine"), *P. taeda* L. ("Loblolly pine") and *P. monticola* Dougl. ("Western white pine"). The samples were taken from seed orchards or grafting trials at the Forest Research Institute in Rotorua. The *P. radiata* clones used were 510, 511, 517, 522, and 525 selected by F.R.I. in 1970 for superior growth attributes. The samples of *P. elliottii* and *P. taeda* were taken from scions of Queensland 'plus' trees grafted onto *P. radiata* root stocks.

2.2 PREPARATION OF PROTEIN EXTRACTS

(Butcher *et al.* 1981.)

A flow diagram for the method of protein extraction is shown in Figure 3. Protein was extracted from mature *Pinus* tissue in a mixture containing 7% polyvinylpolypyrrolidone (PVPP), 0.5% ascorbic acid, 0.3% sodium diethyldithiocarbamate (DIECA), 1% 2-mercaptoethanol (MCE) and 1% triton X-100 in distilled water. This extraction mixture was stored at 6°C and used within 5 days. The fresh or frozen *Pinus* tissue was weighed and chopped with a scalpel into pieces approximately 3 mm in length.

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Fig 2 Shoot tip sample material. Note varying amount of needle material visible.

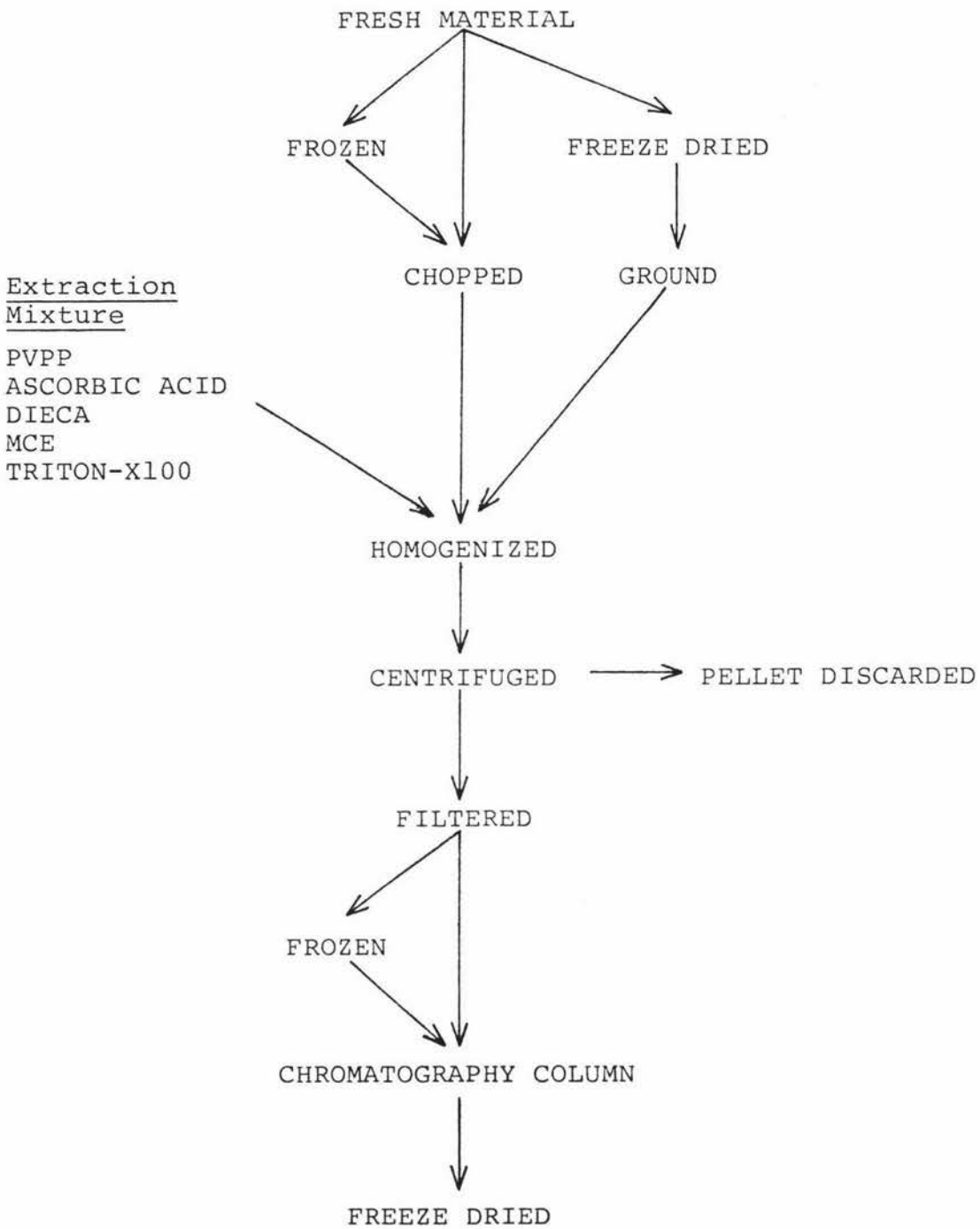

Fig 3 Flow diagram for method of protein extraction

Freeze dried material was powdered in a hammer mill (Glen Creston C580, England) using a 1 mm diameter screen. Freeze dried powder (50 g) or the chopped fresh or frozen material (200 g) was placed immediately into 500 ml of chilled extraction mixture with continuous stirring. When all the material was in the mixture, the stirring bar was removed and the mixture was homogenized with an Ultra-Turrax (type 45, Janke and Kunkel K.G., Staufen i. Breisgau) homogenizer at full speed for 1 min. The slurry was placed in centrifuge buckets and centrifuged at 15,000 g for 30 min at 4°C (MSE High Speed 18, England). The supernatant was then passed through Whatman No. 1 filter paper and sucrose added to the filtrate to make a 20% solution.

The supernatants were either passed through the sephadex column immediately or stored frozen at -17°C.

2.3 COLUMN GEL CHROMATOGRAPHY

Sephadex G-25 (fine) was used to obtain protein material with a molecular weight greater than 5,000. A glass column 300 mm x 100 mm was used to enable volumes of up to 250 ml to be passed through at one time, with good separation of high and low molecular weight material (Figure 4). The column was calibrated using 250 ml of an aqueous solution of blue dextran 2,000 (molecular weight 2,000,000) and rhodamine B (molecular weight 479).

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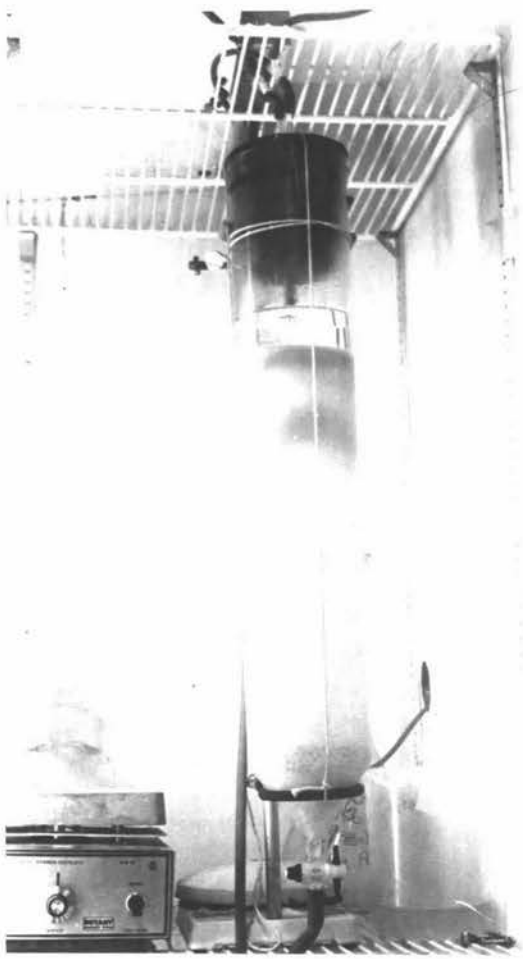


Fig 4 Gel chromatography column. Bands visible in the gel are formed by calibration compounds.

The rhodamine B was separated by at least 540 ml from the void volume containing the blue dextran 2,000. The column was washed between runs with at least 40 ℓ of distilled water. All runs were carried out in a refrigerator at 6°C.

The void volume (600 ml) containing the protein and other high molecular weight material was freeze dried and the dried product was taken up in as little distilled water (usually 3-5 ml) as was required to dissolve the product. This solution was placed in McCartney bottles and stored at -17°C until required.

2.4 PROTEIN QUANTITATION

Protein was assayed using the method of Bradford (1976).

100 mg of coomassie brilliant blue G-250 was dissolved in 50 ml of 95% ethanol. 100 ml of 85% (w/v) orthophosphoric acid was added and the solution made up to 1,000 ml with distilled water.

A standard protein series containing 100, 200, 400, 600, 800 and 1,000 µg bovine serum albumin in distilled water and a solution of the freeze-dried extract of *P. radiata* 517 (1,000 µg/ml distilled water) were prepared. An aliquot (0.1 ml) of each of the protein solutions was pipetted into 10 ml test tubes and 5 ml of the protein dye reagent was added. The mixtures were stirred thoroughly and allowed to stand for at least 2 min., before the absorbance at 595 nm was measured using a recording spectrophotometer (Hitachi, Japan).

2.5 CARBOHYDRATE QUANTITATION

Carbohydrate was assayed using the method of Hodge and Hofreiter (1962).

2.5.1 Stock Solutions -

5% w/v phenol
98% sulphuric acid
50 µg/ml sample solution (*P. radiata* 517)

2.5.2 Carbohydrate Assay -

A standard series of D(+) glucose was prepared, containing 10, 20, 30, 40, and 50 µg glucose per millilitre of distilled water. Aliquots of the standard solutions (0.1 ml) or the sample solution (0.1 ml), and an aliquot of the phenol solution (0.1 ml) were mixed in test tubes which had been previously washed in sulphuric acid. An aliquot of sulphuric acid (5 ml) was then added rapidly so that mixing occurred as quickly as possible. The tubes were allowed to cool for 10-15 mins. before the absorbance was measured at 490 nm (Hitachi Recording Spectrophotometer, Japan).

2.6 DISCONTINUOUS POLYACRYLAMIDE ROD GEL ELECTROPHORESIS

2.6.1 Apparatus -

Electrophoresis and peroxidase isozyme analysis were carried out on Acrylophor apparatus (Pleuger, Belgium), using a Pleuger (CVC-D) power supply.

Glass tubes 90 mm long with an internal diameter of 5 mm were cut. These tubes were thoroughly cleaned before each electrophoresis run and soaked in chromic acid for at least 24 hrs. The apparatus was set up according to the instructions supplied by Pleuger.

2.6.2 Stock Solutions -

The electrophoretic system used was based on that of Ornstein (1964) and Davis (1964) which stacks at pH 8.9 and runs at pH 9.5. Stock solutions were prepared as shown in Table 1. Final concentrations in the gels are shown in Table 2.

2.6.3 Main (Separating) Gel -

For one set of eight gels, 4 ml of stocks A, and C, and 8 ml of stock G were mixed and degassed under vacuum for at least 5 min.

The tubes were closed at one end with a rubber bung and loaded with the degassed acrylamide solution to a point approximately 15 mm below the top of the tube. The final length of the main gel for all electrophoretic systems was approximately 70 mm. The solution in the tube was overlayed with distilled water to form a flat interface with the stacking gel which would be placed on top.

TABLE 1 Polyacrylamide gel stock solutions for discontinuous rod gel electrophoresis

Stock A	In HCl	24 ml
	Tris	18.1 g
	TEMED	0.12 ml
	pH 8.9	
	H_2O	to 100 ml
Stock B	In HCl	48 ml
	Tris	5.98 g
	TEMED	0.46 ml
	pH 6.7	
	H_2O	to 100 ml
Stock C	Acrylamide	28 g
	Bis	0.735 g
	H_2O	to 100 ml
Stock D	Acrylamide	20 g
	Bis	5 g
	H_2O	to 100 ml
Stock E	Riboflavin	4 mg
	H_2O	to 100 ml
Stock F	Sucrose	40 g
	H_2O	to 100 ml
Stock G	Ammonium persulphate	50 mg
	H_2O	to 25 ml
Stock H	Tris	3 g
	Glycine	14.4 g
	pH 8.3	
	H_2O	to 100 ml
	Dilute 10 x for electrophoresis	
Stock J	Bromophenol blue	5 mg
	Glycerol	30 ml
	H_2O	to 100 ml

TABLE 2 Final concentrations of the polyacrylamide gels for electrophoresis

Component	Main Gel %	Stacking Gel %
Acrylamide	7	2.5
Bis	0.18	0.625
Tris	4.525	0.75
TEMED	0.03	0.058
Ammonium persulphate	0.05	-
Riboflavin	-	0.001
Sucrose	-	5
'T	7.2	3.1
'C	2.6	20.

'Maurer (1971)

When polymerization of the main gel was complete the rubber bungs were removed from the bottom of the tubes and the top and bottom gel surfaces were rinsed with distilled water to remove any unpolymerized acrylamide. Excess water was removed by shaking.

2.6.4 Stacking Gel -

For one set of eight gels, 1 ml each of stocks B, D and E, 1 ml of distilled water, and 4 ml of stock F were mixed before degassing under vacuum for at least 5 min.

The stacking gel solutions were loaded on top of the polymerized main gel to a point approximately 5 mm below the top of the tube. This solution was overlayed with distilled water as before and the whole apparatus placed to polymerize under fluorescent light for at least 1 hr. After polymerization was complete, the gels were washed with distilled water as described for the main gel.

2.6.5 Electrophoresis -

Electrophoresis buffer (300 ml) (stock H) was placed in the bottom compartment of the Acrylophor apparatus. With the gel tubes in position in the upper compartment, and the compartment held upside-down, a small volume of the same buffer was placed in the bottom of the tubes.

The upper compartment was then carefully turned right-side-up and placed in position on the bottom compartment. This ensured air was not trapped in the bottom of the tubes and the buffer was in direct contact with the gel. Tracking dye solution (stock J) (50 µl) was layered on top of each gel. The protein sample (100-250 µl depending on the concentration) was then placed on top of the gel and gently mixed with the tracking dye solution. The sample/tracking dye mixture was carefully overlayed with a small volume of the dilute electrophoresis buffer (stock H) until it formed a 'bead' on top of the tube. More dilute buffer (200 ml) was then carefully added to the upper compartment and the lid placed in position.

Electrophoresis was carried out at 2mA per tube until the tracking dye had reached the main gel and then increased to 4mA per tube until the dye was within 5 mm of the bottom of the gel.

When electrophoresis was finished, the gels were removed intact from the tubes. This was accomplished by inserting the needle of a syringe between the gel and the tube and at the same time expressing a small volume of distilled water from the syringe. When this was performed all the way around the gel, the gel was forced gently out of the tube using air pressure from a pasteur pipette bulb.

The position of the tracking dye was marked by inserting a small syringe needle dipped in black drawing ink into the gel in the middle of the tracking dye band. The excess ink was washed off with distilled water and the gel was placed in a staining/destaining tube. (These tubes were numbered 5 ml test tubes which had several holes up and down the length.) The gels were removed from the electrophoresis tubes and placed in the staining solution as quickly as possible after electrophoresis to minimize diffusion of the protein bands.

2.6.6 Protein Stains -

Stain Method 1 (ethanol-acetic acid) method

Proteins were stained and destained using the solutions in Table 3. The gels were left in the stain solution overnight and destained in several changes of destain solution until the background was clear. The gels were removed from the stain tubes and stored in glass test tubes in 10% acetic acid.

Stain Method 2 (perchloric acid) method

If results were required immediately, the method of Reisner *et al.* (1975) was used.

TABLE 3 Staining solutions for polyacrylamide gel electrophoresis

METHOD 1	Protein Stain	
	Coomassie brilliant blue G250	0.5 g
	Ethanol (95%)	40 ml
	Acetic acid (glacial)	7 ml
	H_2O	to 100 ml
	Destain Solution	
	Ethanol (95%)	250 ml
	Acetic acid (glacial)	70 ml
	H_2O	to 1000 ml
METHOD 2	Coomassie brilliant blue G250	0.4 g
	Perchloric acid	3.5 ml
	H_2O	to 100 ml

This stain solution is shown in Table 3. This is a rapid method as the gels do not require destaining, although better results were obtained if the background was destained in 10% acetic acid. The gels could be stored in 10% acetic acid for several months before the stain faded.

2.7 ISOELECTRIC FOCUSSING

2.7.1 Apparatus -

Isoelectric focussing and acid phosphatase isozyme analysis were carried out on LKB 2117 'Multiphor' apparatus (LKB Sweden) using a LKB 2103 power supply. The apparatus was set up as follows. The left-hand buffer tank (cathode) was filled with 1,200 ml of 1 M sodium hydroxide, and the right-hand buffer tank (anode) with 1,200 ml of 1 M orthophosphoric acid. The glass cooling plate was placed in position and coated with kerosene. A sample applicator template supplied by LKB for use with LKB 'PAG' plages was placed in position and coated with kerosene.

2.7.2 Prepared Gels -

Ampholine PAG plates (LKB, Sweden) with a pH range of 3.5-9.5 (LKB 1804-101) were used when available.

The length of gel required (depending on the number of samples to be run) was cut from one of the gels supplied using a scalpel. The gel package was cut around the gel leaving approximately 10 mm on each side of the gel. The transparent cover was then removed from the gel. The gel was removed from the silvered backing and placed on the apparatus by using forceps on the protruding edge of the cellophane gel backing. Final concentrations of the gel are shown in Table 4.

2.7.3 Laboratory Made Gels -

Laboratory made gels were cast using the method of Görg *et al.* (1978). Two glass plates were cut measuring 120 mm by 80 mm enabling gels measuring 110 mm by 70 mm to be cast. Both plates were scrubbed with an abrasive cleaner ('Ajax') and rinsed with tap and then distilled water before casting each gel. The first plate was dipped in a 1% solution of triton X-100 and allowed to drain. A piece of cellophane sheeting measuring approximately 140 mm by 100 mm was cut and soaked in distilled water for at least 2 min. The sheet was placed on the second glass plate making sure air was not trapped between the cellophane and the glass plate. The cellophane was smoothed out and folded around the bottom of the plate.

TABLE 4 Final concentrations of the LKB 'PAG' plates

'T	5%
'C	3%
Ampholine	2.4%

TABLE 5 Final concentrations of the polyacrylamide gels for isoelectric focussing

Component	Concentration %
Acrylamide	6.0
Bis	0.2
Ampholine	2.0
Glycerol	0.125
TEMED	0.013
Riboflavin	0.2
'T	6.1
'C	2.56

'Maurer (1971)

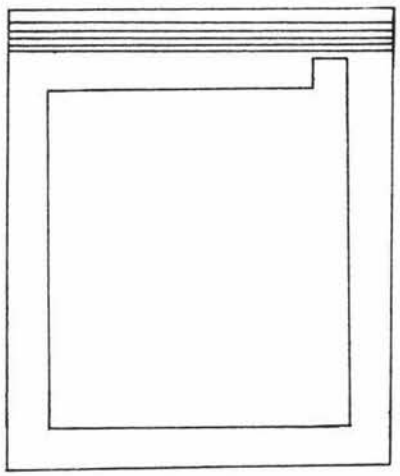
The second glass plate had the edges ground so that it would not damage the cellophane. Both plates were placed in a glassware drier and dried for 10-15 min. A gasket was made from six layers of 'Parafilm' giving a gel thickness of approximately 0.72 mm (Figure 5A). When both plates were dry, the gasket was sandwiched between them (Figure 5B) and clamped in position using plastic clothes pegs on the sides and top, and 'bulldog' clips on the bottom. The 'bulldog' clips enabled the mould to stand upright for casting.

2.7.4 Stock Solutions -

The isoelectric focussing gel system used was modified from the LKB method (LKB Application Note 250). Stock solutions were prepared as shown in Table 6. Final concentrations of the gel are shown in Table 5.

For one gel, 2.0 ml of stock A, 1.7 ml of stock C, 0.32 ml of Ampholine solution, 0.4 ml of stock E, 10 µl TEMED, and 3.58 ml of distilled water were mixed in a small glass vial and degassed for at least 5 min. The solution was taken up in a disposable 10 ml syringe and the gel cast by inserting the syringe needle into the gap in the mould gasket and filling the mould. Care was taken to ensure that air was not trapped in the mould by tilting the apparatus to fill the last portion of the mould and by tapping the glass plates if necessary.

A



B

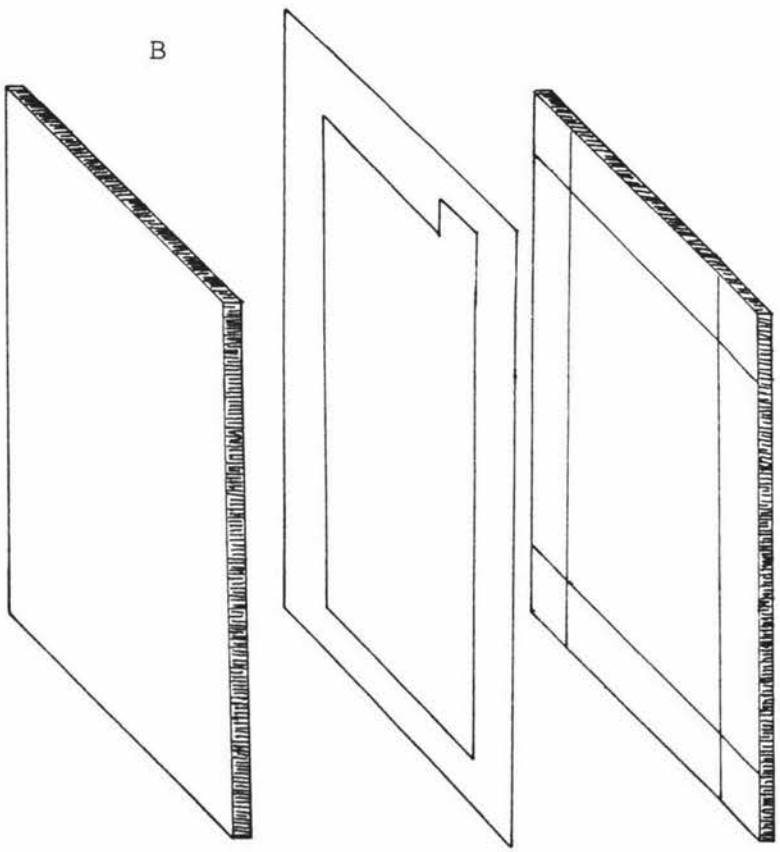


Fig 5 A Isoelectric focussing gel gasket for laboratory made gels.

B Exploded view of gel mould for laboratory isoelectric focussing gels.

TABLE 6 Stock solutions for isoelectric focussing gels

Stock A	Glycerol	50 ml
	H_2O	to 100 ml
Stock C	Acrylamide	28 g
	Bis	0.735 g
	H_2O	to 100 ml
Stock E	Riboflavin	0.004 g
	H_2O	to 100 ml
Ampholine solution (LKB)		40%

The mould was placed in fluorescent light to polymerize the gel (approximately 20 min). Care was taken to ensure that the level of the solution in the mould remained high and "topping up" was sometimes necessary.

When polymerisation was complete, the mould was placed in a refrigerator at 6^{o}C for at least 12 hr but still in fluorescent light. When the Multiphor apparatus had been prepared as described previously, the mould was removed from the refrigerator and the clamps removed. The mould was prised apart by inserting the tip of a scalpel blade into the gap in the gasket and twisting the scalpel slightly. This lifts the glass plate from the mould leaving the gasket and the gel on the second glass plate. The gasket was removed carefully so as not to tear the cellophane. A scalpel was used to cut the cellophane around the gel leaving approximately 4 mm of cellophane on each edge. The gel could then be handled by using forceps on the protruding edges of the cellophane.

2.7.5 Isoelectric Focussing -

The laboratory made gel or 'PAG' plate was placed in position on the 'Multiphor' apparatus and all air was excluded from beneath the gel. Electrode strips, supplied by LKB with the 'PAG' plates, were soaked in the respective electrode solution (sodium hydroxide or phosphoric acid) and placed on the gel lining up along the template.

Six sheets of Whatman No. 1 filter paper were cut to the size required and three used for each electrode wick. The wicks were first soaked in the respective electrode solution and laid carefully on top of the electrode strip. The other end of the wicks made contact with the electrode solution. A small piece of rubber sheeting 10 mm wide, 3 mm deep, and the same length as the electrode strip was placed on top of the electrode wicks to ensure good contact. Care was taken to ensure the electrode strips were parallel to each other, otherwise a distorted electrical field could result in a distorted pH gradient. An anticondensation electrophoresis hood was used in all runs.

Isoelectric focussing was carried out at a maximum of 500V and 1 Watt. A constant current of 10 mA was used for a complete ampholine PAG plate, and proportionately less if only parts of a plate were used. For the laboratory made gels, a current of 2 mA was used. Water (15^{o}C) was used for cooling.

Samples were applied using application wicks supplied by LKB, or Whatman No. 1 filter paper wicks measuring 10 mm by 5 mm. The wicks were dipped in the protein solution, and laid on the gel at the cathode end.

A myoglobin solution (5 mg/ml) was used as a marker protein so that focussing could be followed visually. A plot of voltage versus time was taken (Figure 6) and focussing was stopped when the voltage had levelled off. When focussing was complete, the gels were fixed and stained.

2.7.6 Protein Stain for Isoelectric Focussing Gels -

Fixing, staining, and destaining solutions were prepared as in Table 7. Immediately focussing was finished, the gel was removed from the apparatus, and placed in sufficient fixing solution to completely immerse the gel. After fixing for 1 hr, the gel was placed in destain solution for at least 1 hr or left overnight. The gel was then placed in stain solution and stained until the gel was an intense blue. The gel was destained in several changes of destain until the background was clear.

2.8 ISOZYME ANALYSIS

The term isozyme is used here "to refer to multiple molecular forms of an enzyme, with similar or identical catalytic activities occurring in the same organism" (Scandalios 1969).

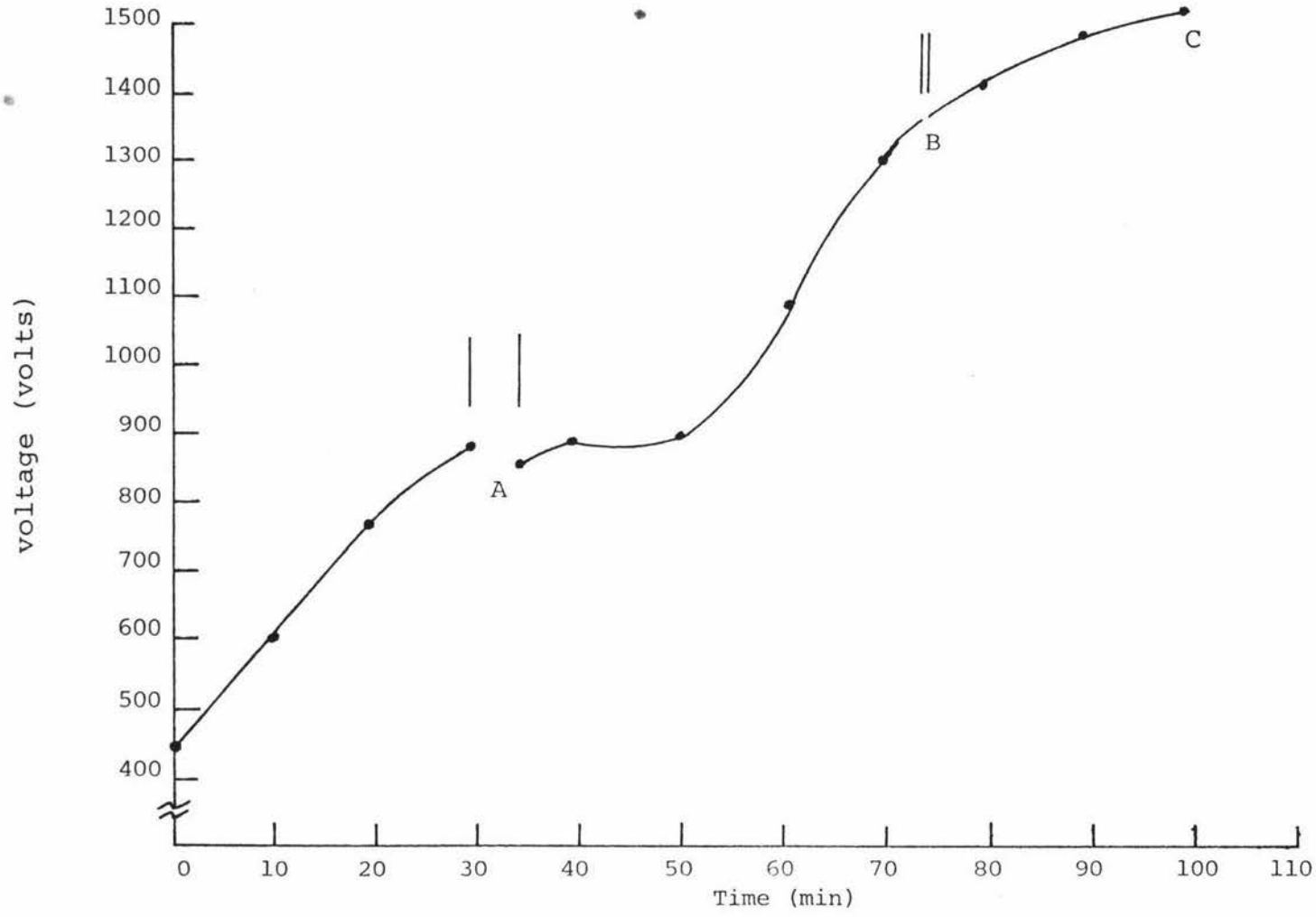


Fig 6 Voltage versus time graph typical for isoelectric focussing on laboratory made gels. (A) - application of samples (B) - removal of wicks (C) - focussing complete

TABLE 7 Staining solutions for isoelectric focussing

Fixing solution	Sulphosalicyclic acid	17.3 g
	Trichloroacetic acid	57.5 g
	H_2O	to 500 ml
Stain	Coomassie brilliant blue R250	0.46 g
	Destain solution	400 ml
Destain	Ethanol (95%)	500 ml
	Acetic acid (glacial)	160 ml
	H_2O	to 2000 ml

2.8.1 Peroxidase -

The method used for visualising peroxidase isozymes was based on that of Copes (D. Copes pers. comm.) which is a modification of the method of Brewbaker *et al.* (1968).

Stock solutions were prepared as shown in Table 8. Standard electrophoresis was carried out as described previously. When electrophoresis was finished and the gels had been removed from the tubes, the gels were placed in a solution containing 2 ml of stock D and 100 ml of stock C. The staining time depended on the concentration of the protein in the sample and production of suitable bands varied between 20 min and 2 hr. After staining, the gels were placed in storage test tubes and stored in distilled water as the product of the enzyme reaction is insoluble in water but appears to diffuse when stored in 10% acetic acid. The gels could be stored for up to six months depending on the initial stain density.

2.8.2 Acid Phosphatase -

The method used was an overlay method modified from that of Ross (1976). Stock solutions were prepared as shown in Table 9.

TABLE 8 Stock solutions for peroxidase staining in polyacrylamide gel electrophoresis

Stock A	3,3' - Dimethoxybenzidine	100 mg
	Ethanol (95%)	30 ml
Stock B	1 M NaOH	200 ml
	1 M Acetic acid adjust pH to 4.0	approx. 900 ml
Stock C	Stock A	30 ml
	Stock B	70 ml
Stock D	Hydrogen peroxide	3 ml
	H_2O	to 100 ml

TABLE 9 Stock solutions for acid phosphatase staining in isoelectric focussing gels

Stock A	0.1 M Sodium acetate - HCl buffer pH 5.2	
Stock B	0.4 M Sodium acetate - HCl buffer pH 5.2	
Stock C	10% Magnesium chloride	
Stock D	α Napthyl acid phosphate	0.01 g
	Stock A	70 ml
	dissolve then add -	
	Stock C	2 ml
	Fast blue RR	70 mg
	agar	1 g
	distilled water	30 ml
	boil several minutes and allow to cool slightly	

Isoelectric focussing was carried out as described previously. When focussing had finished, the gel was removed from the apparatus and placed in sufficient of stock B to cover the gel. The gel was left to wash for 5 minutes to remove excess ampholines and adjust the pH. The gel was allowed to drain before being placed on a clean glass plate. When stock E had reached approximately 70°C, stock D was added and the mixture quickly stirred before being layered over the gel. The gel and overlay were incubated at 30-35°C for several hours. When staining appeared to be at a maximum, the overlay was removed and the gel photographed. The stain is not permanent but is visible for several weeks.

2.9 IMMUNOLOGICAL ASSAYS

2.9.1 Formation of Antibodies -

Protin extracts of *P. radiata* 511 and *P. elliottii* (extract 1) were prepared as described previously except that the freeze-dried product was not taken up in distilled water. For antibody production, 20 mg of the freeze dried product was taken up in 0.5 ml of 0.15 M sodium chloride (isotonic) solution and emulsified with an equal volume of Freunds complete adjuvant using a modified double ended needle (Figure 7A), attached to two syringes (Figure 7B).

A

B

Fig 7 Apparatus for emulsifying Freunds adjuvant with samples.

A double ended needle
B apparatus ready for use

The solution was emulsified by passing it repeatedly between the two syringes. When each emulsion was considered ready (that is, the viscosity was felt to suddenly increase) it was injected intramuscularly into the rear leg of a New Zealand White Rabbit. This procedure was carried out for duplicates of each extract so that duplicate antiserum preparations could be prepared.

This first injection régime was followed fourteen days later with further aliquots from the same extracts emulsified in Freunds incomplete adjuvant. Blood was collected before the first injection and fourteen days after the second injection. If further antiserum was required, the second injection and blood collection régime was repeated.

2.9.2 Blood Collection -

Hair covering 10 mm of a vein on the back and close to the margin of an ear of the rabbit was removed by plucking. When the vein was cleared, the inside of the ear was swabbed with xylol to stimulate blood flow to the ear. The vein was then punctured with a blood lancet and the blood collected in a test tube. The blood was allowed to stand for 1.5 hr at room temperature followed by 15 min at 37^{o}C.

The coagulated blood was refrigerated overnight and the serum collected by centrifugation at 1,500 g for 10 min. A small volume (10 drops) of 1% thimerosal (sodium [(o-carboxyphenyl) thio] ethylmercury) was added to prevent bacterial contamination and the serum was stored at 4°C.

2.9.3 Preparation of Agarose Gels on Glass Plates -

Stock solutions were prepared as shown in Table 10. The tris-barbiturate buffer pH 8.6 (stock A) is diluted 1:4 before use.

For eight gel plates, 1.0 g agarose was added to 100 ml of dilute stock A. This solution was heated in a water bath to 90°C. When the agarose had melted, 12 ml aliquots were pipetted onto the cleaned glass plates set on a levelled horizontal table (LKB 2117-404) for casting. The poured plates were stored on damp paper towels in a closed container at 6°C.

2.9.4 Immunodiffusion -

Ouchterlony double diffusion technique was followed as described in the LKB Application Note 249. Agarose gels on glass plates were prepared as described previously.

TABLE 10 Stock solutions for agarose gels used in Ouchterlony double diffusion and immunoelectrophoresis analysis

Stock A	Diethylbarbituric acid	22.4 g
	Tris	44.3 g
	Calcium lactate	0.553 g
	Sodium azide	0.650 g
	H_2O	to 1 litre
Stock B	Bromophenol blue	0.005 g
	H_2O	to 100 ml

The plates were placed in the plate holder supplied in the LKB 2117-401 'Multiphor' immunoelectrophoresis kit. Four groups of seven wells in the pattern shown in Figure 8 were punched in each plate using a 4.0 mm diameter well punch and a double diffusion template supplied in the LKB kit. Antiserum (15 µl) was pipetted into the central well and 15 µl of the protein extracts were pipetted into the outer wells. The plates were placed on damp paper towels in a closed container and left to diffuse overnight at room temperature. The gels were pressed, washed, stained, and destained as described in sections 2.9.6 and 2.9.7.

2.9.5 Immunoelectrophoresis -

Grabar and Williams immunoelectrophoresis was followed as described in the LKB Application Note 249. Agarose gels on glass plates were prepared as described previously and placed on the plate holder supplied in the LKB 2117-401 'Multiphor' immunoelectrophoresis kit. The central well for each sample application point in the immunoelectrophoresis template supplied by LKB was punched using a 4.0 mm diameter gel punch. An extra well in line with the sample wells was punched on the edge of each gel.

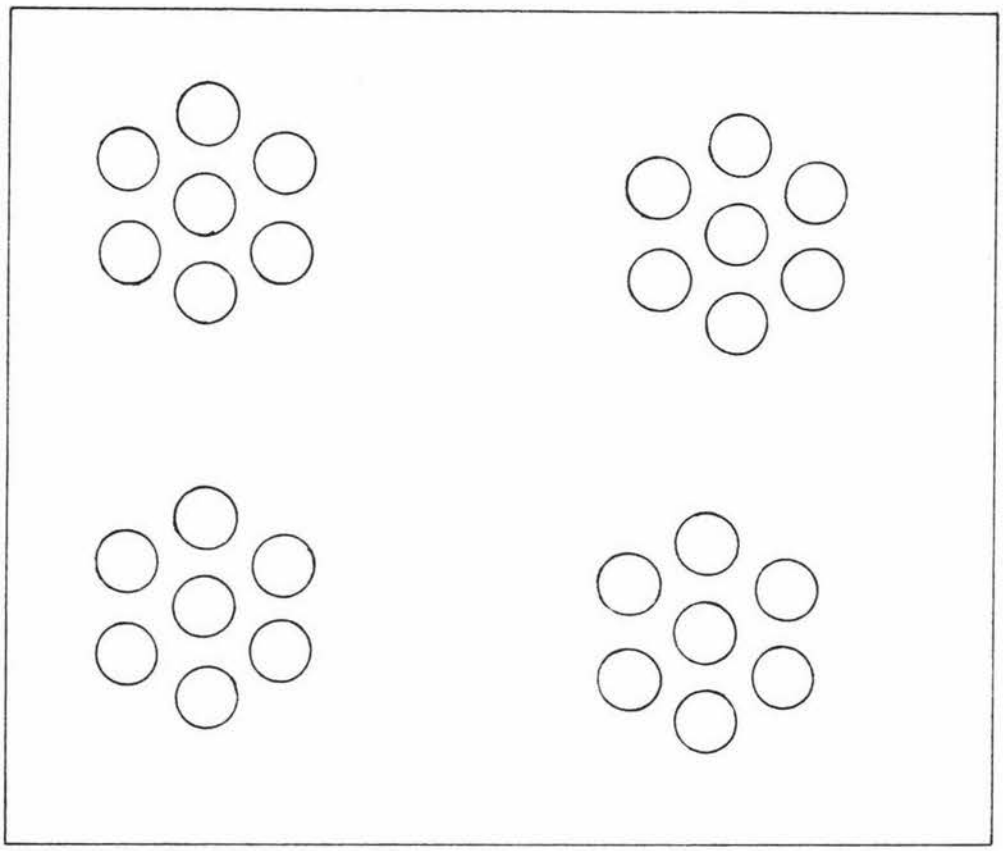


Fig 8 Pattern of wells used in double diffusion analysis. Antiserum was placed in the central well and the extracts to be compared were placed in pairs in the outer wells.

The sides of the antiserum troughs were cut to mark their position and the gel transferred to the cooling plate of the 'Multiphor' apparatus, with the sample wells close to the cathode.

The buffer tanks were filled with 1 litre of dilute stock A (Table 10). Twelve pieces of Whatman No. 1 filter paper cut to the correct size were soaked in the electrophoresis buffer. Six of these were laid on the edges of the gel close to the buffer tanks with 10 mm of the gel in contact. Care was taken to ensure the wicks were parallel with each other. When both wicks were in place, 15 µl of the protein samples to be electrophoresed were pipetted into the sample wells, and tracking dye solution (10 µl) (stock B) was pipetted into the extra well so that the progress of electrophoresis could be followed visually.

Electrophoresis was carried out at 5 V/cm, using tap water (15^{o}C) for cooling, until the tracking dye had reached the end of the antiserum troughs.

When electrophoresis was finished, the gel plates were removed from the 'Multiphor' apparatus and placed on the horizontal table. The antiserum troughs were then removed from the gels and 100-200 µl of antiserum was pipetted into them. The plates were placed on damp paper towels in a closed container and left to diffuse overnight at room temperature.

2.9.6 Pressing and Drying -

When the diffusion of protein and antibody from immunodiffusion or immunoelectrophoresis procedures was finished, the gels were washed with distilled water and placed on a hard surface. Several layers of filter paper were placed on top of the gel followed by a glass plate and a heavy weight (approximately 1 kg). This procedure was repeated with fresh filter paper after 3 min. When the gel had been pressed for another 3 min. it was dried with a portable hair drier until it was a thin film on the glass plate.

2.9.7 Staining -

Solutions for washing, staining, and destaining were prepared as shown in Table 11. The pressed and dried gels were washed for at least 1.5 hr in two changes of the sodium chloride solution, after which the gels were again dried with the portable hair drier. The gels were then placed in the stain solution for 10 min., washed under running tap water, and destained in the destaining solution until the background was clear.

TABLE 11 Washing, staining, and destaining solutions for agarose gels

Washing solution:	0.1M Sodium chloride	
Staining solution:	Coomassie brilliant blue R-250	1 g
	Ethanol	90 ml
	Acetic acid	20 ml
	H_2O	90 ml
Destaining solution:	Ethanol	90 ml
	Acetic acid	20 ml
	H_2O	90 ml

3. RESULTS

The freeze dried product from one extract (*P. radiata* 517) was analysed for protein and carbohydrate content to determine sample purity and to estimate the required sample loadings for each of the techniques. This product represented 0.1% of the initial fresh weight (dry weight of sample material = 34.8% fresh weight) and consisted of 25% protein and 70% carbohydrate. This analysis was not performed on all the samples as each analysis required a relatively large proportion of the extract and insufficient fresh material was available.

3.1 DISCONTINUOUS POLYACRYLAMIDE ROD GEL ELECTROPHORESIS

Typical gels with protein samples separated by electrophoresis are shown in Figure 9. The ethanolacetic acid stained gels (stain method 1) were scanned twice on an ultra violet scanner (Joyce Loebl, England) at 265 nm and recorded on a chart recorder (Rikadenki Kogyo, Japan) with the gels turned 90^{o} between scans. The action of turning the gels ensures that only true peaks, (peaks present on both scans) will be scored, and any peaks which may have been caused by gel irregularities or particulate matter will be discounted. A typical scan recording is shown in Figure 10 where the vertical numbered bars represent peaks present on both recordings.

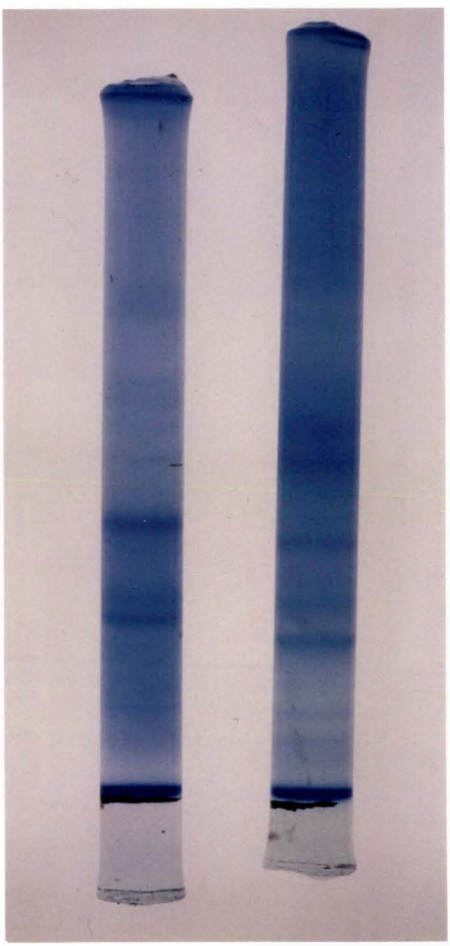
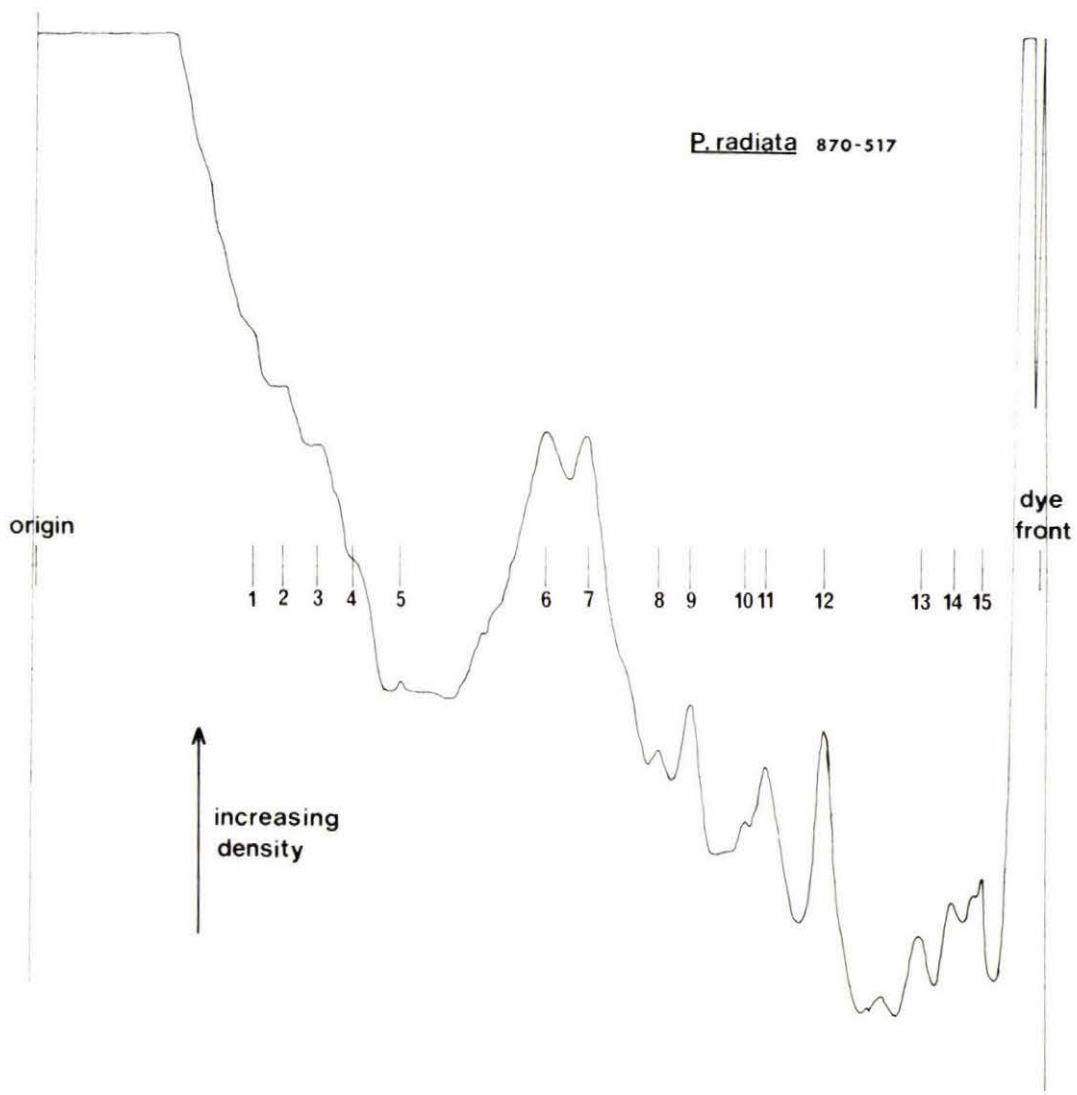


Fig 9 Typical discontinuous polyacrylamide rod gels after electrophoresis. Extracts shown are two *P. radiata* clones.

Fig 10 Ultraviolet scan recording for the extract of *P. radiata* 517. The sample was separated by electrophoresis in a discontinuous polyacrylamide rod gel.


P. radiata 870-517
origin
dye front
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
increasing density

The distance migrated by each of the bands and the dye front was obtained by both measurement from the scan recordings and measurement direct from the gels. The results of the measurements were expressed as a ratio where:

$$Rm = \text{relative mobility of a band during electrophoresis} = \frac{\text{distance migrated by a band}}{\text{distance migrated by the dye front}}$$

Results obtained using both measurements for the same gel are shown in Table 12.

The scanner/recorder system used was incapable of recording the complete range of densities present in the gels hence the ratios for all other samples were measured by direct observation of the gels. These results are shown in Table 13. From the Rm values, electrophoregrams can be drawn so that individual gels can be compared directly. The electrophoregram for the five clones of *P. radiata* is shown in Figure 11, and the electrophoregram for each species of *Pinus* sampled is shown in Figure 12.

The degree of electrophoretic similarity between the different extracts was determined by calculating the "similarity index" (Ziegenfus and Clarkson, 1971; Shechter and de Wet, 1975; Ladizinsky and Hymowitz, 1979) for each pair of gels where similarity index

$$(SI) = \frac{h \times 100}{h + n}$$

h = number of homologous bands

n = number of nonhomologous bands

TABLE 12 Comparison of Rm values obtained by direct measurement from scan recordings and polyacrylamide gels

P. radiata 517	
Scan	Gel
	0.05
	0.12
0.22	0.22
0.25	0.25
0.29	0.29
0.32	0.32
0.37	0.37
	0.45
0.51	0.51
0.55	0.55
	0.59
0.63	0.63
0.66	0.66
	0.69
0.71	0.71
0.73	0.73
0.78	0.78
	0.81
	0.84
0.88	0.88
0.92	0.92
	0.93
0.95	0.94

TABLE 13 Rm values for four species of *Pinus* obtained by direct measurement from the gels

P. radiata					*P. elliottii*		*P. taeda*	*P. monticola*
510	511	517	522	525	ext. 1	ext. 2		
0.04	0.27	0.05	0.06	0.16	0.14	0.05	0.10	0.10
0.13	0.32	0.12	0.12	0.20	0.22	0.12	0.20	0.13
0.16	0.35	0.22	0.15	0.25	0.29	0.14	0.29	0.21
0.20	0.38	0.25	0.21	0.27	0.36	0.21	0.41	0.27
0.23	0.51	0.29	0.25	0.32	0.43	0.28	0.45	0.31
0.29	0.55	0.32	0.27	0.34	0.52	0.34	0.51	0.37
0.31	0.65	0.37	0.31	0.37	0.57	0.40	0.55	0.42
0.41	0.68	0.45	0.36	0.42	0.59	0.46	0.57	0.46
0.45	0.72	0.51	0.38	0.45	0.66	0.50	0.62	0.50
0.48	0.78	0.55	0.43	0.51	0.69	0.53	0.65	0.58
0.52	0.80	0.59	0.45	0.59	0.71	0.57	0.71	0.69
0.55	0.87	0.63	0.52	0.62	0.74	0.62	0.73	0.73
0.61	0.91	0.66	0.54	0.68	0.76	0.65	0.83	0.81
0.65	0.96	0.69	0.59	0.71	0.78	0.68	0.86	0.90
0.71		0.71	0.63	0.74	0.82	0.70	0.94	
0.74		0.73	0.67	0.78	0.87	0.74		
0.79		0.78	0.71	0.82	0.93	0.76		
0.80		0.81	0.72	0.85	0.97	0.82		
0.82		0.84	0.78	0.87		0.87		
0.84		0.88	0.88	0.90		0.91		
0.88		0.92	0.91	0.95		0.92		
0.91		0.93	0.92	0.97		0.97		
0.93		0.94	0.94					

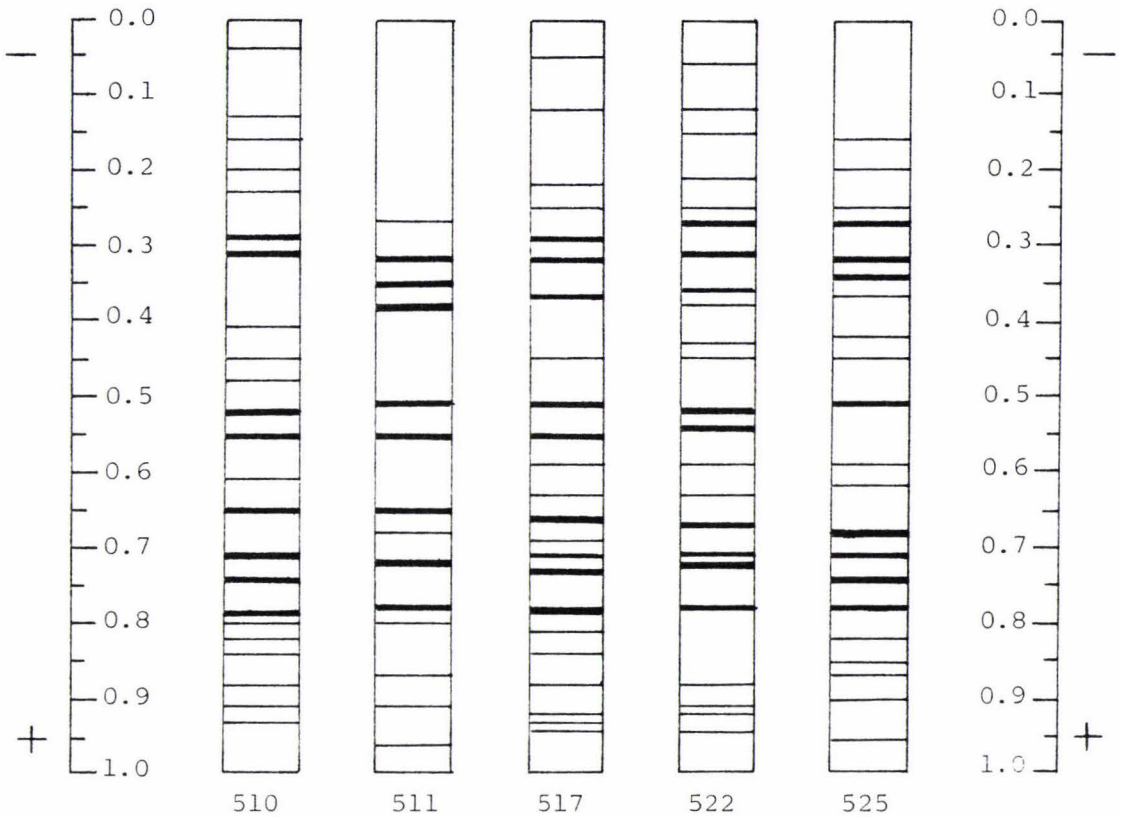


Fig 11 Electrophoregram of five *P. radiata* clones.

Samples were separated by discontinuous polyacrylamide rod gel electrophoresis.

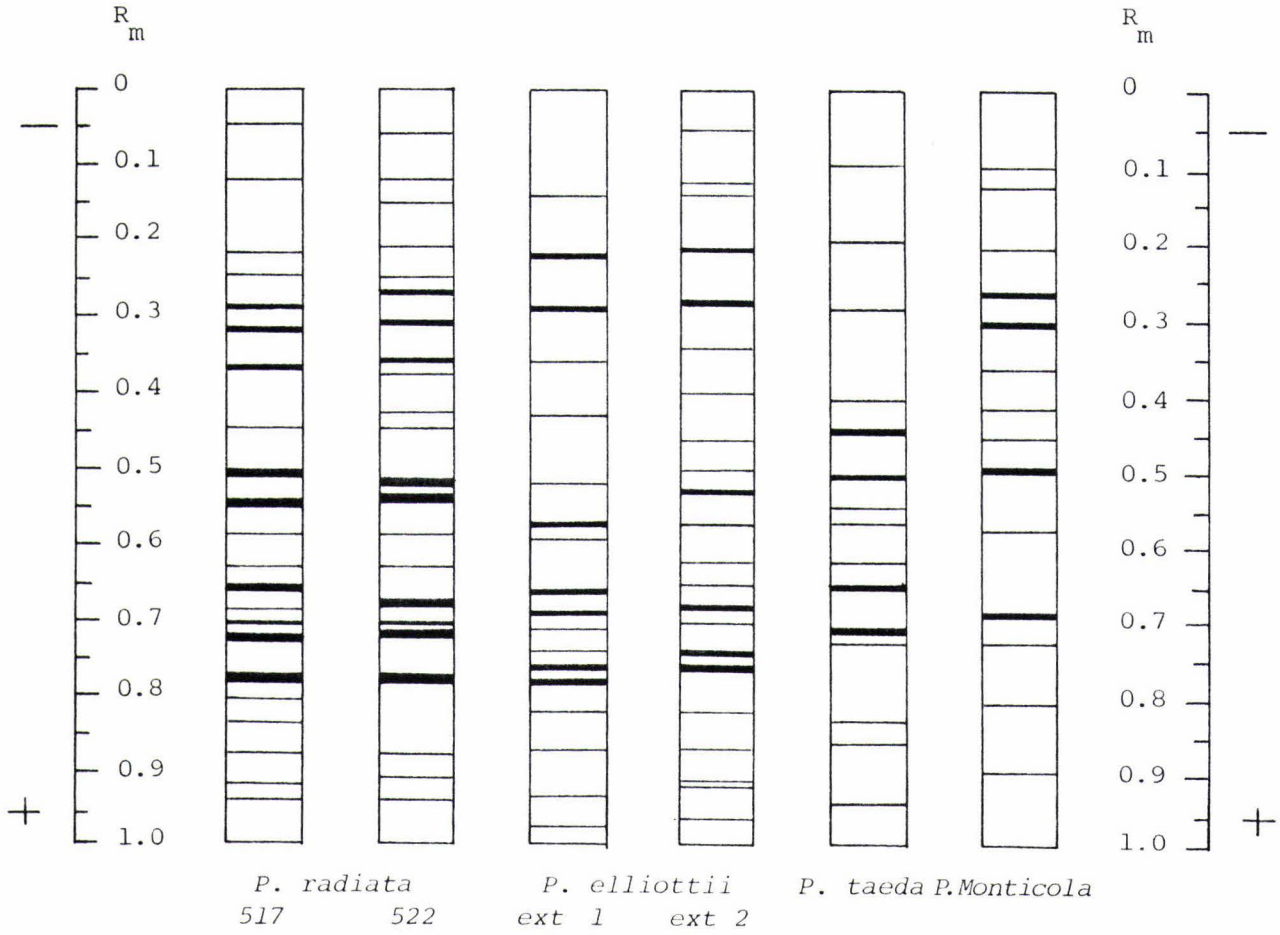


Fig 12 Electrophoregram for four species of *Pinus*. Samples were separated by discontinuous polyacrylamide gel electrophoresis.

A computer program (Appendix I) was written to calculate the similarity index for each pair of gels. The Rm value (X 100) for each band was entered into the microcomputer (Sord M100 ACE, Tokyo, Japan) and the program counts any pair of bands that fall within 0.02 Rm units (see section on peroxidase isozymes for explanation of the use of 0.02 Rm units). Examples of the printout from the computer are shown in Appendix II. The similarity index for each pair of gels calculated using this program are shown in Table 14.

To test how meaningful the results for the similarity indices are, the computer program also generates and ranks groups of random numbers between 1 and 99 inclusive. The program was set up so that it would generate the same number of random numbers as there were bands in each gel. For example, eighteen bands were detected in the extract of *P. elliottii* extract 1, and fifteen bands detected in the extract of *P. taeda*. The computer was therefore instructed to generate two series of random numbers; ten groups of eighteen numbers and ten groups of fifteen numbers. The program took one group of numbers from each series and counted the number of values within two units (0.02 x 100) from which the similarity index was calculated. The procedure was then repeated for the other groups. The results for the simulated comparison for *P. elliottii* extract 1 and *P. taeda* is shown in Appendix III. Using this analysis, each combination of numbers simulating the number of bands in each extract were run and the average similarity indices calculated (Table 15). This table is set out using the same format as Table 14.

TABLE 14 Similarity indices for gel data from table 13
Indices were calculated using the computer program in Appendix I

		P. radiata					*P. elliottii*		*P. taeda*	*P. monticola*
		510	511	517	522	525	ext 1	ext 2		
P. radiata	510		37.03	70.37	70.37	60.71	51.85	66.66	52.0	42.30
	511			54.16	54.16	50.00	60.00	56.52	31.81	40.00
	517				76.92	66.66	64.00	73.07	58.33	48.00
	522					73.07	46.42	73.07	58.33	54.16
	525						65.00	62.96	48.00	50.00
P. elliottii	ext 1							60.00	50.00	45.45
	ext 2								68.18	50.00
P. taeda										52.63

TABLE 15 Average similarity index for random numbers
See text for details

		No. Bands	*P. radiata*					*P. elliottii*		*P. taeda*	*P. Monticola*
			510	511	517	522	525	ext 1	ext 2		
No. Bands			23	14	23	23	22	18	22	15	14
P. radiata	510	23		36.63	41.02	41.02	39.07	42.15	39.07	38.37	36.63
	511	14			36.63	36.63	34.78	36.53	34.78	29.64	30.94
	517	23				41.02	39.07	42.15	39.07	38.37	36.63
	522	23					39.07	42.15	39.07	38.37	36.63
	525	22						38.89	44.12	33.59	34.78
P. elliottii	ext 1	18							38.89	35.73	36.53
	ext 2	22								33.59	34.78
P. taeda		15									29.64

Comparing Tables 14 and 15, it can be seen that the values on Table 14 are generally higher than the values expected from the random matching of pairs of bands, and the highest values on the table tend to be for the intraspecific comparisons (for example, *P. radiata* 517 x *P. radiata* 522). The values for interspecific comparisons are generally lower than those for intraspecific comparisons. Similarity indices for *P. monticola* are only slightly higher than would be expected from random matching.

A basic assumption, inherent in the analysis of similarity indices, is that values for intra-specific comparisons are expected to be higher than values for interspecific comparisons, thus indicating the closer relationship between clones. Two extracts yeild values (Table 14) which do not follow this assumption. Values for *P. elliottii* extract 2 are higher than expected possibly reflecting the inaccuracies in the electrophoretic technique and measurement of band position. The values for *P. radiata* 511 tend to be lower than those for other *P. radiata* clones. Difficulty was experienced in staining gels of this clone and bands near the origin could not be differentiated (see Table 13). The values for *P. radiata* 511 were therefore excluded from the following calculations.

Chi squared analysis (Appendix IV) was performed using similarity indices calculated from random numbers (Table 15) as the expected values, and similarity indices from the gel data (Table 14) as the observed values.

Data for each species comparison were grouped and all differences were found to be significant at the 0.5% level, indicating that the pairing of bands in the gels were probably caused by factors other than random chance.

To summarise Table 14, the values for each species comparison were averaged so that an overall comparison between the species could be made (Table 16). The highest average values obtained were for intraspecific comparisons. The values for the interspecific comparisons suggest that *P. elliottii* and *P. taeda* are closely related. (*P. elliottii* x *P. elliottii* = 60.00 compared to *P. elliottii* x *P. taeda* = 59.09.) It also appears that *P. radiata* is more closely related to *P. elliottii* than to *P. taeda*. However, the result for *P. elliottii* x *P. radiata* is probably higher than would be expected as already noted.

A high degree of variation in electrophoresis data is apparent (Table 14) and this is reflected in the average similarity index values on Table 16. This variation produces inconsistent results, for example, a close relationship between *P. elliottii* and *P. radiata* is suggested when *P. elliottii* is compared to *P. radiata* (62.88 compared to an intraspecific comparison of 60.00) while a more distant relationship is suggested when *P. radiata* is compared to *P. elliottii* (62.88 compared to an intraspecific comparison of 69.68).

TABLE 16 Average similarity indices for four *Pinus* species using rod gel electrophoresis

Values are percentages

	P. radiata	*P. elliottii*	*P. taeda*	*P. monticola*
P. radiata	69.68	62.88	54.17	48.62
P. elliottii		60.00	59.09	47.73
P. taeda				52.63

This high variation suggests that the differences in similarity index between *P. radiata*, *P. elliottii*, and *P. taeda* are probably not significant indicating electrophoresis may not be useful for detecting small differences between extracts. The data does however, indicate a consistently distant relationship between *P. monticola* and the other species, supporting the division of the genus *Pinus* into two subgenera. A close relationship is suggested between *P. elliottii* and *P. taeda* but no evidence of a close relationship between *P. radiata* and *P. taeda* was found. Thus, in spite of the inconsistencies, more evidence was found in support of hypothesis 1 (Figure 1A) than hypothesis 2 (Figure 1B).

3.2 ISOELECTRIC FOCUSSING

A typical isoelectric focussing gel showing extracts of four *P. radiata* clones, *P. elliottii* extract 1, extracts of two species of *Carmichaelia*, and a myoglobin standard is shown in Figure 13. The gel is orientated so that the anode (pH 3.5) is at the top. The majority of the densely stained bands for the *Pinus* extracts are clustered at the anodic end of the gel. The pH of the gel in this region, and hence the isoelectric points of the major protein components of the *Pinus* extracts, are in the range pH 4-5 (Figure 14). Fewer bands are apparent in the middle and at the cathode end of the gel (pH 7.0-9.5).

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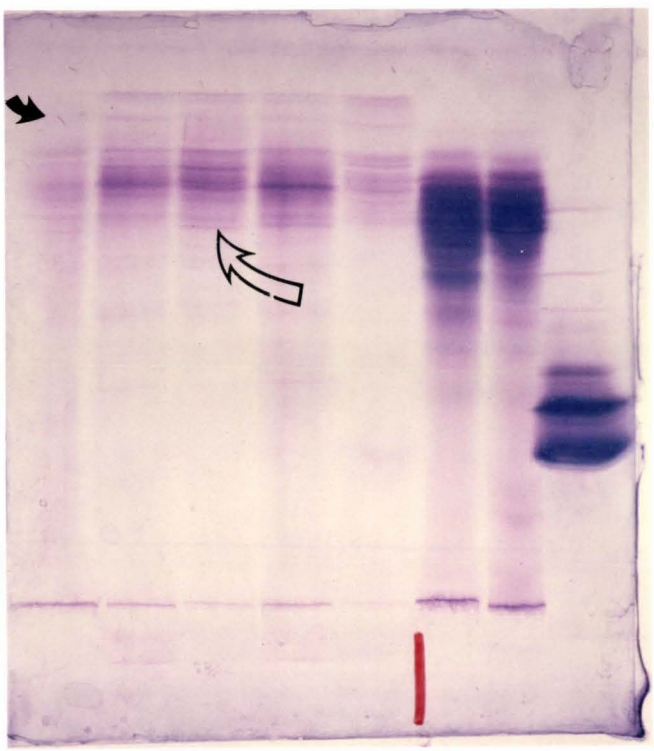
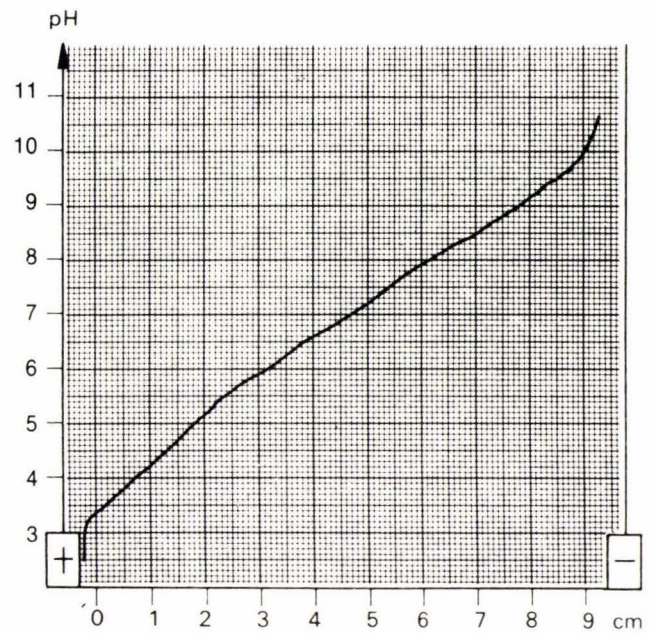


Fig 13 Typical flat bed polyacrylamide gel after isoelectric focussing. Extracts shown are (from left) *P. elliottii* extract 1, *P. radiata* 525, *P. radiata* 522, *P. radiata* 517, *P. radiata* 511, two *Carmichaelea* species, and a myoglobin standard.

LKB 1804-101, pH 3.5–9.5
Anode-electrode solution: 1 M H_3PO_4
Cathode-electrode solution: 1 M NaOH
LKB 2103-settings at 10 °C: P=30 W; U=1500 V; I=50 mA
Time for experiment: 1.5 hours



A typical pH 3.5–9.5 gradient

Fig 14 Typical pH gradient for a pH 3.5 - 9.5 isoelectric focussing gel.

(LKB 'PAG' plate information sheet).

This is true also of the *Carmichaelia* extracts (extracted at pH 8.4) although the major concentration of proteins for these extracts is shifted slightly toward the cathode. As the myoglobin standard (pI = 7.0, Lehninger 1970) focussed in the middle of the gel, it suggests the lack of bands in the *Pinus* extracts in this area is not an artifact caused by some peculiarity in the gel.

Isoelectric focussing enables proteins differing by only a few hundredths of a pH unit in their isoelectric points (LKB Application Note 250) to be separated in the same gel. Hence when two bands occupy the same position on the gel, they almost certainly represent the same protein. This is illustrated by the similarity in banding pattern of the four *P. radiata* clones in Figure 13. Measurement of band position necessary in techniques using discrete gels for each sample (for example, Rm values in rod gel electrophoresis) are not required using flat bed isoelectric focussing as similarity in band position can be observed directly from the gel.

While the overall banding pattern for the four *P. radiata* clones seen in Figure 13 is similar, some differences can be seen. For example, two bands detected in *P. radiata* 522 (hollow arrow) do not appear to be in the same position as bands present in either *P. radiata* 511 or *P. radiata* 525. Band density also appears to be different for the same band in different clones.

The most densely stained band in *P. radiata* 517 appears more densely stained than the comparable band in *P. radiata* 522 even though the amount of protein loaded onto the gel was the same. A band present in *P. radiata* 525 and *P. radiata* 522 appears more densely stained than the comparable band in *P. radiata* 511 or *P. radiata* 517. Differences in the protein complement between the two *Pinus* species are also apparent. For example, two bands in the extract of *P. elliottii* (small arrow) do not appear to be present in *P. radiata* 525 although a band lying between the two *P. elliottii* bands can be seen. Thus the technique of isoelectric focussing enables interspecific and intraspecific differences to be detected.

The number of protein bands detected in the extracts using isoelectric focussing varied between thirteen (*P. taeda* extract 1) and twenty-seven (*P. radiata* 517 and 522). (Table 17.) The number of bands which appear to be common between extracts varied between four (for example, *P. elliottii* extract 1 x *P. monticola*) and twenty-three (*P. radiata* 517 x *P. radiata* 522) (Table 18). The comparisons showing the greatest number of common bands on Table 18 are the intraspecific comparisons. Conversely, the lowest number of common bands are generally detected in interspecific comparisons suggesting that the number of bands in common to two extracts is a measure of the relationship of those extracts.

TABLE 17 Number of protein bands detected in four *Pinus* species using isoelectric focussing.

Extract	Number of Bands
P. radiata 511	23
P. radiata 517	27
P. radiata 522	27
P. radiata 525	25
P. elliottii extract 1	18
P. elliottii extract 2	14
P. taeda extract 1	13
P. taeda extract 2	15
P. monticola	19

TABLE 18 Number of common protein bands between extracts of four *Pinus* species using isoelectric focussing

		P. radiata				*P. elliottii*		*P. taeda*		*P. monticola*
		511	517	522	525	ext 1	ext 2	ext 1	ext 2	
P. radiata	511		16	15	16	8	6	4	6	7
	517			23	20	9	6	5	7	7
	522				22	10	6	4	7	7
	525					11	7	8	6	7
P. elliottii	ext 1						12	8	8	4
	ext 2							11	10	5
P. taeda	ext 1								12	5
	ext 2									5

From Table 18, similarity indices (p. 52) can be calculated (Table 19). The highest value for a similarity index was obtained from the intraspecific comparison while the lowest values were generally obtained from the interspecific comparisons. However relatively high similarity indices were obtained for comparisons of *P. elliottii* extracts with *P. taeda* extracts, suggesting a close relationship between these two species although the 'degree' of this relationship varied depending on which of the *P. elliottii* extracts was considered.

To summarise the data on Table 19, all intraspecific and interspecific comparisons between the different extracts were averaged so that an overall comparison between the species would be made (Table 20). Once again the highest values obtained were for the intraspecific comparisons and again, the lowest values were for the interspecific comparisons. In spite of this trend, the similarity index for the comparison between *P. elliottii* and *P. taeda* was substantially higher than other interspecific comparisons, again suggesting a close relationship between these two species. The similarity indices for comparisons with the extract of *P. monticola* are generally the lowest suggesting this species is distantly related to the other species.

Data from isoelectric focussing therefore support the division of the genus *Pinus* into two subgenera.

TABLE 19 Similarity indices for data from isoelectric focussing

		P. radiata				*P. elliottii*		*P. taeda*		*P. monticola*
		511	517	522	525	ext 1	ext 2	ext 1	ext 2	
P. radiata	511		47.06	42.86	50.00	24.24	19.35	12.5	18.75	20.00
	517			74.19	62.5	25.00	17.14	14.28	20.00	17.95
	522				73.33	28.57	17.14	11.11	20.00	17.95
	525					34.38	21.88	26.67	17.65	18.92
P. elliottii	ext 1						60.00	34.78	32.00	12.12
	ext 2							68.75	52.63	17.86
P. taeda	ext 1								75.00	18.52
	ext 2									17.24

TABLE 20 Average similarity indices for four species of *Pinus* using isoelectric focussing

Values are percentages

	P. radiata	*P. elliottii*	*P. taeda*	*P. monticola*
P. radiata	58.32	23.46	17.62	18.71
P. elliottii		60.00	47.04	14.99
P. taeda			75.00	17.88

These data also supports hypothesis one (Figure 1A) by suggesting a relatively high relationship between *P. elliottii* and *P. taeda*.

3.3 PEROXIDASE ISOZYMES

A protein extract of *P. radiata* 517 was electrophoresed and stained for peroxidase activity on three separate occasions; 10th November 1980, 4th June 1981, and 26th June 1981. A typical gel is shown in Figure 15. The relative mobility of each band for each gel run was obtained by direct measurement from the gels (Table 21) and an electrophoregram for each gel drawn (Figure 16). The width of each band represents the relative stain density of the band compared to other bands in the same gel.

An estimation of the 'error' in Rm values between different gel runs was obtained by expressing as a percentage the maximum variation in Rm value for the most variable band over the three runs (Table 21). The variation in the position of bands is caused by slight changes in the conditions of electrophoresis such as gel composition and temperature differences. No trends in the variation of band position, the number of bands detected, or in changes of stain density could be detected, indicating that the sample is stable for considerable lengths of time when stored as described in the Methods.

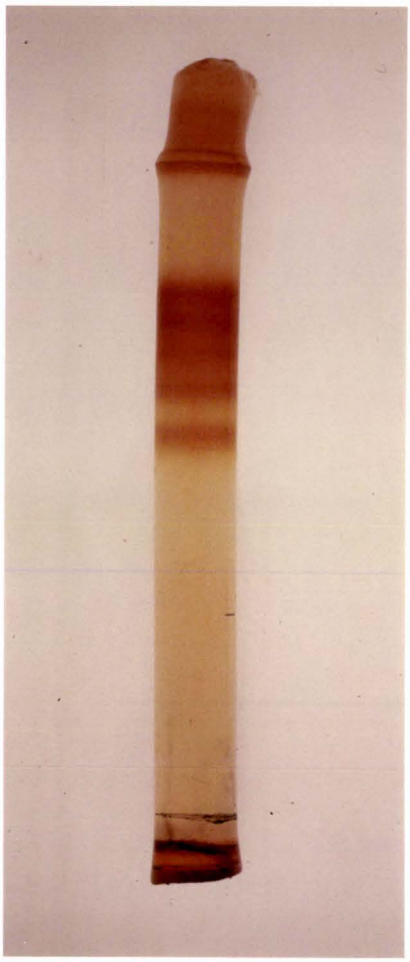


Fig 15 Typical peroxidase stained discontinuous polyacrylamide rod gel. Extract shown is *P. radiata* 517.

TABLE 21 Relative mobility of peroxidase isozyme bands for *P. radiata* 517

BAND	DATE			"ERROR" %
	10.11.80	4.6.81	26.6.81	
A	0.15	0.16	0.14	0.02
B	0.21	0.21	0.19	0.02
C	0.30	0.28	0.29	0.02
D	0.36	0.35	0.35	0.01
E	0.44	0.43	0.42	0.02

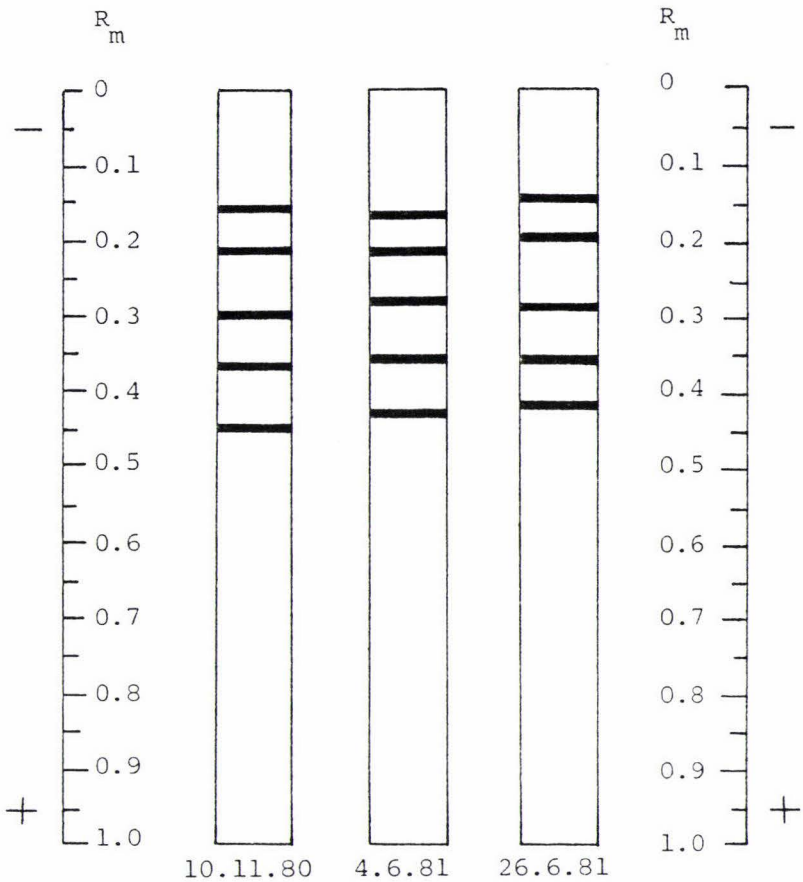


Fig 16 Peroxidase isozyme electrophoregram for *P. radiata* 517.

Discontinuous polyacrylamide rod gel electrophoresis was performed on the dates shown.

Bands in different gels were considered to represent the same isozyme if they had similar stain intensity and an Rm value within the 'error' of 0.02 Rm units. For example, group A, for the isozymes of the five *P. radiata* clones (Table 22, Figure 17) includes bands with an Rm value of 0.16± 0.01 Rm units. The peroxidase isozyme bands for the five clones of *P. radiata* have been divided into four 'classes' designated A, B, C and D, according to stain density, band position, and the presence or absence of a particular band in any sample. Two bands are included in classes A, B and D, and one band in class C. The band pattern suggests that each band within the classes A, B, and D, represent "allozymes" (Guries and Ledig 1978) or "allelic isozymes" (Scandalios 1969), and each class represents a different locus. The "allozymes" appear to be monomeric, that is, two bands are present in a heterozygote (Scandalios 1969; Rudin 1977), but this cannot be fully determined without testing for segregation in haploid tissue, (for example, the megametophyte tissue). The same band pattern has been found in other enzyme systems in conifers, for example, esterase (Rudin and Rasmuson 1973), ribonuclease II (Mejnartowicz and Bergmann 1977), leucine amino peptidase (Rudin 1977) and leucine amino peptidase and phosphoglucomutase (Adams and Joly 1980a). Assuming that the different bands within classes A, B, and D do represent allozymes, the five *P. radiata* clones can be represented as follows:

TABLE 22 Rm values of peroxidase isozymes for 5 clones of *P. radiata*
Samples were separated by discontinuous polyacrylamide rod gel electrophoresis

Group	Clone: 510	511	517	522	525
A1			0.16	0.17	0.15
A2	0.20	0.19	0.21	0.21	0.20
B1	0.25			0.27	0.26
B2	0.29	0.28	0.28	0.30	0.30
C	0.36	0.36	0.35	0.37	0.36
D1	0.43		0.43	0.43	
D2		0.46		0.47	

Values were grouped as described in the text.

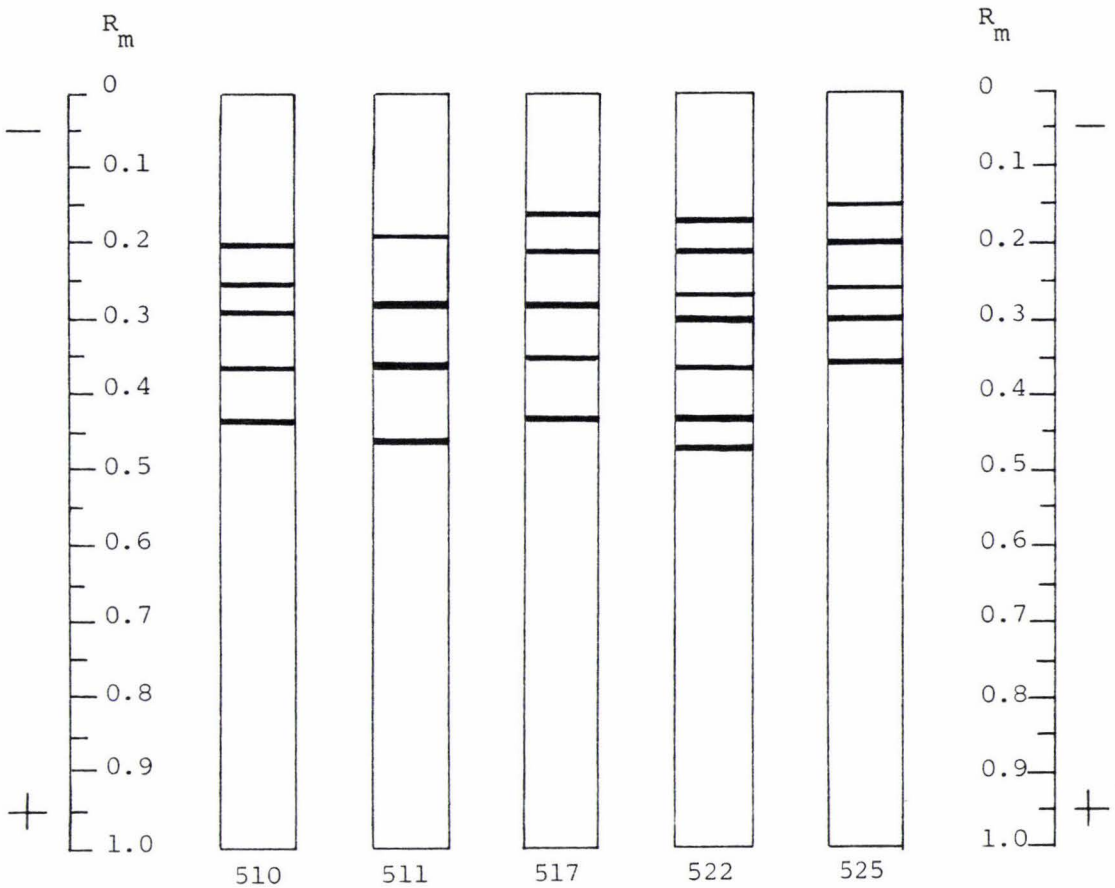


Fig 17

Peroxidase isozyme electrophoregram for five *P. radiata* clones.

Samples were separated by discontinuous polyacrylamide rod gel electrophoresis.

P. radiata 510 = A_2 A_2, B_1 B_2, C, D_1 D_1
P. radiata 511 = A_2 A_2, B_2 B_2, C, D_2 D_2
P. radiata 517 = A_1 A_2, B_2 B_2, C, D_1 D_1
P. radiata 522 = A_1 A_1, B_1 B_2, C, D_1 D_2
P. radiata 525 = A_1 A_2, B_1 B_2, C

The stain for *P. radiata* 525 was not clear in the lower part of the gel, hence the presence or absence of band in class D could not be determined.

Extracts of two individuals of *P. elliottii*, *P. taeda*, and *P. monticola* were analysed for peroxidase activity. At least three bands were detected in each sample with a maximum number of nine found in *P. taeda* extract 2. The range in the number of peroxidase bands between species agrees with that recorded by other workers (Juo and Stotzky 1973). Rm values were obtained for each band in each extract by direct observation (Table 23). An electrophoregram for each species was drawn from Table 23 and these were compared to electrophoregrams for two clones of *P. radiata* (Figure 18).

Good agreement was found in some band positions between samples of the same species in Figure 18. Each pair of extracts have at least three pairs of bands in common [*P. radiata* bands A_2, B_2, C, D_1 (Table 22); *P. elliottii* bands A, B, C; *P. taeda* bands A, F, I; and *P. monticola* bands A, C, D (Table 23)].

TABLE 23 Rm values of peroxidase isozymes for four species of *Pinus*. Samples were separated by discontinuous rod gel electophoresis

P. radiata			*P. elliottii*			*P. taeda*			*P. monticola*		
Band	517	522	Band	Extract 1	Extract 2	Band	Extract 1	Extract 2	Band	Extract 2	Extract 2
A1	0.16	0.17	A1		0.02	A	0.05	0.06	A	0.19	0.17
A2	0.21	0.21	A2	0.04		B		0.22	B	0.28	
B1		0.27	B	0.26	0.26	C		0.25	C	0.30	0.30
B2	0.28	0.30	C	0.31	0.32	D1	0.29		D	0.36	0.36
C	0.35	0.37	D	0.34		D2		0.30			
D1	0.43	0.43				D3	0.31				
D2		0.47				E		0.35			
						F	0.40	0.40			
						G		0.44			
						H		0.49			
						I	0.69	0.70			

Values were arranged as described in the text.

Fig 18 Peroxidase isozyme electrophoregram for four *Pinus* species. Samples were separated by discontinuous polyacrylamide rod gel electrophoresis.

The variation in band numbers between species and between samples of the same species may represent differences in peroxidase activity at different stages of development (Ramaiah *et al.* 1971; Conkle 1971b) or the presence of allozymes (for example, bands D_1, D_2 and D_3 in *P. taeda*, and as discussed for *P. radiata*).

Although relatively large differences in band patterns are evident between the different species, in agreement with other workers (Juo and Stotzky 1973), some bands appear to be common between species. A zone of activity around Rm=0.30 is present in all samples, possibly including allozymes with Rm values of 0.28±0.01 (present in *P. radiata* 510, 517; *P. taeda* extracts 1 and 2, and *P. monticola* extract 1) and 0.31±0.01 (present in *P. elliottii* extracts 1 and 2, *P. taeda* extracts 1 and 2, and *P. monticola* extracts 1 and 2). Another band (Rm=0.25±0.01) is present in *P. radiata*, *P. elliottii*, and *P. taeda* but not present in *P. monticola*. A very slow migrating band is found in *P. elliottii* and *P. taeda* which may be present as two allozymes (Rm=0.02 in *P. elliottii* extract 2; and Rm=0.05±0.01 present in *P. elliottii* extract 1 and *P. taeda* extract 1 and 2). A unique band (0.70±0.01) was found in *P. taeda*. Juo and Stotzky (1973) found similar interspecific variation in band numbers and position in eight *Pinus* species. These workers did not show actual values for Rm but approximate values can be obtained using the scale published with their figure (Juo and Stotzky 1973, Figure 1). (Table 24.)

TABLE 24 Approximate values of Rm for peroxidase isozymes

(Juo and Stotzky, 1973)

P. radiata	*P. elliottii*	*P. taeda*
0.18	0.20	0.16
0.28	0.26	0.28
0.41	0.30	0.41
0.59	0.44	
	0.48	0.57
	0.60	0.71
	0.65	
	0.75	
	0.80	

Values were estimated using a scale published with the data.

While band numbers and the position of some bands resemble the numbers and positions of bands found in some extracts used in this study, no overall pattern similarity was found.

The data from peroxidase isozyme patterns show considerable differences between individuals of the same species (*P. taeda* extracts 1 and 2) making interspecific comparisons difficult. However, differences in banding patterns between species in different subgenera and between species within a subgenus can be shown. The banding pattern for *P. elliottii* and *P. taeda* appear to be similar but neither of these species show a strong resemblance to *P. radiata*. The pattern for *P. monticola* however, shows least resemblance to the other species. The results from peroxidase isozyme analysis gives some support to the division of the genus *Pinus* into two subgenera, and suggests a close relationship between *P. elliottii* and *P. taeda* (hypothesis one, Figure 1A).

3.4 ACID PHOSPHATASE

Results of acid phosphatase isozymes detected using isoelectric focussing of four species of *Pinus* are shown in Figure 19. The gel, stained specifically for acid phosphatase isozymes, was placed in a photographic enlarger and the bands drawn directly onto paper (Figure 20). Both Figure 19 and 20 are orientated so that the anode (pH 3.5) is at the top. Samples were applied near the cathode (pH 9.5).

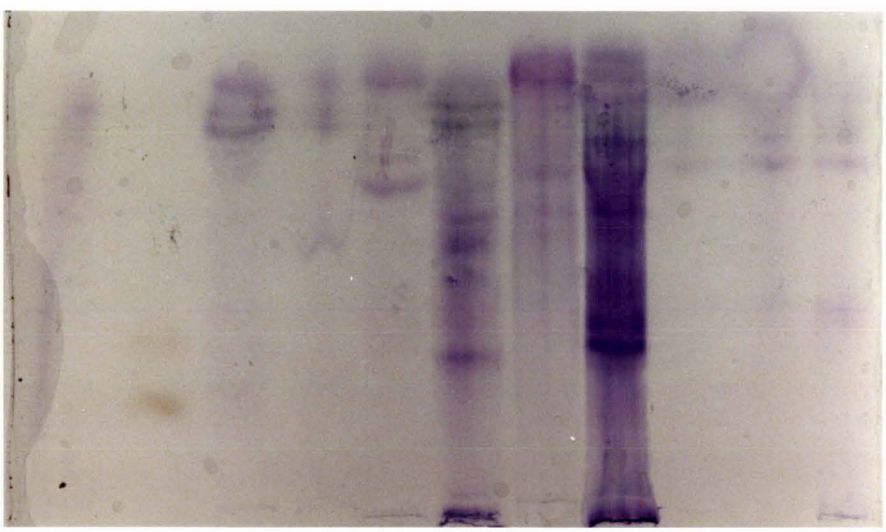


Fig 19 Acid phosphatase isoelectric focussing gel. Extracts shown are (from left); *P. monticola* extract 2, *P. monticola* extract 1, *P. taeda* extract 2, *P. taeda* extract 1, *P. elliottii* extract 2, *P. elliottii* extract 1, *P. radiata* 522, *P. radiata* 517, *P. radiata* 511.

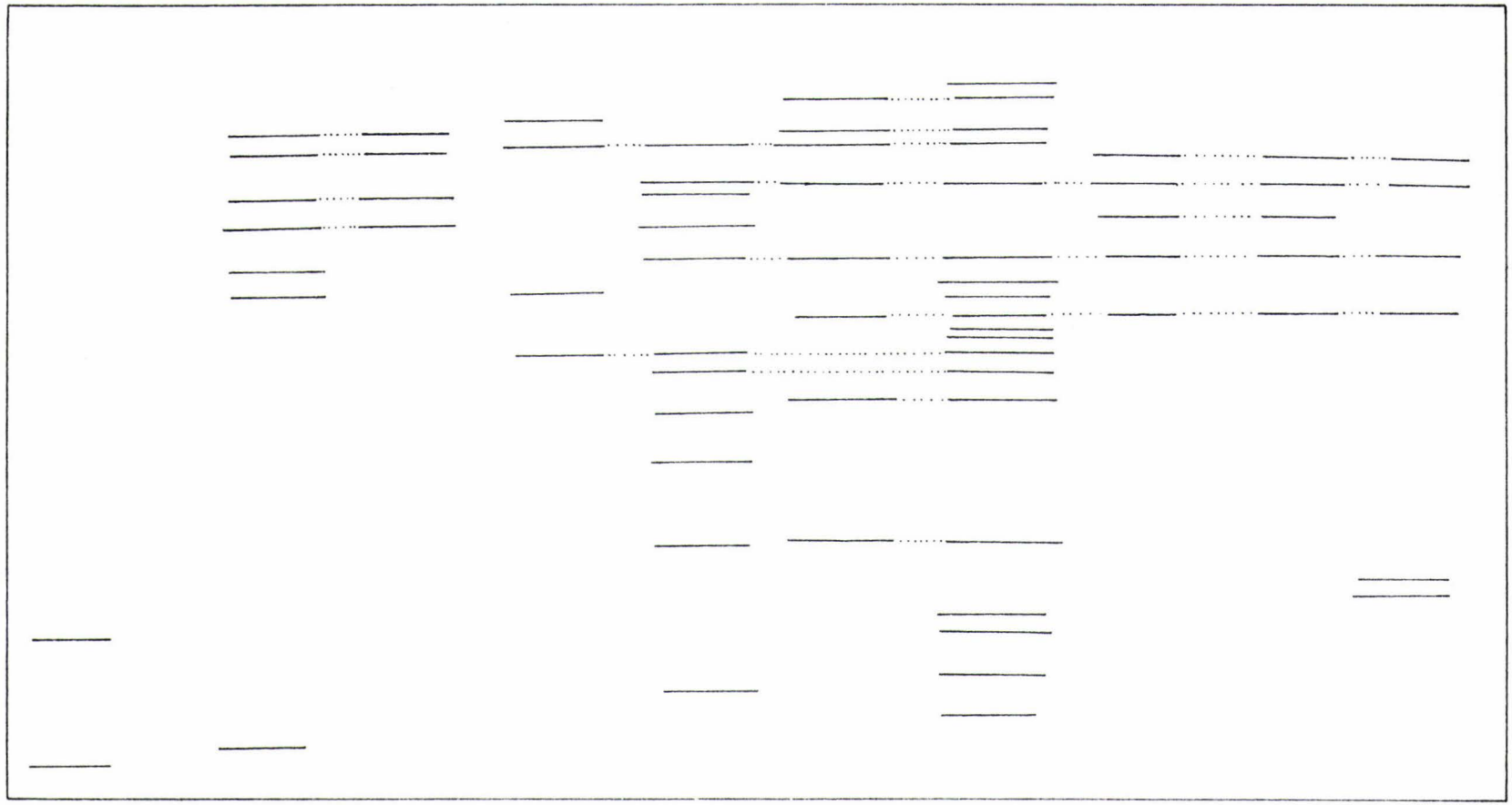


Fig 20 Isozymes of Acid Phosphatase detected in four species of *Pinus* after isoelectric focussing.

The number of acid phosphatase isozymes detected using isoelectric focussing is considerably greater than the number detected in *Pinus* using gel electrophoresis by other authors (Hamaker and Snyder 1973; Adams and Jolly, 1980a).

This technique allows a direct comparison between extracts as similar bands focus at the same position in the gel. For example, bands in the *P. radiata* clones can be seen to line up at the same point (Figure 19 and 20). Four bands appear to be common to all three *P. radiata* clones as indicated by the dotted lines (Figure 20). One band is found in only two of the clones (517 and 522) while two bands close to the cathode were detected in *P. radiata* 511 only. Nineteen bands were detected in *P. elliottii* extract 1 and eight in *P. elliottii* extract 2. All the bands detected in extract 2 appeared to be present in extract 1. Eleven bands were detected in *P. taeda* extract 1 and four in *P. taeda* extract 2, but only two appeared to be present in both extracts. Four bands were detected in *P. monticola* extract 1 and these appeared to be present in *P. monticola* extract 2. An extra three bands were detected in *P. monticola* extract 2.

Three bands appear to be common to all *P. radiata* clones and *P. elliottii* extracts. Two of these were also detected in *P. taeda* extract 1. Another three bands were found to be common to both *P. taeda* and *P. elliottii*. None of the bands in *P. monticola* appeared to be similar to bands in the other three species.

Isoelectric focussing, applied to acid phosphatase isozymes, suggest that *P. monticola* is distantly related to the other species supporting the division of the genus *Pinus* into the subgenera. Two bands were found present in all three Diploxylon species (although they were not detected in *P. taeda* extract 2 in this run) suggesting these bands may be useful in determining the subgeneric status of *Pinus* species. The three extra bands in common between *P. elliottii* and *P. taeda* suggest a closer relationship between these species than between *P. elliottii* and *P. radiata* (one extra band). No bands were found to be common to only *P. radiata* and *P. taeda*. The results therefore support hypothesis one (Figure 1A) and suggest a closer relationship between *P. elliottii* and *P. taeda* than between *P. taeda* and *P. radiata*.

3.5 OUCHTERLONY DOUBLE - DIFFUSION ANALYSIS

Typical double diffusion results are shown in Figures 21 to 28. The number of precipitin bands for each extract when challenged with antiserum to *P. radiata* 511 or *P. elliottii* extract 1 were counted. The maximum number of bands observed in any one test, and the average number of bands in all tests, were calculated (Table 25 A, B). The number and position of bands observed in a double diffusion test indicates how closely related the test extract is to the antigen extract or to another test extract. (El-Lakany *et al.*, 1977.)

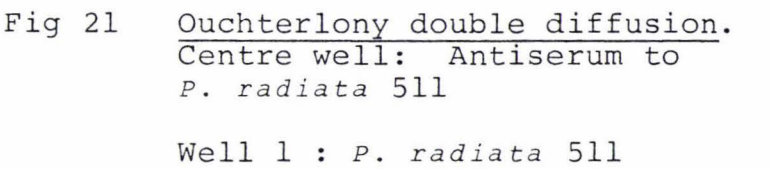


Fig 21 Ouchterlony double diffusion.
Centre well: Antiserum to *P. radiata* 511

Well 1 : *P. radiata* 511

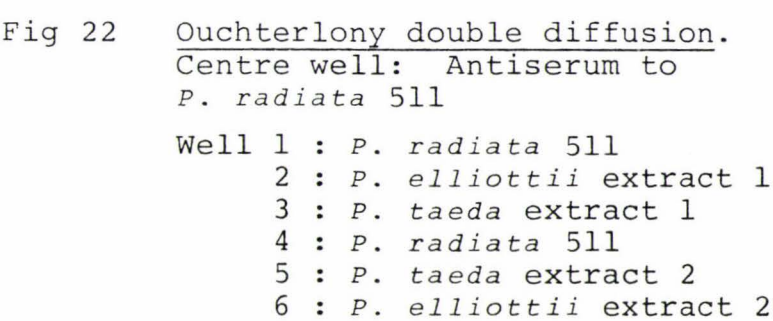
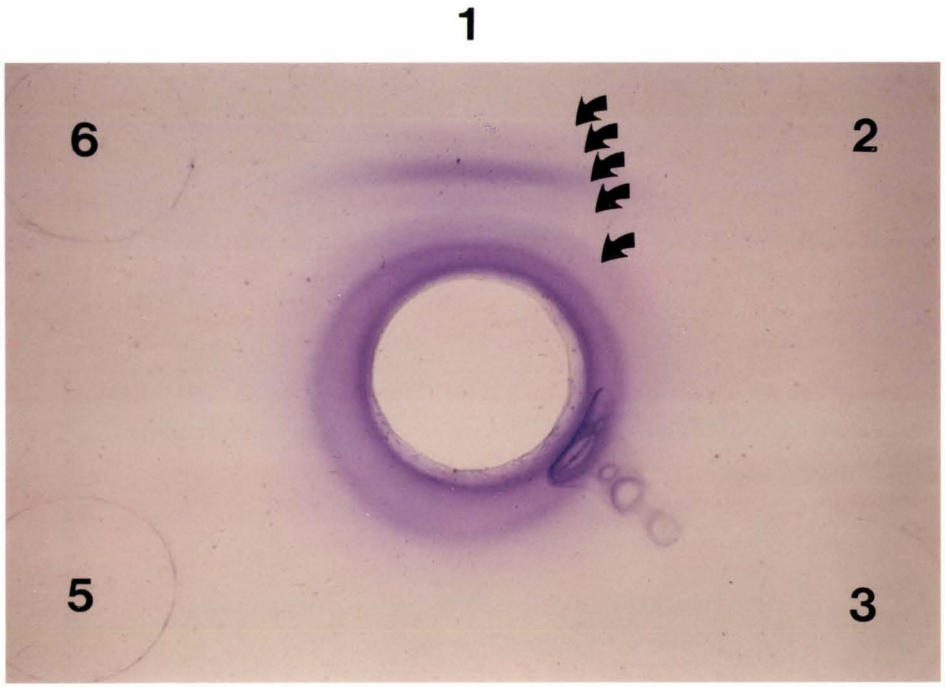


Fig 22 Ouchterlony double diffusion.
Centre well: Antiserum to *P. radiata* 511

Well 1 : *P. radiata* 511
2 : *P. elliottii* extract 1
3 : *P. taeda* extract 1
4 : *P. radiata* 511
5 : *P. taeda* extract 2
6 : *P. elliottii* extract 2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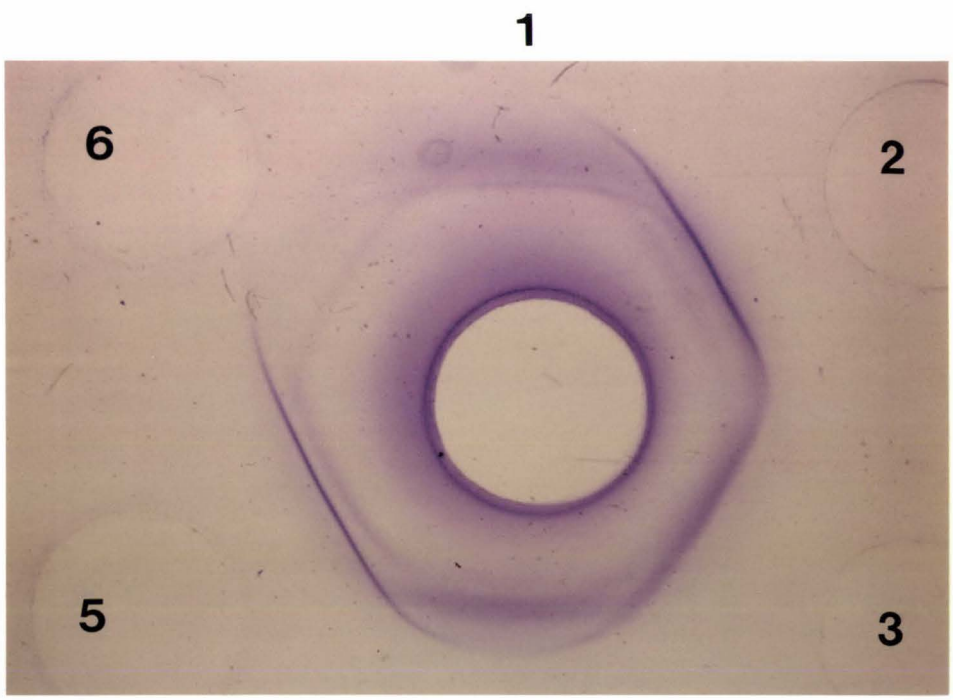
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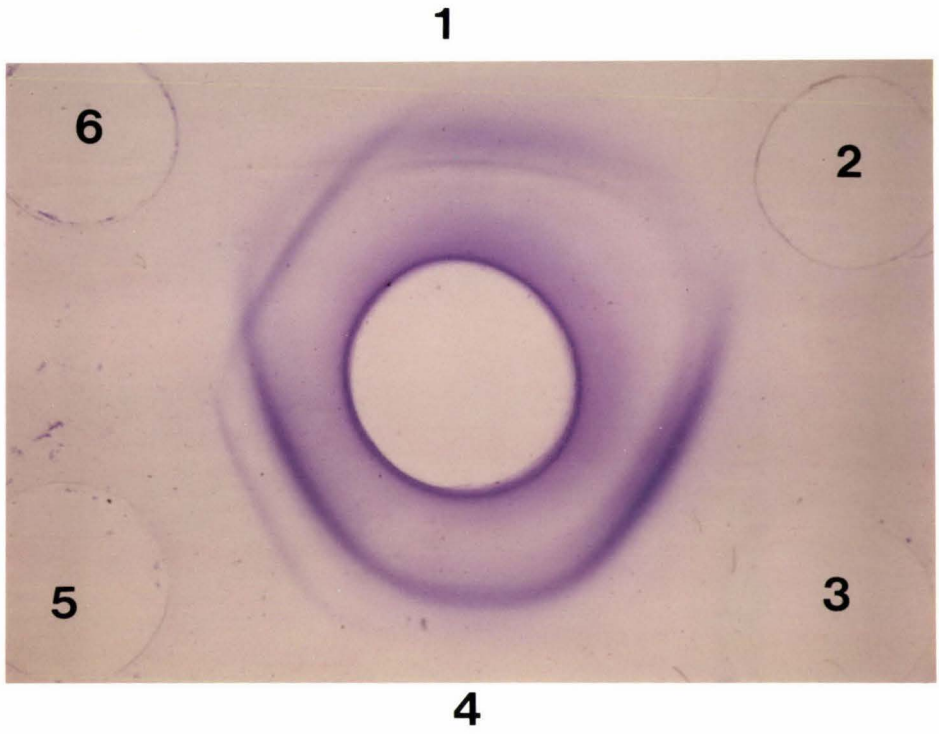
Fig 23 Ouchterlony double diffusion.
Centre well: Antiserum to *P. radiata* 511

Well 1 : *P. radiata* 511
2 : *P. monticola* extract 1
3 : *P. radiata* 522
4 : *P. radiata* 511
5 : *P. radiata* 525
6 : *P. monticola* extract 2

Fig 24 Ouchterlony double diffusion.
Centre well: Antiserum to *P. radiata* 511

Well 1 : *P. elliottii* extract 1
2 : *P. radiata* 522
3 : *P. taeda* extract 1
4 : *P. elliottii* extract 1
5 : *P. taeda* extract 2
6 : *P. radiata* 525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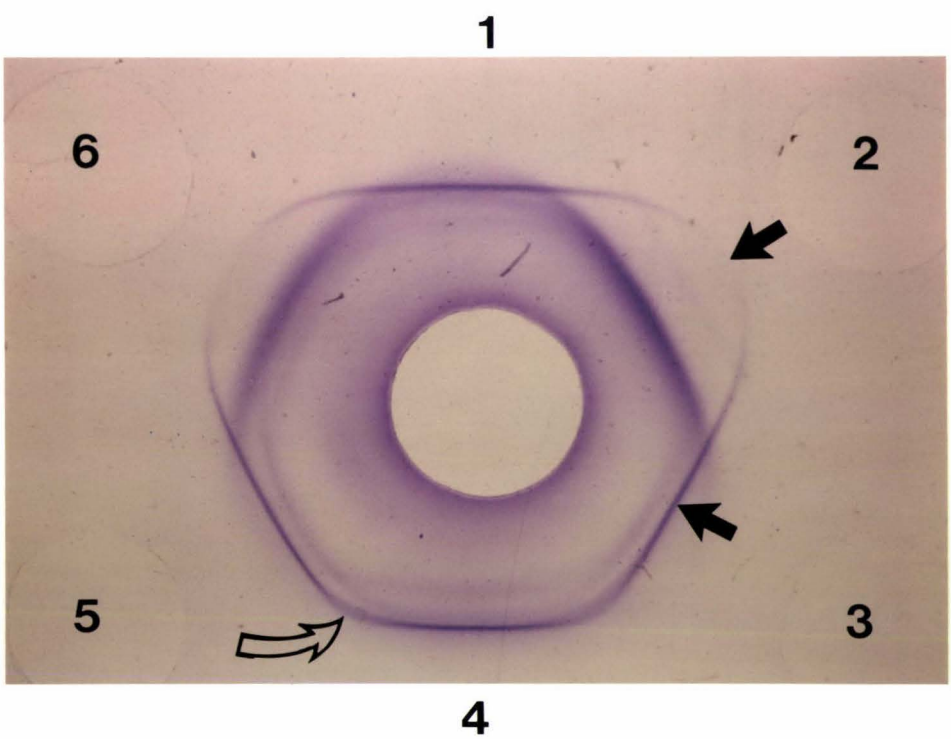
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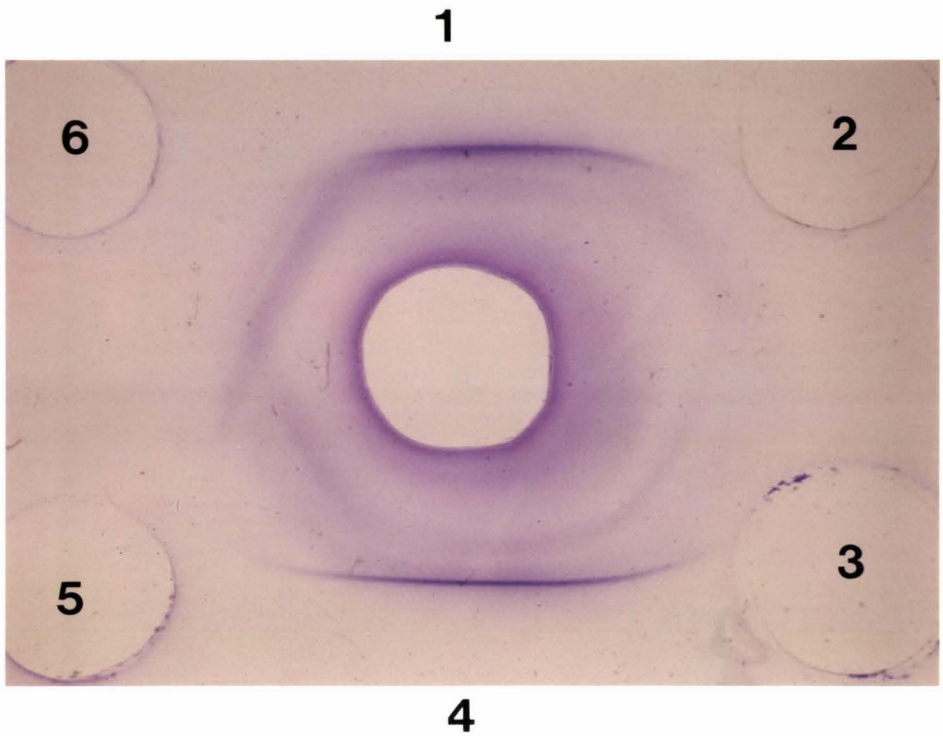
Fig 25 Ouchterlony double diffusion.
Centre well: Antiserum to *P. radiata* 511

Well 1 : *P. elliottii* extract 1
2 : *P. monticola* extract 1
3 : *P. elliottii* extract 2
4 : *P. elliottii* extract 1
5 : *P. elliottii* extract 2
6 : *P. monticola* extract 2

Fig 26 Ouchterlony double diffusion.
Centre well: Antiserum to *P. elliottii* extract 1

Well 1 : *P. radiata* 511
2 : *P. elliottii* extract 1
3 : *P. taeda* extract 1
4 : *P. radiata* 511
5 : *P. taeda* extract 2
6 : *P. elliottii* extract 2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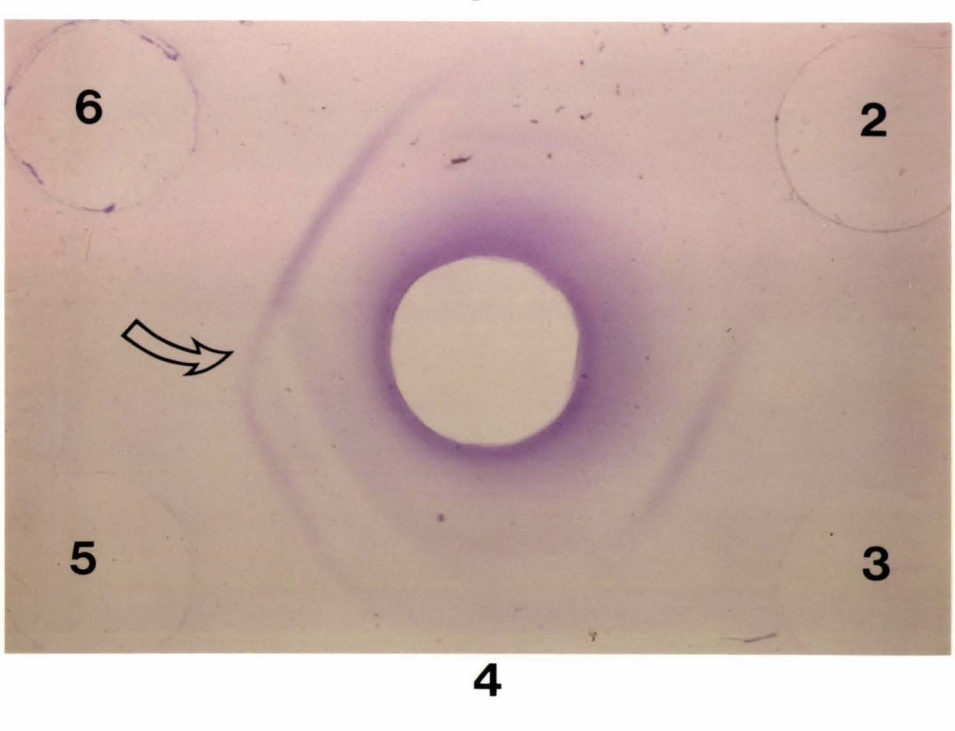
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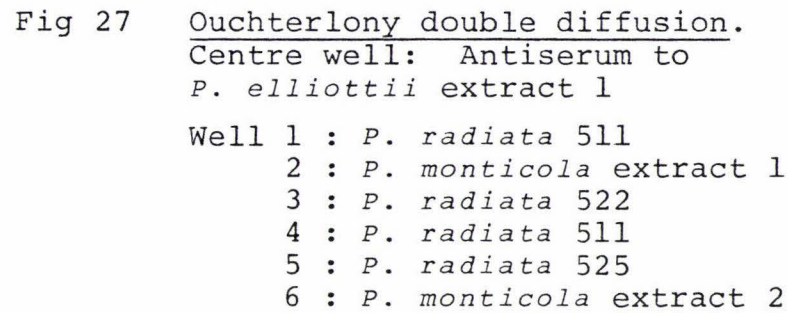


Fig 27 Ouchterlony double diffusion.
Centre well: Antiserum to *P. elliottii* extract 1

Well 1 : *P. radiata* 511
2 : *P. monticola* extract 1
3 : *P. radiata* 522
4 : *P. radiata* 511
5 : *P. radiata* 525
6 : *P. monticola* extract 2

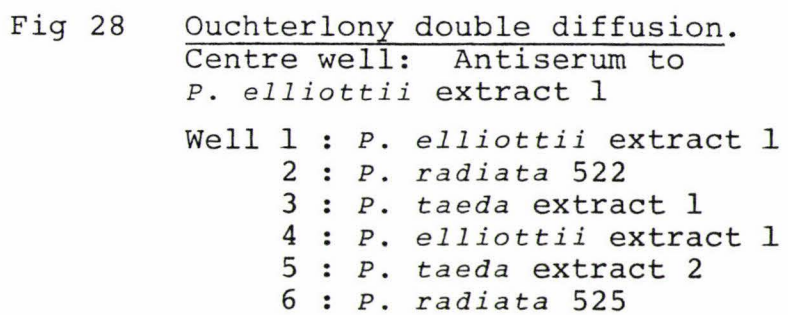
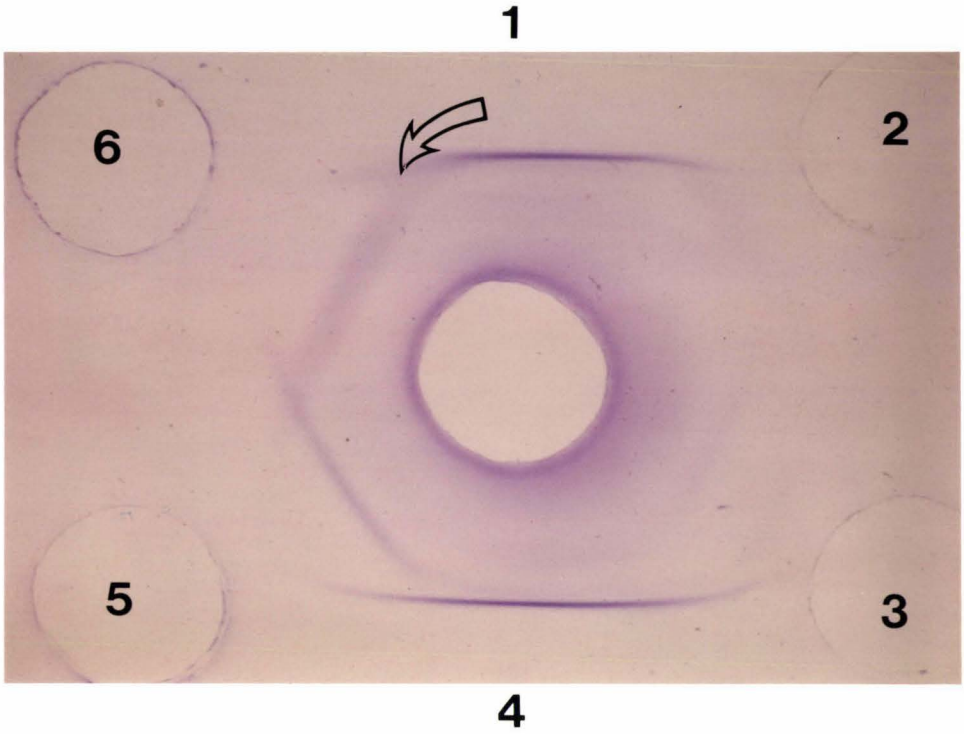


Fig 28 Ouchterlony double diffusion.
Centre well: Antiserum to *P. elliottii* extract 1

Well 1 : *P. elliottii* extract 1
2 : *P. radiata* 522
3 : *P. taeda* extract 1
4 : *P. elliottii* extract 1
5 : *P. taeda* extract 2
6 : *P. radiata* 525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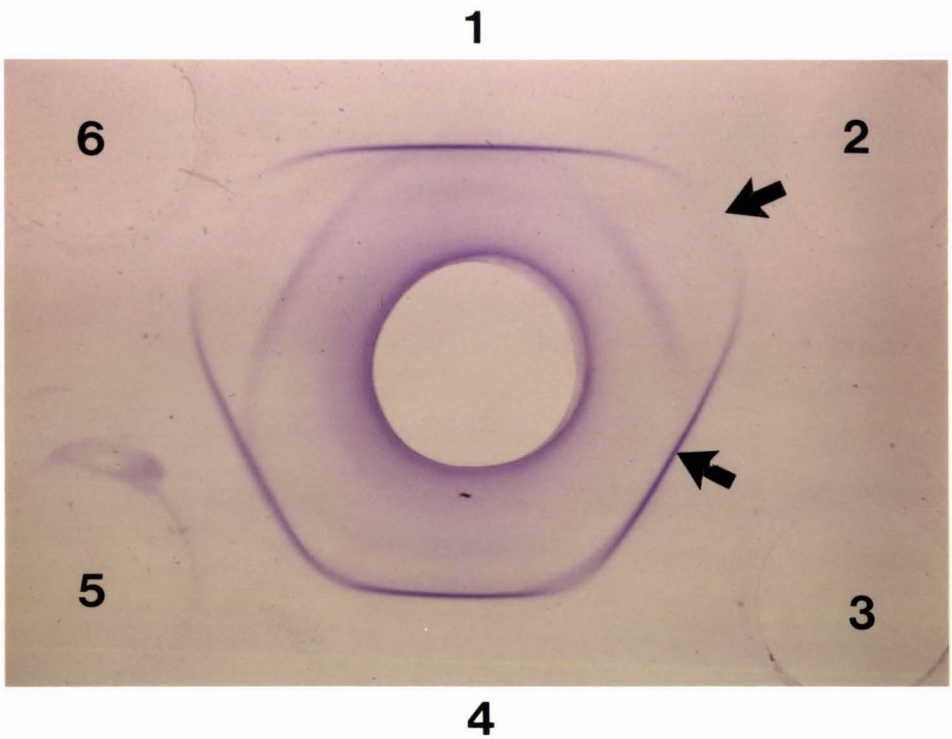
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TABLE 25 Number of bands observed in Ouchterlony double diffusion plates for four species of *Pinus*

A	Antisera to *P. radiata* 511								
Species	*P. radiata* 511	*P. radiata* 522	*P. radiata* 525	*P. elliottii* extract 1	*P. elliottii* extract 2	*P. taeda* extract 1	*P. taeda* extract 2	*P. monticola* extract 1	*P. monticola* extract 2
Maximum number of bands	5	4	4	4	3	3	3	2	2
Average for each species	3.78			3.75		2.75		2.0	

B	Antisera to *P. elliottii* extract 1								
Species	*P. radiata* 511	*P. radiata* 522	*P. radiata* 525	*P. elliottii* extract 1	*P. elliottii* extract 2	*P. taeda* extract 1	*P. taeda* extract 2	*P. monticola* extract 1	*P. monticola* extract 2
Maximum number of bands	2	3	3	4	4	3	2	3	2
Average for each species	2.5			3.75		2.2		2.3	

However, precipitin band position is at least in part determined by the relative concentration of antibody and test extract antigen, hence the same precipitin band may be found in a different position for a different extract, depending on the relative concentration of the antigen in the two extracts. (Dr K.M. Moriarty pers. comm.) This phenomenon is illustrated in Figures 24 and 28 where the major precipitin band dark arrows) is not found at a constant distance from the centre well even though the antigen is present in all wells (in different concentrations). In some instances, the relative position of bands for one extract are reversed in order of another extract (Figure 23, extracts of *P. radiata* 511 and *P. monticola* extract 2). In this analysis, the position of the band is not taken into account and only the number of bands is scored.

The identity reaction between the parent antigen extract (*P. radiata* 511) and its specific antiserum without interference from other extracts is shown in Figure 21. Five bands were detected (arrowed). The 'ring' around the central well is most likely formed from haemoglobin released into the serum which has not washed from the gel. The position and shape of the bands are also in part determined by the relative size, and hence diffusive mobility, of the antigen and respective antibody. Band three (the centre band) is almost level suggesting the effect of relative concentration and diffusive mobility of antigen and antibody were approximately equal.

The shape of band five (nearest to the central well) however, suggests that either antigen is in excess of antibody, or the antigen is highly mobile compared to the antibody.

The presence of an extra band in this reaction (although faint) which was not found in other *P. radiata* clones, suggests that the band represents a clone-specific antigen. Other *P. radiata* clones developed a maximum of four bands as did *P. elliottii* extract 1. Three bands were observed in *P. elliottii* extract 2, *P. taeda* extract 1, and *P. taeda* extract 2; and two bands in both *P. monticola* extracts. These data suggest the three Diploxylon species (*P. radiata, P. elliottii,* and *P. taeda*) are more closely related (that is, show a greater number of precipitin bands) to each other, than to the Haploxylon species *P. monticola*. The results suggest that as expected, the three *P. radiata* clones are closely related with a more distant relationship suggested between *P. radiata* and the other two Diploxylon species.

The average number of bands for each species (taken from several replicates and combining the results of all clones for one species) also suggests a closer relationship between the Diploxylon species (2.75 to 3.78) than between these species and *P. monticola* (2.0). A closer relationship is suggested between *P. radiata* and *P. elliottii* (3.75) than between *P. radiata* and *P. taeda* (2.75).

These data support the division of the genus *Pinus* into two subgenera (Figures 1A and 1B) but does not support either of the relationships hypothesised within the subgenus Diploxylon. These data indicate a closer relationship between *P. radiata* and *P. elliottii* than either of these to *P. taeda*.

The data on Table 25B are less conclusive than that for Table 25A. The probable cause is the low antibody titre produced against *P. elliottii* extract 1 resulting in a lower stain density and fewer bands able to be scored (compare Figures 22 and 26). However, these data do show one extra band in the parent material suggesting the presence of an antigen unique to this species. The maximum number of bands scored in the three non-parent extracts do not show a difference, and the difference in the average band numbers do not appear to be significant.

The analysis of precipitin band position attempts to score bands common to both extracts detecting the presence of homologous antigens. However, as already noted, position alone is not a good indicator of homology. The degree of homology for antigens in two extracts can be scored by analysing the degree of "spurring" of a precipitin line between two extracts. (Goodman and Moore, 1971.) (Appendix V.) Here, spurs are scored on a scale from zero (no spur, complete homology), to five (large spur, little homology).

Spurs assigned with scores of zero, three, and five in this analysis, are illustrated in Figures 24, 26, and 27 respectively (light arrows). Only spurs produced by antiserum from the same animal were used as different animals may recognise non-identity of antigens to different degrees, that is, produce a different spur size for the same test. (Dr K.M. Moriarty, pers. comm.)

Replicate diffusions for each pair of extracts challenged with antiserum to *P. radiata* 511 and *P. elliottii* extract 1 were performed and the spur size of all visible spurs in all replicates noted. The average spur size score is presented in Tables 26A (for *P. radiata* 511 antiserum) and 26B (for *P. elliottii* extract 1 antiserum). While all interspecific tests were performed, some replicate tests using each clone or extract were not, as shown by the dashes.

Data on Table 26A suggest a close antigenic relationship within a species (score for *P. radiata* to *P. radiata* = zero), and between *P. elliottii* and *P. taeda* (scores of zero and three). Only a distant antigenic relationship occurs between *P. radiata* and *P. elliottii* (scores of 4 and 5) and between *P. radiata* and *P. taeda* (scores of 3.5, 4, and 5). Scores for *P. monticola* suggest that this species is distantly related to all others sampled. These data support the division of the genus *Pinus* into two subgenera and suggest a close relationship between *P. elliottii* and *P. taeda*.

TABLE 26A Average spur size score on precipitin bands in Ouchterlony double diffusion plates.

Samples were challenged with antisera to *P. radiata* 511

	P. radiata 511	*P. radiata* 522	*P. radiata* 525	*P. elliottii* extract 1	*P. elliottii* extract 2	*P. taeda* extract 1	*P. taeda* extract 2	*P. monticola* extract 1	*P. monticola* extract 2
P. radiata 511		0	0	5	4	3.5	3.5	5	5
P. radiata 522			-	4	-	5	-	5	-
P. radiata 525				4	-	-	4	-	5
P. elliottii extract 1					0	0	0	5	-
P. elliottii extract 2						-	3	-	5

TABLE 26B Average spur size score on precipitin bands in Ouchterlony double diffusion plates.

Samples were challenged with antisera to *P. elliottii* extract 1

	P. radiata 511	*P. radiata* 522	*P. radiata* 525	*P. elliottii* extract 1	*P. elliottii* extract 2	*P. taeda* extract 1	*P. taeda* extract 2	*P. monticola* extract 1	*P. monticola* extract 2
P. radiata 511		0	0	4	4	4	2	5	5
P. radiata 522			-	5	4	4	4	5	5
P. radiata 525				5	5	4	4	-	5
P. elliottii extract 1					2	0	0	4	5
P. elliottii extract 2						-	1	-	-

The data on Table 26A therefore support the first hypothesis illustrated in Figure 1A.

Data on Table 26B for extracts challenged with antiserum to *P. elliottii* extract 1 show similar results to Table 26A. A close relationship between *P. elliottii* and *P. taeda* is suggested (scores of 0 and 1) but only a distant relationship is suggested between *P. radiata* and *P. elliottii* (scores of 4 and 5) and *P. radiata* and *P. taeda* (scores of 2 and 4). Scores for *P. monticola* suggest that this species is distantly related to the other species sampled. Data on this table thus also support the first hypothesis illustrated in Figure 1A.

The values for all extracts of each species challenged with both antisera (Table 26) were averaged (Table 27). These data suggest that while *P. elliottii* and *P. taeda* are the most closely related species (0.67), *P. radiata* is more closely related to *P. taeda* (3.8) than to *P. elliottii* (4.4). However, neither *P. radiata* nor *P. elliottii* is closely related to *P. monticola* (5.0, 4.75 respectively).

3.6 IMMUNOELECTROPHORESIS

A typical immunoelectrophoresis gel is shown in Figure 29A. An aliquot from the extract of *P. radiata* 511 was placed in the sample well and electrophoresed in the direction indicated by the arrow although at the pH at which the gel was run, some proteins will be positively charged and move toward the anode.

TABLE 27 Average spur size score on precipitin bands in Ouchterlony double diffusion plates.

Values were averaged for each species.

	P. radiata	*P. elliottii*	*P. taeda*	*P. monticola*
P. radiata	0	4.4	3.8	5.0
P. elliottii		0	0.67	4.75

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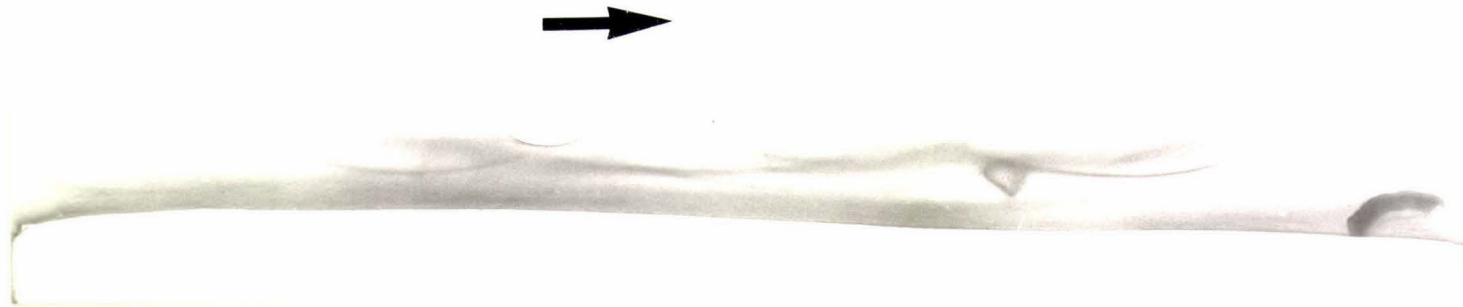

Fig 29A Typical immunoelectrophoresis gel. The extract of *P. radiata* 511 was electrophoresed in the direction indicated by the arrow, and challenged with antiserum (in the trough at the bottom of the figure) prepared against the same extract. Note some antigens have moved toward the cathode.

The extract was challenged with antiserum to the same extract and the plate developed as described in the methods. A diagram of this and other immunoelectrophoresis results was drawn by placing the 'immunoplate' in a photographic enlarger and tracing the precipitin arcs directly onto paper, as illustrated in Figure 29B. The results of other comparisons are shown in Figures 30 to 45.

Any precipitin arc which develops when an extract is challenged with an antiserum to another species indicates the presence of related antigens (Gell 1968). Thus precipitin arcs produced when an extract is challenged with antisera to *P. radiata* 511 indicates the presence of an identical or similar antigen to an antigen present in *P. radiata* 511 (otherwise, the specific antibody could not have been produced). This implies that the number of precipitin arcs formed is a measure of the relationship of that extract to the extract used to produce the antiserum (Smith 1976). The greater the number of precipitin arcs formed, the closer the antigen complement of the extract will be to the antiserum extract.

The greatest number of precipitin arcs formed when extracts were challenged with antiserum to *P. radiata* 511, was for the 'parent extract' that is, *P. radiata* 511. (Table 28.) Fewer bands were found when the other *P. radiata* clones were challenged with the same antiserum suggesting the presence of some clone specific antigens.

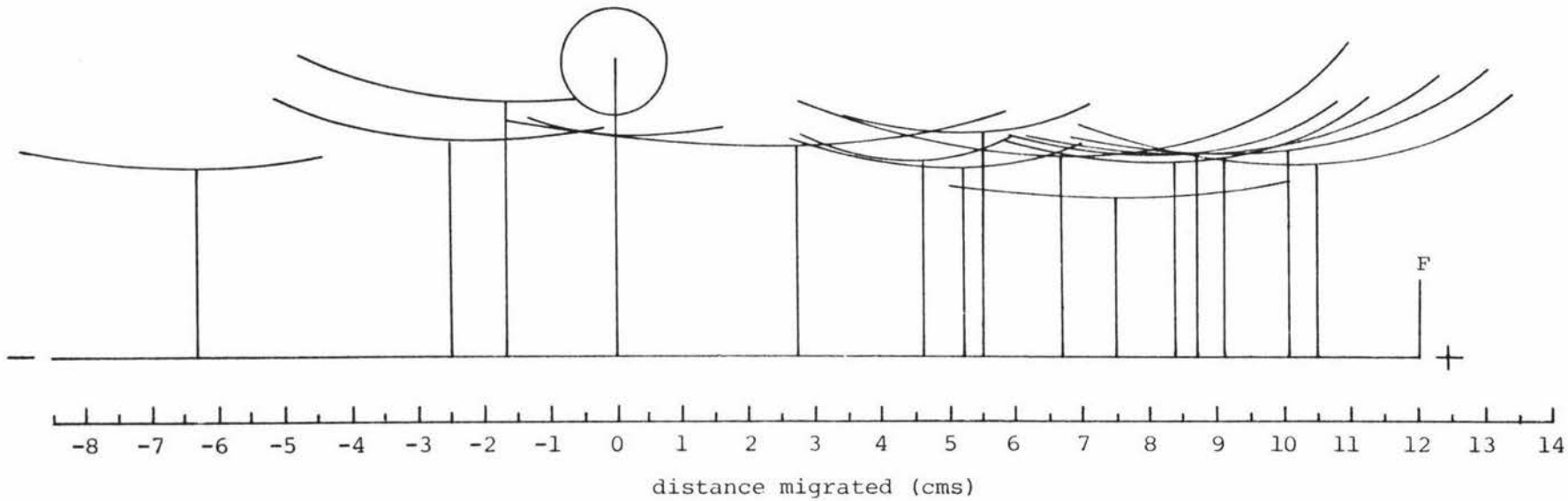

Fig 29B Immunoelectrophoregram for *P. radiata* 511 challenged with antisera prepared against *P. radiata* 511. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

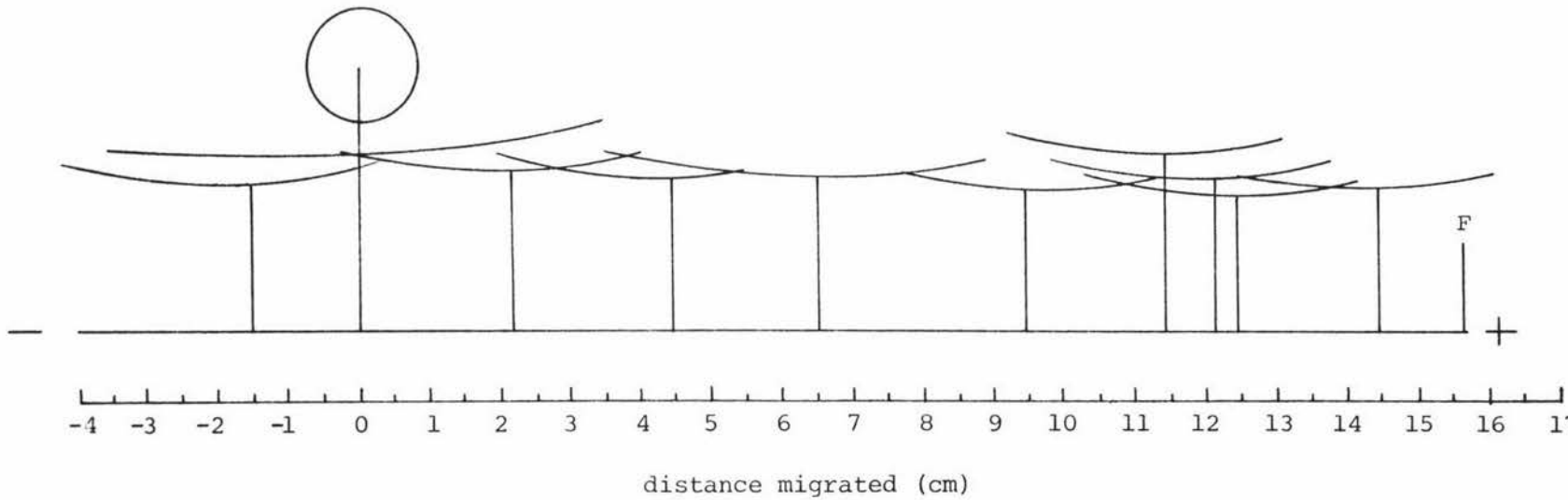

Fig 30 Immunoelectrophoregram for *P. radiata* 517 challenged with antisera prepared against *P. radiata* 511. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

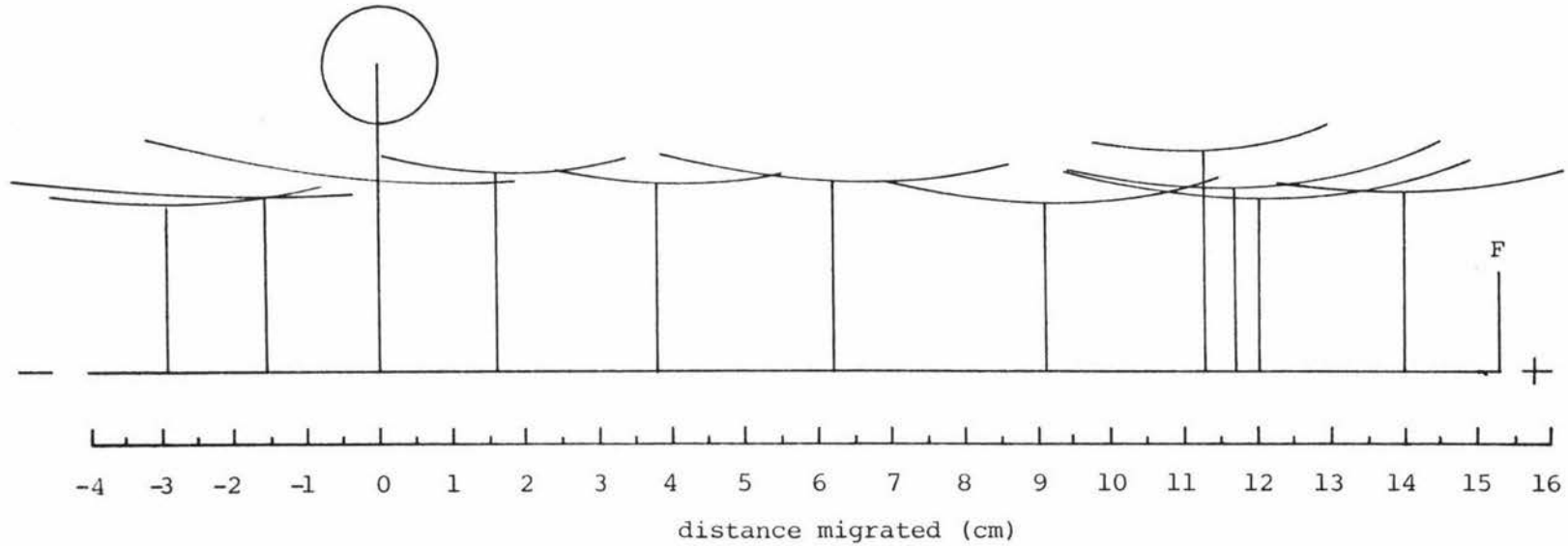

Fig 31 Immunoelectrophoregram for *P. radiata* 522 challenged with antisera prepared against *P. radiata* 511. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

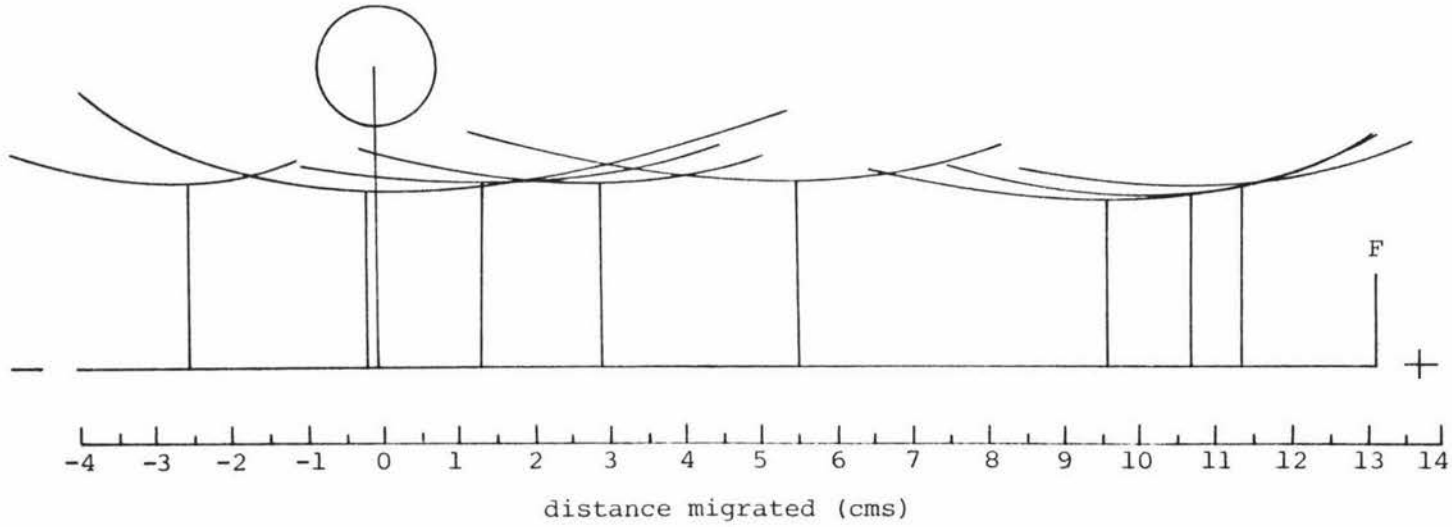

Fig 32 Immunoelectrophoregram for *P. elliottii* extract 1 challenged with antisera prepared against *P. radiata* 511. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

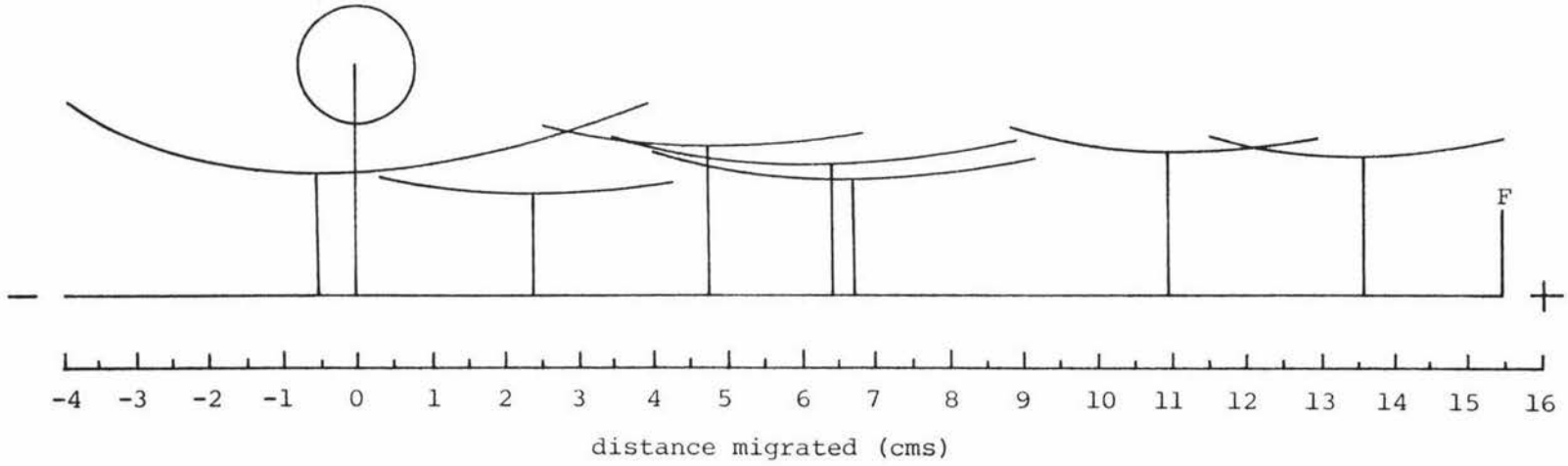

Fig 33 Immunoelectrophoregram for *P. elliottii* extract 2 challenged with antisera prepared against *P. radiata* 511. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

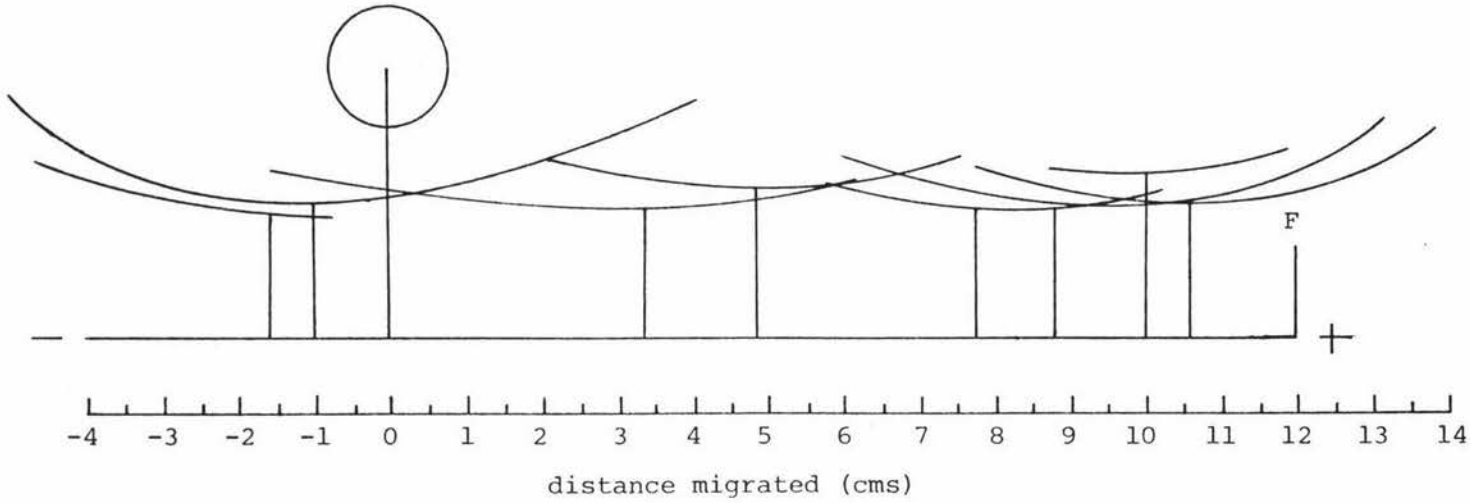

Fig 34 Immunoelectrophoregram for *P. taeda* extract 1 challenged with antisera prepared against *P. radiata* 511. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

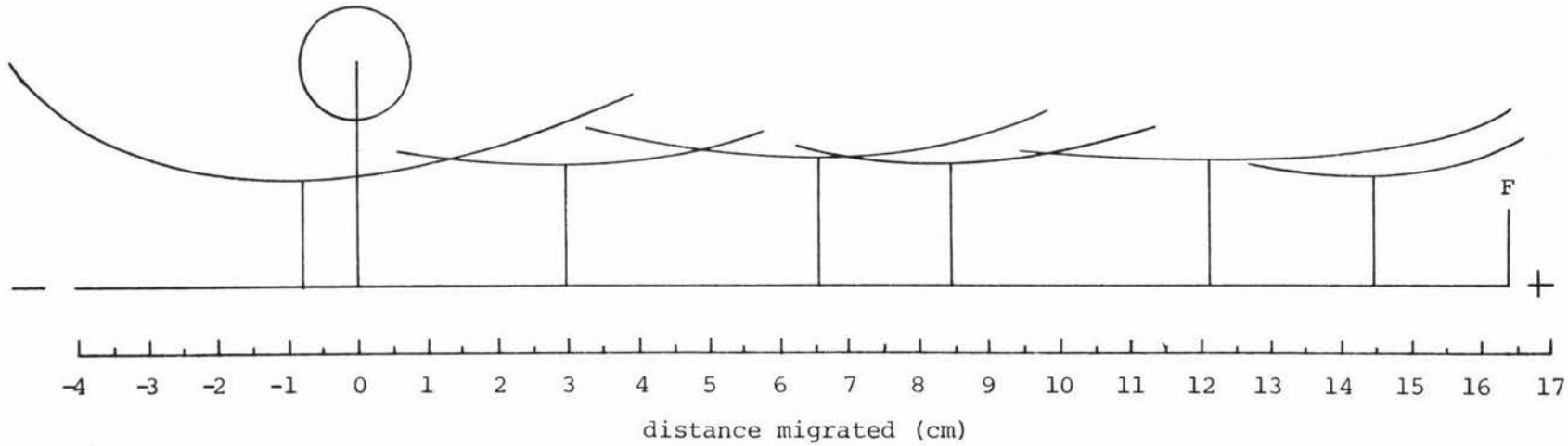

Fig 35 Immunoelectrophoregram for *P. taeda* extract 2 challenged with antisera prepared against *P. radiata* 511. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

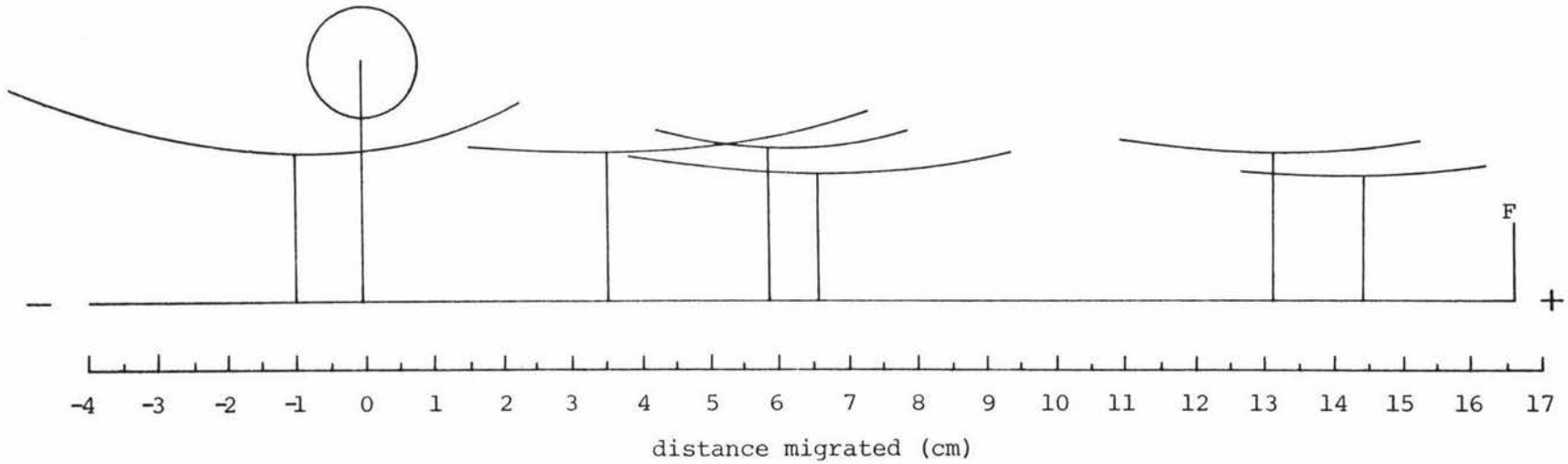

Fig 36 Immunoelectrophoregram for *P. monticola* extract 1 challenged with antisera prepared against *P. radiata* 511. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

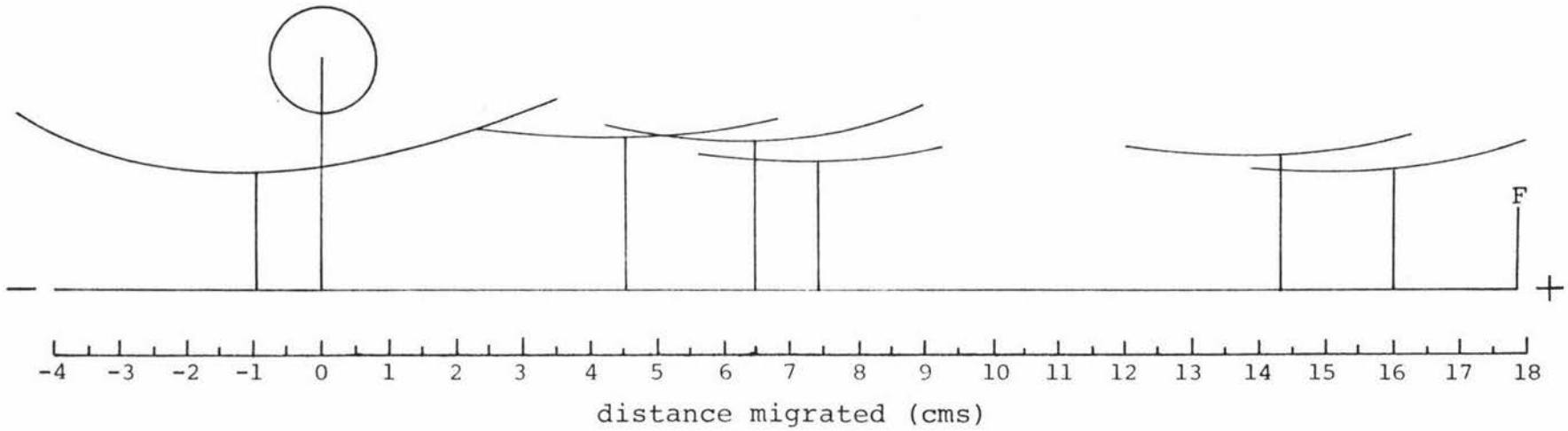

Fig 37 Immunoelectrophoregram for *P. monticola* extract 2 challenged with antisera prepared against *P. radiata* 511. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

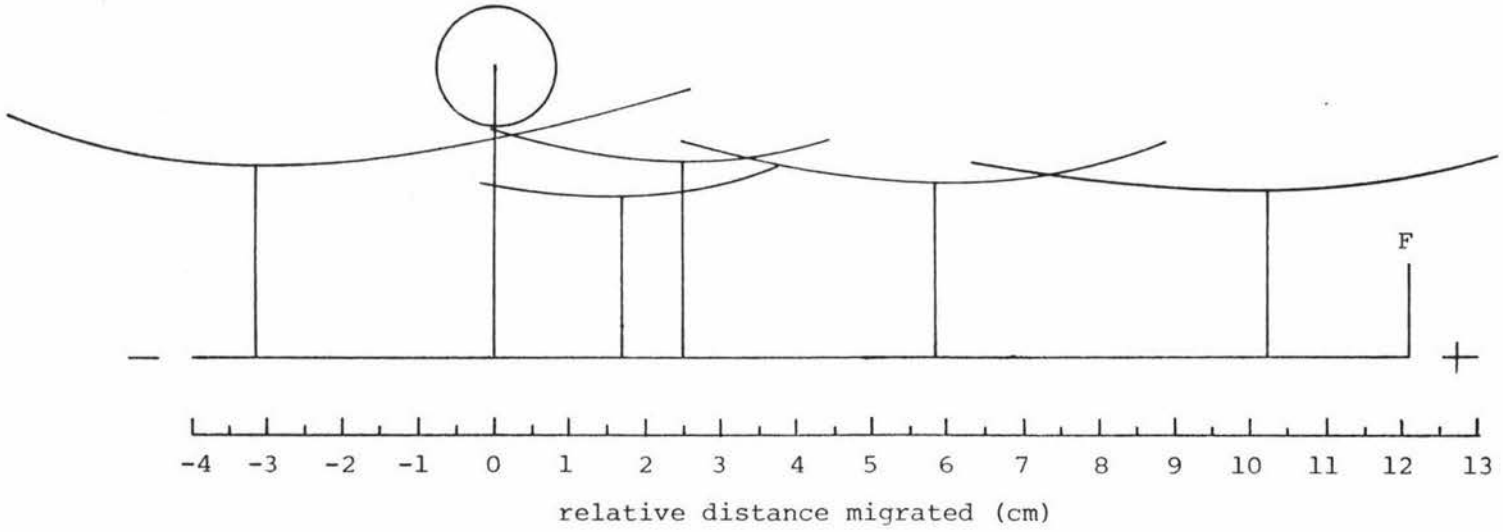

Fig 38 Immunoelectrophoregram for *P. radiata* 511 challenged with antisera prepared against *P. elliottii* extract 1. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

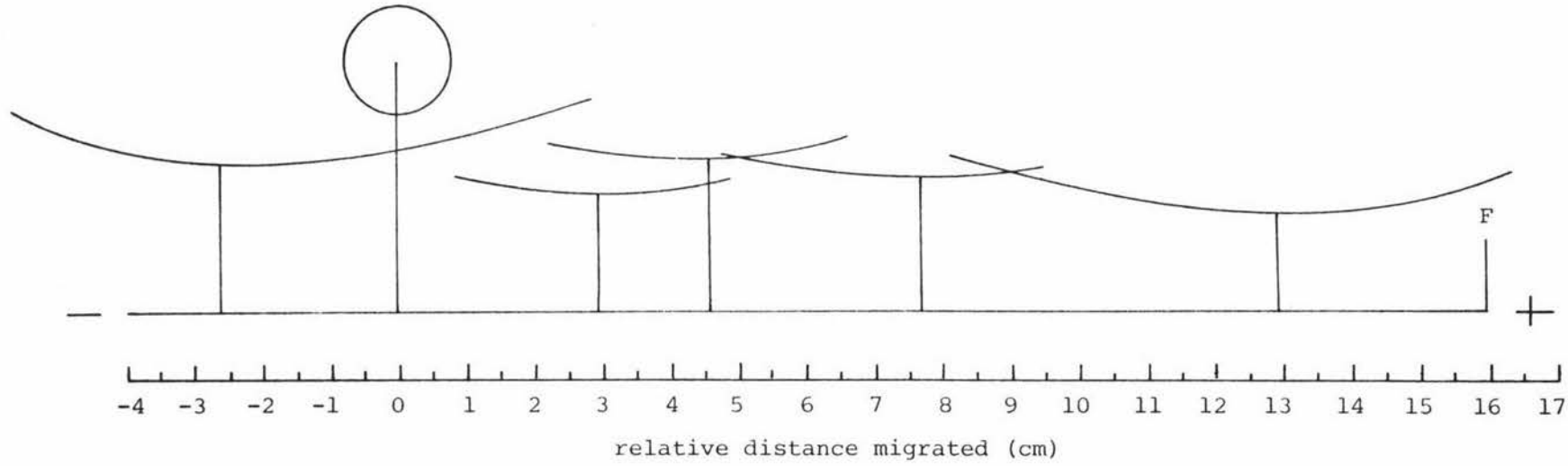

Fig 39 Immunoelectrophoregram for *P. radiata* 517 challenged with antisera prepared against *P. elliottii* extract 1. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

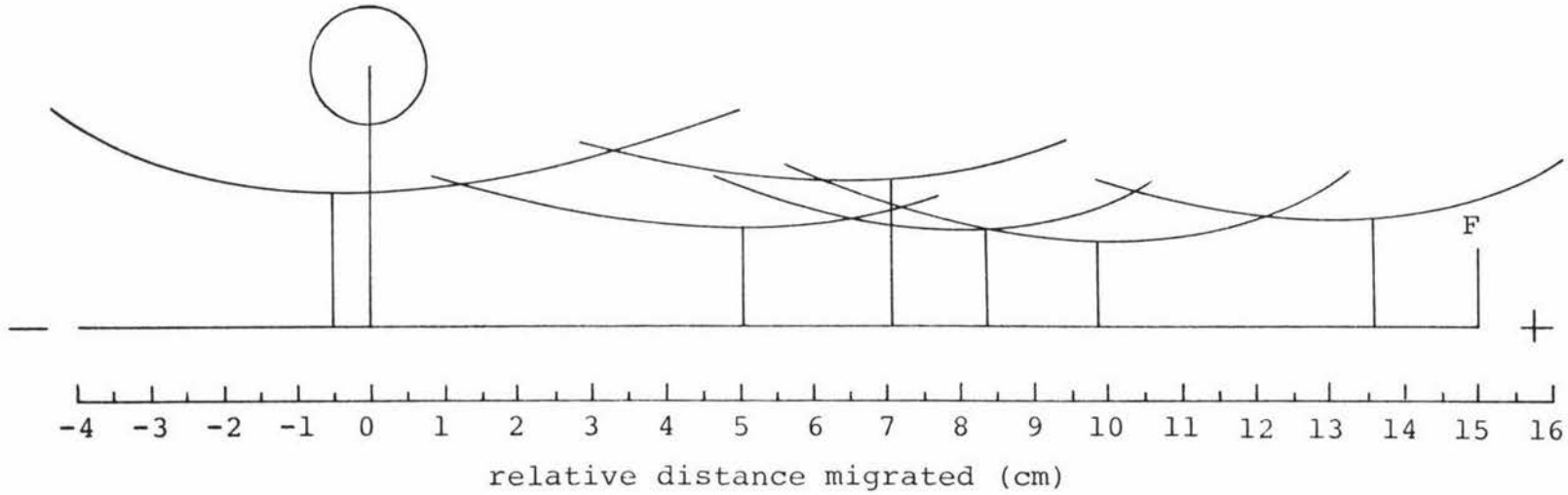

Fig 40 Immunoelectrophoregram for *P. elliottii* extract 1 challenged with antisera prepared against *P. elliottii* extract 1. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

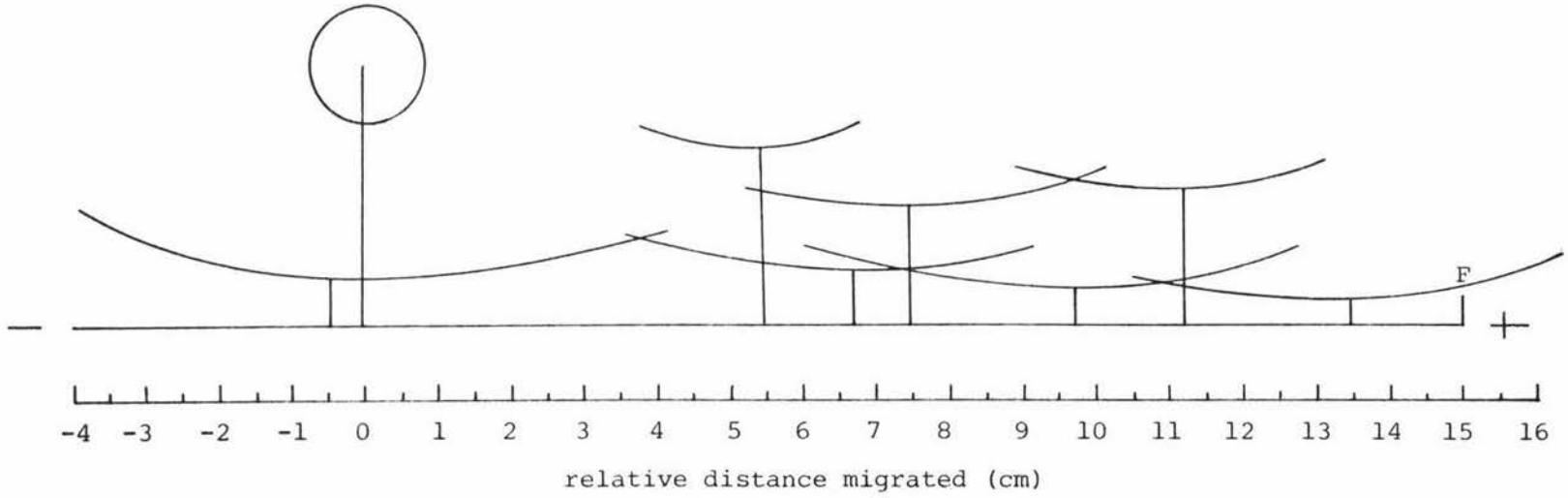

Fig 41 Immunoelectrophoregram for *P. elliottii* extract 2 challenged with antisera prepared against *P. elliottii* extract 1. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

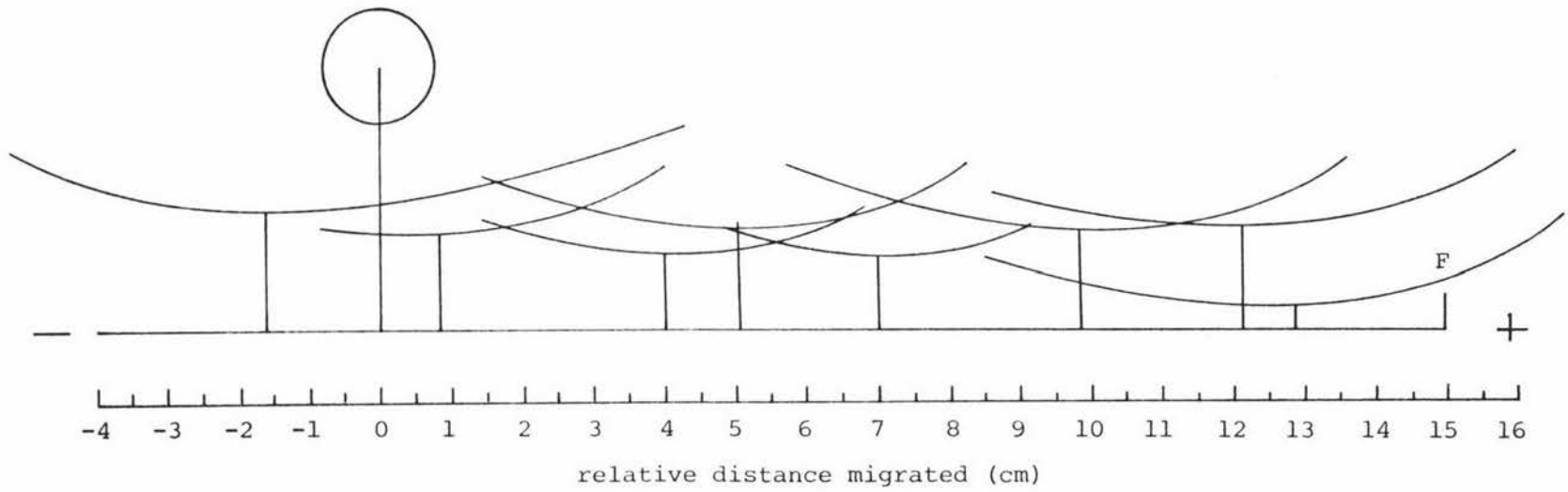

Fig 42 Immunoelectrophoregram for *P. taeda* extract 1 challenged with antisera prepared against *P. elliottii* extract 1. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

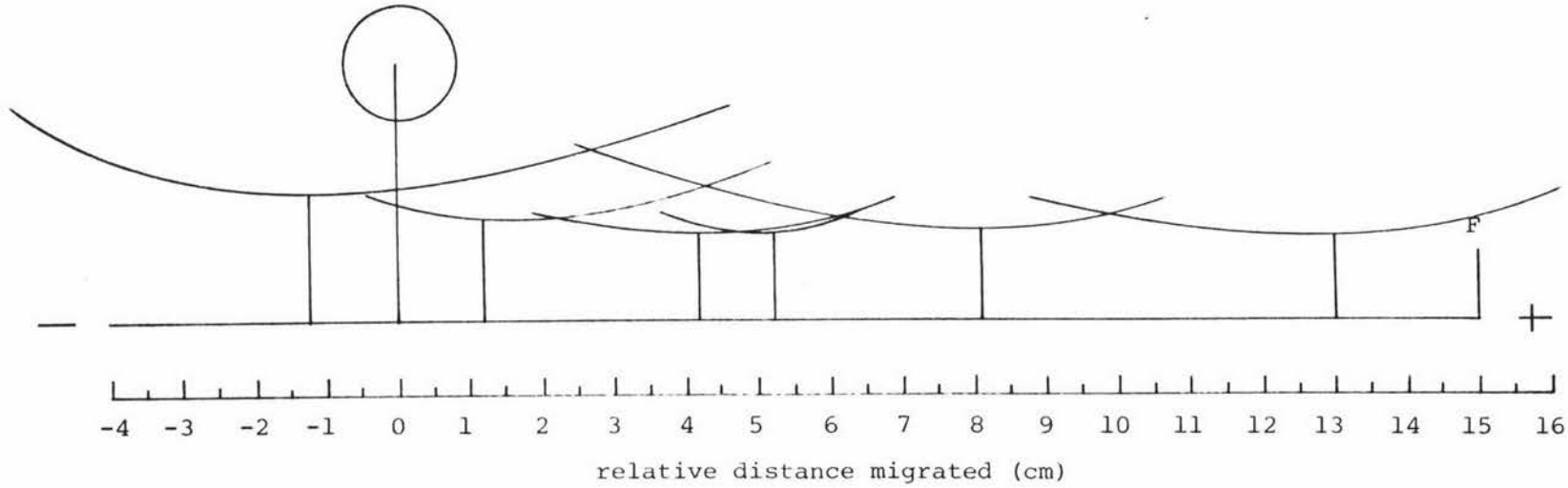

Fig 43 Immunoelectrophoregram for *P. taeda* extract 2 challenged with antisera prepared against *P. elliottii* extract 1. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

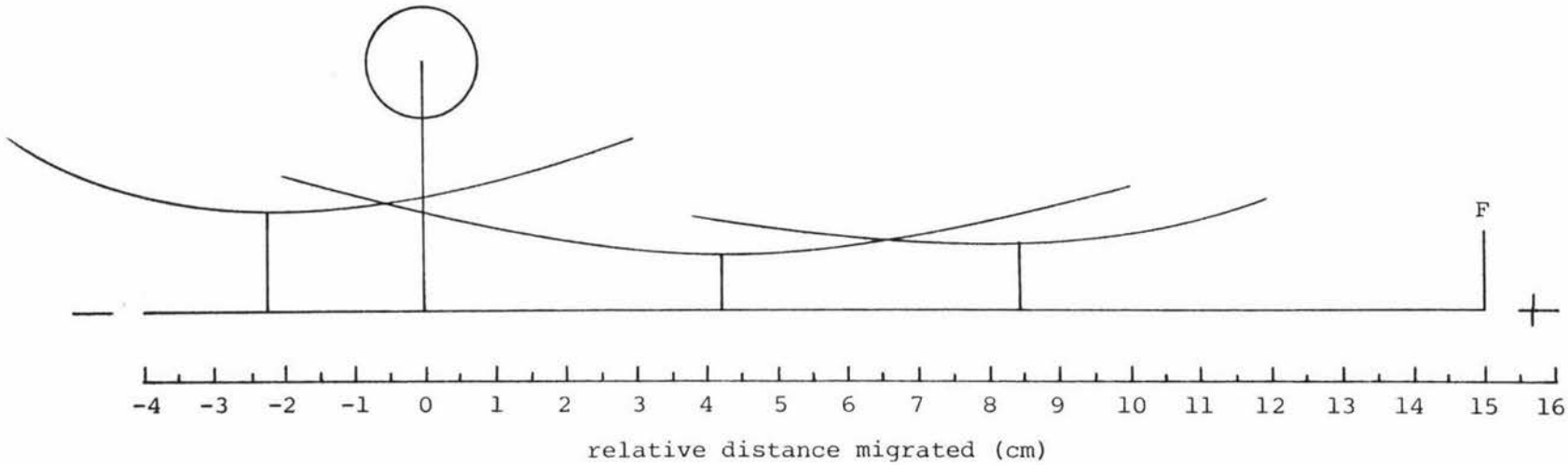

Fig 44 Immunoelectrophoregram for *P. monticola* extract 1 challenged with antisera prepared against *P. elliottii* extract 1. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

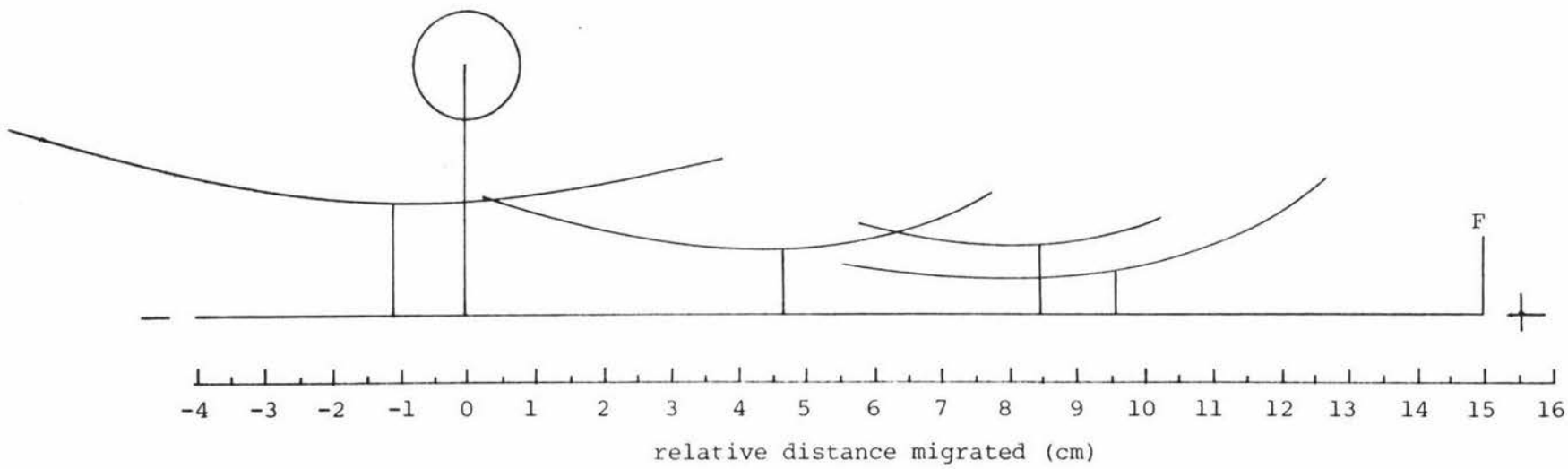

Fig 45 Immunoelectrophoregram for *P. monticola* extract 2 challenged with antisera prepared against *P. elliottii* extract 1. Sample was applied in the sample well (large circle) at the origin. Dye front migrated to 'F'.

TABLE 28 Number of precipitin arcs detected in immunoelectrophoresis. Samples were challenged with antisera to *P. radiata* 511

Extract	Number of Precipitin Arcs
P. radiata 511	15
P. radiata 517	10
P. radiata 522	11
P. elliottii extract 1	8
P. elliottii extract 2	7
P. taeda extract 1	8
P. taeda extract 2	6
P. monticola extract 1	6
P. monticola extract 2	6

Approximately the same number of precipitin arcs formed when *P. elliottii* and *P. taeda* were challenged with this antiserum suggesting that these species are equally distantly related to *P. radiata*. The smallest number of precipitin arcs were produced in response to extracts of *P. monticola*, suggesting this species is the most distantly related.

For species challenged with antiserum to *P. elliottii* extract 1 (Table 29), the greatest number of precipitin arcs were produced in response to *P. taeda* extract 1. However, as already discussed, antigens detected in species challenged with the antiserum to *P. elliottii* extract 1 must be represented by similar antigens in *P. elliottii* extract 1. As for *P. radiata* 511 (Table 28), the total number of antigens which elicited an antibody response apparently were not detected in the 'parent extract'. The high number of precipitin arcs detected in *P. taeda* suggests a close relationship between this species and *P. elliottii*. Fewer precipitin arcs were found when *P. radiata* extracts were challenged with this antiserum suggesting a distant relationship, while the lowest number of precipitin arcs produced in response to extracts of *P. monticola* suggest this species is the most distantly related to *P. elliottii*.

TABLE 29 Number of precipitin arcs detected in immunoelectrophoresis. Samples were challenged with antisera to *P. elliottii* extract 1

Extract	Number of Precipitin Arcs
P. radiata 511	5
P. radiata 517	5
P. elliottii extract 1	6
P. elliottii extract 2	7
P. taeda extract 1	8
P. taeda extract 2	6
P. monticola extract 1	3
P. monticola extract 2	4

The distance from the centre of the sample well to the centre of each precipitin arc (taken to be the point closest to the antisera trough), and the distance migrated by the dye front (taken to be the middle of the dye band), were measured, and the relative mobility (p. 52) of each precipitin arc calculated (Tables 30 and 31).

Data on Tables 30 and 31 are grouped using band position and information gained from double diffusion analysis. The double diffusion results were taken into account to detect proteins which, while antigenically similar, were not electrophoretically identical. This is illustrated in Figure 46 where the major precipitin arc (heavy precipitin line near the sample well) is displaced to the left in the sample of *P. taeda* when compared to the sample of *P. elliottii*. A major precipitin band can also be seen in double diffusion analysis (Figures 24 and 28) where the major precipitin band detected in *P. elliottii* extract 1 is seen to link with the major precipitin band in *P. taeda* extract 1, indicating antigenic similarity. This band almost certainly includes the component of the major precipitin arc in Figure 46 when stain density and position are considered.

Data on Table 30 suggests that several antigens are present in all the species examined (classes D, F, H, O, and P). Only one class (G) was detected in *P. radiata* and *P. monticola* only. Six classes (C, E, I, J, L, and M) were detected in *P. radiata* and either *P. elliottii* or *P. taeda* or both.

TABLE 30 Relative mobilities of antigens of four species of *Pinus* separated by immunoelectrophoresis
Extracts were challenged with antiserum to *P. radiata 511*

CLASS	*P. radiata*			*P. elliottii*		*P. taeda*		*P. monticola*	
	511	517	522	ext 1	ext 2	ext 1	ext 2	ext 1	ext 2
A	-0.53								
B	-0.21		-0.19						
C	-0.14	-0.10	-0.10	-0.11		-0.13			
D	0.00	0.00	0.00	-0.01	-0.03	-0.08	-0.05	-0.06	-0.06
E	*	0.14	0.10	0.11	0.15				
F	0.22	0.28	0.25	0.25	0.31	0.28	0.18	0.21	0.26
G	0.38							0.35	0.36
H	0.43	0.42	0.41	0.42	0.42	0.40	0.40	0.40	0.42
I	0.45				0.44				
J	0.55						0.52		
K	0.62	0.60	0.60						
L	0.69					0.65			
M	0.73	0.73	0.74	0.73	0.71	0.74	0.75		
N	0.76	0.78	0.79						
O	0.83	0.79	0.79	0.82		0.83		0.79	0.80
P	0.88	0.92	0.92	0.86	0.88	0.88	0.88	0.87	0.89

* Antigen must be present in this extract but was not detected.
Data is grouped as described in the text.

TABLE 31 Relative mobilities of antigens of four *Pinus* species separated by immunoelectrophoresis
Extracts were challenged with antiserum to *P. elliottii* extract 1

	P. radiata		*P. elliottii*		*P. taeda*		*P. monticola*	
CLASS	511	517	ext 1	ext 2	ext 1	ext 2	ext 1	ext 2
A	-0.16	-0.16	*					
B			-0.03	-0.03	-0.11	-0.08	-0.15	-0.07
C			*		0.05	0.08		
D	0.14	0.19	*					
E	0.20	0.29	*		0.27	0.28	0.28	0.32
F			0.34	0.36	0.34	0.35		
G	0.48	0.48	0.47	0.45	0.47	0.54	0.57	0.57
H			0.56	0.50				
I			0.66	0.65	0.66			0.64
J	0.84	0.81	*	0.75	0.81			
K			0.91	0.90	0.86	0.87		

* Antigen must be present in this extract but was not detected.
Data is grouped as described in the text.

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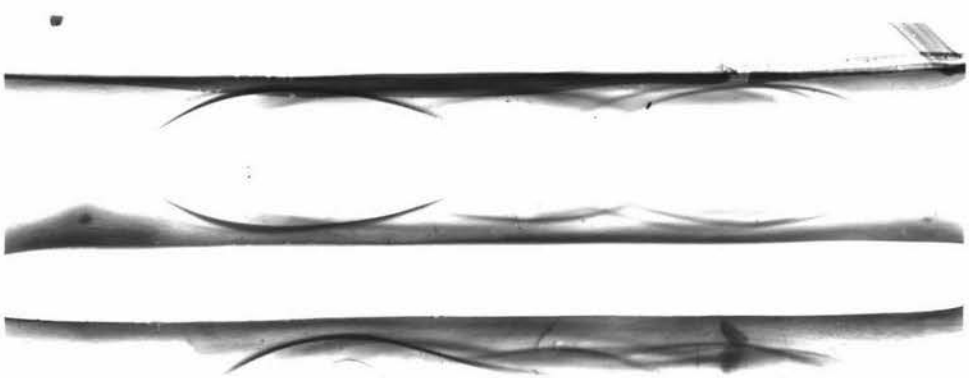


Fig 46 Immunoelectrophoresis gel. Extracts shown are *P. taeda* extract 1 (top) and *P. elliottii* extract 1 challenged with antisera to *P. radiata* 511 (trough).

Three classes (B, K, and N) appear to be restricted to the *P. radiata* extracts while class A was detected in *P. radiata* 511 only. These data suggest that differences in antigenic content between individuals within a species, and between individuals of different species, can be detected using immunological techniques.

A similar pattern is found on Table 31 where two classes (E and G) appear to be represented in all species. One class (H) appears to be present in *P. elliottii* only, while three classes (C, F, and K) appear to be present in *P. elliottii* and *P. taeda* only.

Comparing the relative mobilities of the antigens detected using both antisera (Tables 30 and 31), similarities can be seen. For example, class H (Table 30) and class G (Table 31) would appear to represent the same antigen. Some antigens, detected in one analysis apparently were not detected in the other. For example, on Table 31, the *P. radiata* bands with a relative mobility of 0.00 (Table 30) were not detected when challenged with antiserum to *P. elliottii* extract 1 even though these bands are likely to represent the same antigen as in class B (Table 31). In this case, the bands may have been lost by diffusion into the central trough.

Two classes on Table 30 (E and M) include precipitin arcs produced by *P. elliottii* but these classes do not appear to be represented on Table 31.

Apparently, the antigens, while present in *P. elliottii* extract 1, either did not ellicit an antibody response in the rabbits or (probably) the bands were not detected in the gel. The classes found on Table 30 which are not represented on Table 31, possibly represent antigens present in *P. radiata* but not *P. elliottii*. Conversely, classes on Table 31 not present on Table 30 possibly represent antigens in *P. elliottii* not in *P. radiata*.

The immunoelectrophoretic data (Tables 28 to 31) therefore support the division of the genus *Pinus* into two subgenera. These data also suggest a close relationship between *P. elliottii* and *P. taeda* in support of hypothesis one (Figure 1A). The technique apparently enables both intraspecific and interspecific differences to be detected.

4. DISCUSSION

Conifers are an economically important world-wide group of production forest trees and some conifer species (especially *P. radiata*) are particularly important in New Zealand. It may be initially surprising therefore that such an apparent lack of information on the protein components of conifers has existed in the literature until recently. In a major review, Mirov (1967) does not mention the use of protein analysis even though chapters relating to the chemical and genetic aspects of pines are covered in some depth. The information gained from an analysis of the protein components would be of use in many different areas of conifer research, for example, in taxonomy, breeding, and grafting studies. However, the use of high resolution protein separation and analysis techniques has been hindered by the lack of a suitable protein extraction method, particularly one suitable for adult conifer tissue. The method described in this study, and discussed below, was found to be suitable for use on mature *Pinus* tissue and components of the extracts were able to be separated by high resolution protein analysis techniques.

Proteins have become prominent in the taxonomic studies of many plant groups. For example, Brown (1979) reviews the use of electrophoretic techniques to study enzyme polymorphism in a wide range of plant groups. These electrophoretic techniques have also been used to determine hybrids or provenances in populations.

Copes and Beckwith (1977) used isoenzyme patterns detected in starch gels to determine pure stands of Sitka spruce (*Picea sitchensis*) and White spruce (*Picea glauca*). Similarly, Bergmann (1978), using starch gels, found variations in the allele frequencies for acid phosphatase isozymes in Norway spruce (*Picea abies*) and Lundkvist and Rudin (1977), also using starch gels, detected isozyme variation between four isolated populations of the same species.

Studies of the protein constituents of conifers to date have almost exclusively used starch gels to separate these components for isozyme analysis. Starch gel electrophoresis is a relatively low resolution technique, at best producing diffuse bands, and does not require samples of high purity for separations. For this reason, 'crude' extracts were used in the majority of the studies. Lundkvist and Rudin (1977), for example, homogenised needle or embryo tissue of *Picea abies* in a tris/borate buffer pH 7.4 while Guries and Ledig (1978) ground embryos of *Pinus rigida* in a tris/HCl buffer pH 7.1, before electrophoresis on starch gels.

The majority of workers have concentrated on juvenile tissues where, due to the low concentration of interfering substances, and relatively high concentrations of protein(McMullan and Ebell 1970), distilled water or 'simple' buffers are adequate to produce useful extracts. Conkle (1971 a,b) crushed embryos of *Pinus attenuata* and used the undiluted liquid as the sample extract, while Adams and Joly (1980a) crushed megagametophytes of *P. taeda* in a small quantity of distilled water before electrophoresis.

Guries and Ledig (1978) and Mejnartowicz and Bergmann (1977) used a tris/HCl buffer to demonstrate enzyme activity in seeds of different conifer species.

Protein extracted from juvenile tissue is of limited value in graft incompatability studies or tree breeding programs as the attributes of the adult tree are largely unknown, and, furthermore, protein spectra change between juvenile and adult forms and between trees under different growth conditions. Ramaiah *et al.* (1971) and Conkle (1971, b) demonstrated changes in the banding pattern of soluble protein and isozymes occurring during germination and early growth of *Pinus banksiana* and *P. attenuata* respectively. Van Lear and Smith (1970) demonstrated differences in the total soluble protein and enzyme patterns of *P. elliottii* under different nutritional regimes. A difference in the isoenzyme patterns between different growth forms also occurs, for example, in dwarf and normal Douglas fir (*Pseudotsuga menziesii*) (Copes 1975).

Protein extraction from mature conifer tissue is apparently more difficult than is usual for most plant tissues and techniques suitable for other plant groups were unsuccessful when attempted on Douglas fir (McMullan and Ebell 1970). The unsuccessful methods investigated by these authors included the use of insoluble PVP in a phosphate buffer (pH 7.3) with or without sodium ascorbate and/or dithiothreitol. The successful method included 8M urea in the extracting solution followed by separation of the sample components by electrophoresis on a low pH/3M urea polyacrylamide gel.

Since urea is a good protein denaturing agent and since the interferring substances from the sample tissue are still present (but presumably dissociated from the protein), this method is unlikely to be useful for enzyme assays or immunological analysis. Hamaker and Snyder (1973) extracted protein from needle tissue of *Pinus taeda* and *P. palustris* using an extraction solution which included 15% urea (approximately 2.5M) to assay for several enzymes. However, only nineteen of the fifty-three enzyme systems analysed showed activity and some of these enzyme systems which did not show activity here have been detected in *Pinus* species by other workers; for example, leucine amino peptidase, (Rudin *et al.* 1973).

Several authors have been successful in extracting some protein from mature tissues but their methods either were useful for starch gel systems only, or were very complex. Rudin and Rasmuson (1973); Rudin *et al.* (1973); and Rudin (1975, 1977) extracted proteins from needles of *Pinus sylvestris* using PVP in a pH 7.4 tris/borate/EDTA buffer and separated leucine amino peptidase, esterase, and glutamate-oxalate-transaminase enzymes in a 12% starch gel. Mitton *et al.* (1979) extracted protein under nitrogen using a complex buffer at pH 7.0 which contained ten components including DIECA, 2-mercapto-ethanol, PVP, and ascorbate. Copes (1978) found differences in peroxidase banding patterns between mature tissues of compatible and non-compatible Douglas fir grafts using PVP in a standard starch gel buffer. However, unlike the method described in this study, none of the published methods above meet all of the criteria outlined in the Introduction.

The salient features of the method described in this study are the use of a low pH extraction mixture and the early separation of proteins from other components using gel filtration. PVP (or PVPP) is used in all the published methods cited above (except for the method of Hamaker and Snyder (1973)) to extract protein from mature tissues in conjunction with a neutral or alkaline buffer. Components of the extraction mixture used in this study are similar to the components in the buffered systems used by some other workers. McMullan and Ebell (1970) and Mitton *et al.* (1979) used PVP, ascorbate, and thiols in their extraction buffers (unsuccessfully by McMullan and Ebell), but in conjunction with a high pH buffer.

When the components of the extraction mixture were buffered at pH 7.2 (with 0.1M phosphate buffer) usable extracts were not obtained. Following freeze drying, the product of the buffered extraction mixture was green/brown and remained as an "oily" liquid, unlike the dry white powder obtained when the low pH extraction mixture was used. The oily product could not be separated into protein bands using electrophoresis and usually formed a large smear near the origin of the polyacrylamide gel, a result similar to unsuccessful results reported by other workers (McMullan and Ebell 1970).

The low pH in the extraction mixture is likely to be affecting phenolic binding to PVPP as maximum binding to PVPP was found to occur at a pH of 3.5, and was lowest at alkaline pH's (Andersen and Sowers 1968).

Thus the PVP or PVPP used in the published methods would probably be having a minimal effect on the removal of phenols from the solution.

The use of gel filtration in the present method has enabled the production of extracts which could be stored for several months without detectable deterioration. For example, the freeze dried white powder extracted from *P. radiata* 517 was taken up into distilled water and formed a clear solution which was not discoloured after fifteen months. The solution which had been frozen and thawed more than twenty times, retained enzyme activity indicating that protein extracted using this method is stable and largely free of interferring substances. The analysis for protein and carbohydrate in the freeze dried product suggests that while extraction efficiency for protein is relatively low, only a small amount of material other than protein and carbohydrate was present. The product was also suitable for production of antisera in rabbits. Thus the method presented in this study meets the requirements outlined in the Introduction.

The freeze dried extracts obtained from clonal material of *P. radiata* and individual trees of *P. elliottii*, *P. taeda*, and *P. monticola*, were analysed using four techniques as described in the Methods. To the best of the author's knowledge, only one of these techniques (discontinuous polyacrylamide gel electrophoresis) has been used previously on mature *Pinus* tissue extracts.

McMullan and Ebell (1970) used polyacrylamide slab gels to separate protein components from extracts of six conifers including one *Pinus* species (*P. monticola*). Similarly, Van Lear and Smith (1970) demonstrated differences in soluble protein and enzyme patterns in *P. elliottii* under different nutritional regimes and Hamaker and Snyder (1973), also using rod gels, demonstrated enzyme activity in nineteen out of fifty-three enzymes screened in *P. taeda* and *P. palustris*.

Several authors have used polyacrylamide gel electrophoresis to separate components of extracts from conifer seed material. Feret (1971) detected isozyme variation between individual seedlings of *Picea glauca*; Juo and Stotzky (1973) separated globulins and albumins extracted from seeds of nine conifers including five species of *Pinus*; and Durzan and Chalupa (1968) detected up to eighteen bands in extracts of *Pinus banksiana*.

The results obtained using polyacrylamide rod gel electrophoresis in this study provides information on the relationship between the four species examined. However problems are encountered when using rod gel electrophoresis for taxonomic studies stemming largely from inaccuracies in the measurement of band position in the gel and in basic assumptions made of the technique. The length of each particular gel will be slightly different necessitating the use of the "relative mobility" of each band for a comparison between the gels. This is illustrated in Figure 9 where the differences in the length of gels from different gel runs means that any particular pair of bands will be further apart in the longer gels.

Thus a direct comparison of the gels cannot be made. Following the calculation of the relative mobility values, "idealised" gels can be drawn and direct comparisons made (Figure 11 and 12). However, according to some authors "this type of examination and the listing of Rf (Rm) values of bands are subjective, frequently confusing, and not suitable for large numbers of extracts" (Kersters and De Ley 1975). This view is shared by the author.

An objective measure of band positions for calculation of relative mobility, was obtained using a U.V. scanner (Figure 10). Bands with a low stain density were not detected by the scanner, probably because of high background residual stain (Figure 9). When the relative mobility values for the same gel were calculated from direct visual measurement of band position, more bands were detected including all of the bands detected by the scanner, and the values of relative mobility were identical for all but one band which differed by 0.01 Rm units (Table 12). Thus, while direct visual measurement is more subjective, in this situation the calculation of the relative mobility values is apparently as accurate as those calculated from the scanner measurements.

Inaccuracies also arise from the inability to control all variables in a particular gel run. Each gel run used a different gel mix (but usually from the same stock solution) and will probably be run under slightly varied conditions of, for example, temperatures or power, irrespective of the control measures used.

This is illustrated by differences detected in the position of the same peroxidase bands of *P. radiata* 517 (Figure 16, Table 21). To account for such inaccuracies in the method, the variation in the peroxidase band position was used to set the limits for band similarity. Thus, bands within 0.02 Rm units (Table 21) were considered to represent the same protein.

Using this method, resolution will be lowered because, while there is no reason to suggest that different proteins will not have relative mobilities within 0.02 Rm units, they will be counted as the same band. There is a high probability that bands within 0.02 Rm units for extracts of closely related samples do represent the same protein, (for example, bands detected in the different clones of *P. radiata*) but this probability decreases for extracts of distantly related samples.

Care must also be expressed in reaching conclusions from the analysis of 'similarity'. Direct evidence for differences between the components of two extracts is obtained when two bands move to different positions in the gels, but if two bands occupy the same position one may not conclude that they represent identical proteins. This problem has been described as the "hidden heterogeneity" (Johnson 1977).

The problems associated with comparisons between different samples using rod gel electrophoresis has been approached in several ways.

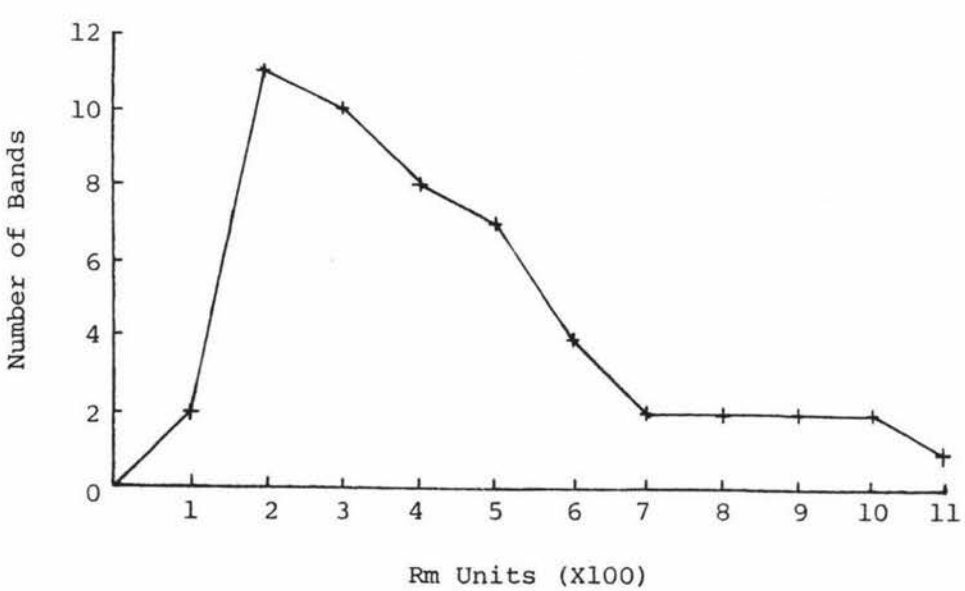
Ziegenfus and Clarkson (1971) and Shechter and de Wet (1975) used the mean Rm values from many duplicate gel runs to compare bands in different gels, yet Shechter and de Wet still considered bands within 0.5 Rm units to represent the same proteins. Other workers create "artificial hybrids" by combining two samples and separating them on the same gel (Ladizinsky and Hymowitz 1979). Here, if two bands present on the different gels separate into two bands on the "hybrid" gel, then evidence for differences between the proteins is obtained. However, if only one band is detected in this "hybrid" gel, it does not necessarily indicate that only one protein is present because the problem of "hidden heterogeneity" still exists, that is, the Rm differences between the two proteins may be too small to be detected by electrophoresis. Quantitative differences in the stain density of different bands has also been used (Johnson 1972), although these differences are probably less significant than differences in band position (Ladizinsky and Hymowitz 1979).

In this study, a micro-computer was used to analyse the results of polyacrylamide rod gel electrophoresis strictly and objectively by using the similarities in band position (that is, within 0.02 Rm units) but taking no account of the stain density of the bands. For the real gels, when the Rm is calculated, each band is, in effect, assigned to one of a possible one hundred classes in an 'ideal' gel. Variation is, therefore, assumed to be discontinuous (Johnson 1977). Thus, if two gels each have twenty-five bands all representing <u>different</u> proteins, one in every four 'band classes' must be filled so that when gels are compared, some bands will fall within the 0.02 "variation".

These matches will of course be due to chance pairing and illustrates the problem the program attempts to control. A 'null hypothesis' can be formalised, that none of the protein bands seen in any pair of gels arises because of genetic similarities in the proteins. This hypothesis can be rejected if matching of bands in real gels is significantly higher than that obtained by the random matching.

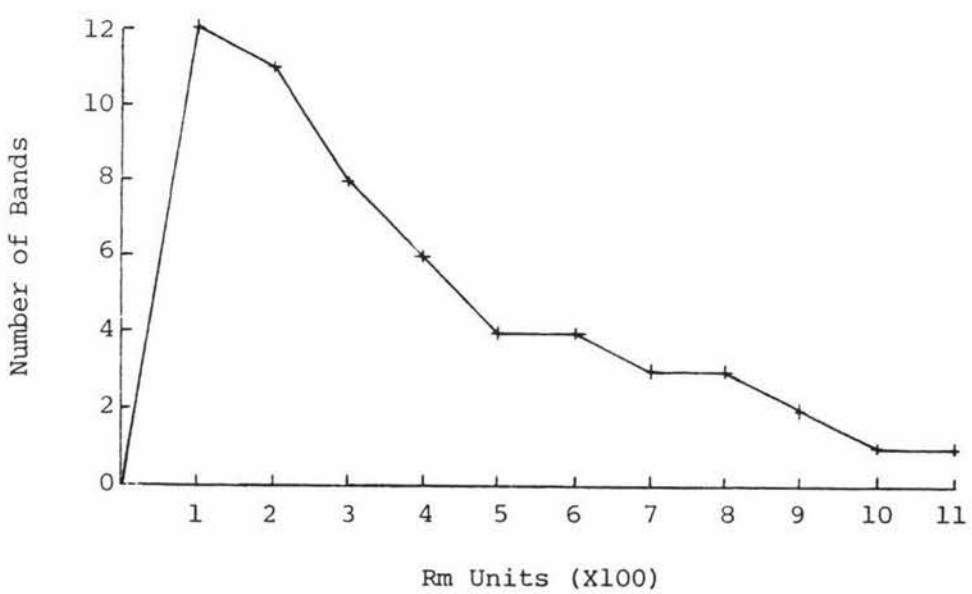
A computer program (Appendix I) was written to model the electrophoresis gels so that possible pairings due to random chance could be estimated. The program generates random numbers between 1 and 100 which represent the relative mobility values (X 100) of bands in the 'model' gel.

To ensure the simulation modelled the real gels closely, the band pattern of the real gels were analysed by calculating the distances between each of the bands in the gel. The number of times each distance occurs is an estimate or 'index' of the pattern or spread of bands. The result is illustrated graphically in the diagram below:



This diagram demonstrates that very few bands were only one Rm unit apart, due to the inability to resolve bands very close together, and because of the multiplication factor in producing the idealised gels. For example, a gel of 5.0 cm is converted to and 'idealised gel' of 10.0 cm; thus bands differing by 0.5 mm would need to be resolved to get a distance of 0.01 Rm unit in the ideal gel, but the technique does not generally allow resolution at this level.

The computer program generates random numbers with a spread pattern as shown below. In contrast to the spread pattern obtained from the real gels, this figure indicates that the modelled data produces "bands" one Rm unit apart more frequently.



To model the gels more closely, the program was altered so that it generates fewer "bands" one Rm unit apart. The spread pattern was then similar to that obtained from the real gels but the "similarity indices" calculated from the altered program data differed by only c.2% from the unaltered program data. While this analysis merely models the real situation (where band position is not determined by random chance) it does give a base line measure which will be more important when the relationship between the samples is distant. Pairs of gels thus generated were compared using the same strict analysis as for the real gels, and the "similarity indices" (due to random pairing) (Table 15) were used as a measure for significance when analysing the data from the real gels.

The results obtained when components of the extracts of five *P. radiata* clones and individuals of *P. elliottii*, *P. taeda* and *P. monticola* were separated by electrophoresis (Table 13) demonstrate a large variation in the number of bands detected. The low number of bands detected in *P. taeda* and *P. monticola* is probably due to smaller sample loadings. While sufficient material was available from *P. radiata* clone material (that is, from several trees) only a limited amount of material was available from the individual trees of the other species, as the trees were small and required for seed production. This, combined with the low protein extraction efficiency made less material available for analysis. The low numbers of bands detected in *P. radiata* 511 resulted from a lack of stain definition in the top portion of the gels in all the samples separated.

Although the bands were sometimes present, they were not taken into account because of a lack of duplication.

The results for electrophoresis of the five clones of *P. radiata* (Table 13, Figure 11) indicate a close similarity of banding pattern and stain density. Some bands appear to be common to all five clones while other bands appear to be present only in one, or a few clones. Individual differences are also apparent in some of the extracts, for example, a band at 0.48 is apparently present in *P. radiata* 510 only.

The similarity in the results between the clones of *P. radiata* is reflected in the results obtained for the *P. elliottii* extracts (Table 13), where fifteen bands appear to be common (within 0.02) and the pattern of stain density in the gels is similar (Figure 12). Less similarity is observed when individuals of different species are compared for band position or stain density.

The results of the computer analysis of the electrophoretic data (Table 14) generally show the same trends. Here, the highest values for similarity index are observed for the intraspecific comparison but high values are also obtained for comparison between the *P. radiata* clones 517 and 522, and *P. elliottii* extract 2; and *P. taeda* and *P. elliottii* extract 2. These high values may be influenced by the increased number of bands detected in *P. elliottii* extract 2. A wide variation for the similarity index values within a species is apparent; for example, the values for intraspecific comparisons of *P. radiata* on Table 14 vary between 60.71 to 76.92 (excluding comparisons with *P. radiata* 511).

Similarly, a wide variation is demonstrated in interspecific comparisons, for example, between the *P. radiata* clones and *P. elliottii* extract 1. Unexpected results are also apparent in this table, for example, in the interspecific comparisons between *P. radiata* clones and the *P. elliottii* extracts, where values are higher than those obtained from the intraspecific comparisons for *P. elliottii*.

The high values obtained are probably due to an overestimation because of a high number of false matchings. Relatively high values are obtained for the average similarity indices from the "model" gels (Table 15), and some values for the actual data (Table 14) are very close to that obtained by random chance. Hence, a large proportion of the bands common to any two gels could have arisen by the random pairing of bands, particularly for interspecific comparisons where the sampled materials are likely to be distantly related. This high degree of random matching is demonstrated in Table 32, where the number of pairs of matching bands expected by random chance alone are given in brackets.

The average similarity index values (Table 16) were calculated so that a comparison of the different species could be made. Here again, the highest values were generally found for intraspecific comparisons although high values were also obtained for comparisons between *P. elliottii* and *P. taeda* and *P. elliottii* and *P. radiata*. In all comparisons, the lowest values were obtained for comparisonswith *P. monticola*.

TABLE 32 Number of bands common to each pair of extracts after components were separated by electrophoresis

(Determined by computer, Appendix I)

		P. radiata					*P. elliottii*		*P. taeda*	*P. monticola*
		510	511	517	522	525	ext 1	ext 2		
P. radiata	510		10(10)	19(13)	19(13)	17(13)	14(12)	18(13)	13(11)	11(10)
	511			13(10)	13(10)	12 (9)	12 (9)	13 (9)	7 (7)	8 (6)
	517				20(13)	18(13)	16(12)	20(13)	14(11)	12(10)
	522					19(13)	13(12)	20(13)	14(11)	13(10)
	525						15(11)	17(12)	12 (9)	12 (9)
P. elliottii	ext 1							15(11)	11 (9)	10 (9)
	ext 2								15 (9)	12 (9)
P. taeda										10 (7)

Values in brackets are the mean number of matching bands expected by random chance alone.

Certain statistical analysis may be applicable to test for significance of data in the form discussed above. Lawson *et al.* (1975) used a probability function to determine the probability of getting "X" or more matches between any gels by random chance alone. This method was not applicable to this study as it requires a number of independent tests to be carried out using one technique, for example, the nine enzyme system determined using electrophoresis by Lawson *et al.* Standard statistical measures, for example t and z tests, were not found to be applicable to this situation because of a lack of data on the standard deviation of the observed data. One measure of significance was applied to data on Tables 14 and 15 (Chi-squared analysis, Appendix IV) although this too is not strictly applicable as the randomly generated gels do not represent completely independent experiments, and it was not possible to set up contingency tables. The randomly generated "gels" do model the real gels, as discussed previously, and the chi-squared test does show a difference between the similarity indices obtained from the generated and real data, significant at the 1% level.

Chi-squared analysis was also performed on the number of bands common to pairs of gels (Table 13). The results here suggest that the level of significance obtained in the previous chi-squared analysis is overestimated by the calculation of the similarity indices. For example, the values for the comparison of the number of bands common to *P. monticola* and *P. radiata* were <u>not</u> significant at the 10% level, that is, the number of pairs of common bands were not significantly greater than could be expected by random chance alone.

Although discontinuous polyacrylamide rod gel electrophoresis is a relatively high resolution technique, problems arise when the discrete gels are compared for taxonomic purposes. These problems are compounded, in this study, by the relatively high number of bands detected here when compared to the number detected by other workers using the similarity index analysis. Ziegenfus and Clarkson (1971) detected between 11 and 15 bands in seed extracts of certain *Acer* species, but unlike the bands detected in this study, (which were sharp and less than 0.01 Rm unit wide) the bands detected by these workers were often very broad (over 0.05 Rm units) and apparently diffuse. The high number of bands detected here increases the chance of random matching of bands, and hence the overestimating the relationship between individuals. Similarly, because of variations between different electrophoretic runs, the use of the 0.02 "error" has compounded the problem of "hidden heterogeneity" by reducing the potential resolution of the technique. "Serious error may occur in all such investigations when similar Rf (Rm) in a gel does not reflect genetic identity" (Johnson 1977). Errors may also occur when electrophoretic data is used to detect similarities between protein bands, rather than to detect differences.

The electrophoretic data presented here demonstrates a relatively wide variation in the number and position of bands detected in a sample between different runs, and between samples of the same species. The data presented here (and possibly by other authors) may include large errors in overestimating the relationships between extracts of distantly related species, or underestimating the intraspecific relationships.

For example, the highest intraspecific similarity index value detected using the data presented here was 76.92, whereas Ziegenfus and Clarkson (1971) combined seeds of several individuals of *Acer*, and assigned intraspecific values of 100.

The data presented here support the division of the genus *Pinus* into two subgenera, as the lowest values for similarity index were found for comparisons of each species with *P. monticola*. Similarly, a high value was obtained for comparisons between *P. elliottii* and *P. radiata*, and *P. elliottii* and *P. taeda* supporting the classification of these species in the one subgenus. However, the data are too unreliable and variable to determine the relationship between these three species with any degree of confidence. The technique did provide evidence for individual differences between the *P. radiata* clones and the technique would probably be most useful in analysing such differences rather than the similarities, between extracts of closely related sample material.

The protein components of extracts from four *P. radiata* clones and individuals from *P. elliottii*, *P. taeda* and *P. monticola* were also separated using isoelectric focussing. This protein separation technique is performed in a flat bed polyacrylamide gel after a continuous pH gradient has been developed utilising the properties of amphoteric substances. Greatly increased band resolution (compared to starch and polyacrylamide gel electrophoresis) results from the use of the flat bed gel, the continuous pH gradient, and the focussing effect of the amphoteric substances, so that proteins differing by only a few hundredths of a pH unit in their isoelectric points can be separated.

The isoelectric point results from the total amino acid content present in the individual proteins, and is defined as the pH at which the net charge of the protein is zero. It is therefore unlikely that two completely different proteins with a different amino acid composition, will have isoelectric points within the range of a "few hundredths of a pH unit", and hence the problem of "hidden heterogeneity" is greatly reduced. Protein bands are compared by direct observation in the flat bed gel avoiding the need for measurements, assignment of bands to discrete classes, and calculation of relative mobility values, as is necessary when comparing bands in discrete gels (for example, in rod gel electrophoresis). The increased resolution, the continuous nature of the pH gradient, and the direct observation greatly reduces the possibilities for errors associated with discrete gel electrophoretic techniques.

The development of a stable pH gradient is critical to the success of the technique and hence reagents and samples are required to be of high purity. Acrylamide with a very low acrylic acid content (<0.1%) in conjunction with ion exchange resins, for example amberlite which absorbs acrylic acid, is used in many situations, and salt free protein samples often purified by dialysis are required. Highly purified samples from mature conifer tissue have apparently been previously unavailable.

The number of bands detected in extracts using isoelectric focussing (Table 17) varied between thirteen and seventeen, similar to the range detected by electrophoresis.

A large increase in the number of bands in the *P. radiata* 511 extract, and a decrease in the number of bands in the *P. elliottii* extract 2 was observed (from 14 to 23, and 22 to 14 respectively). The increased resolution and lower stain densities of bands in the isoelectric focussing gel, when compared to electrophoresis, suggests that many of the densely stained bands detected in electrophoresis have been separated into several components using isoelectric focussing, resulting in an increase in the number but decrease in the stain density of bands (Figure 13 compared to Figure 9). Thus some bands may be too faint to be detected at this sample loading.

The numbers of bands common to two extracts (Table 18) varied from four to twenty-three. The greatest number of bands in common were detected in intraspecific comparisons, from 12 to 23, and were generally lowest for interspecific comparisons. A higher number of common bands were found for comparisons between *P. elliottii* and *P. taeda* extracts than for other interspecific comparisons.

These trends are reflected in the values obtained for the similarity indices (Table 19). Again the highest values are obtained for the intraspecific comparisons while the lowest values were generally obtained for interspecific comparisons. The similarity indices for comparisons between *P. elliottii* and *P. taeda* extracts were higher than those found for the other interspecific comparisons.

The average similarity indices (Table 20) also demonstrate the relatively high values obtained from intraspecific comparison and the generally low values obtained for interspecific comparisons. A higher value is found for the comparison between *P. elliottii* and *P. taeda*. The relatively low similarity indices obtained from intraspecific comparisons using this high resolution technique, compared to the findings of some other workers, (for example, Ziegenfus and Clarkson 1971) suggests that reliability would be further increased by comparing many individuals of a species and this would appear to be a necessary pre-requisite for interspecific comparison.

Polygonal representations (Ziegenfus and Clarkson 1971) were used to graph the average similarity indices from Table 20 (Figures 47A, B, C). Figure 47A represents the relationship of each species to *P. radiata*, Figure 47B to *P. elliottii*, and Figure 47C to *P. taeda*. These diagrams demonstrate the close relationships implied within a species, and the relatively distant relationship implied between *P. monticola* and the other species. Figures 47B and C demonstrate the close relationship suggested between *P. taeda* and *P. elliottii*. *P. radiata* is apparently distantly related to the other species but is slightly closer to *P. elliottii* and *P. taeda* than to *P. monticola* (Figure 47B).

The isoelectric focussing data indicate the technique is useful for determining differences between individuals or clones of a particular species in band numbers, band position, and stain density.

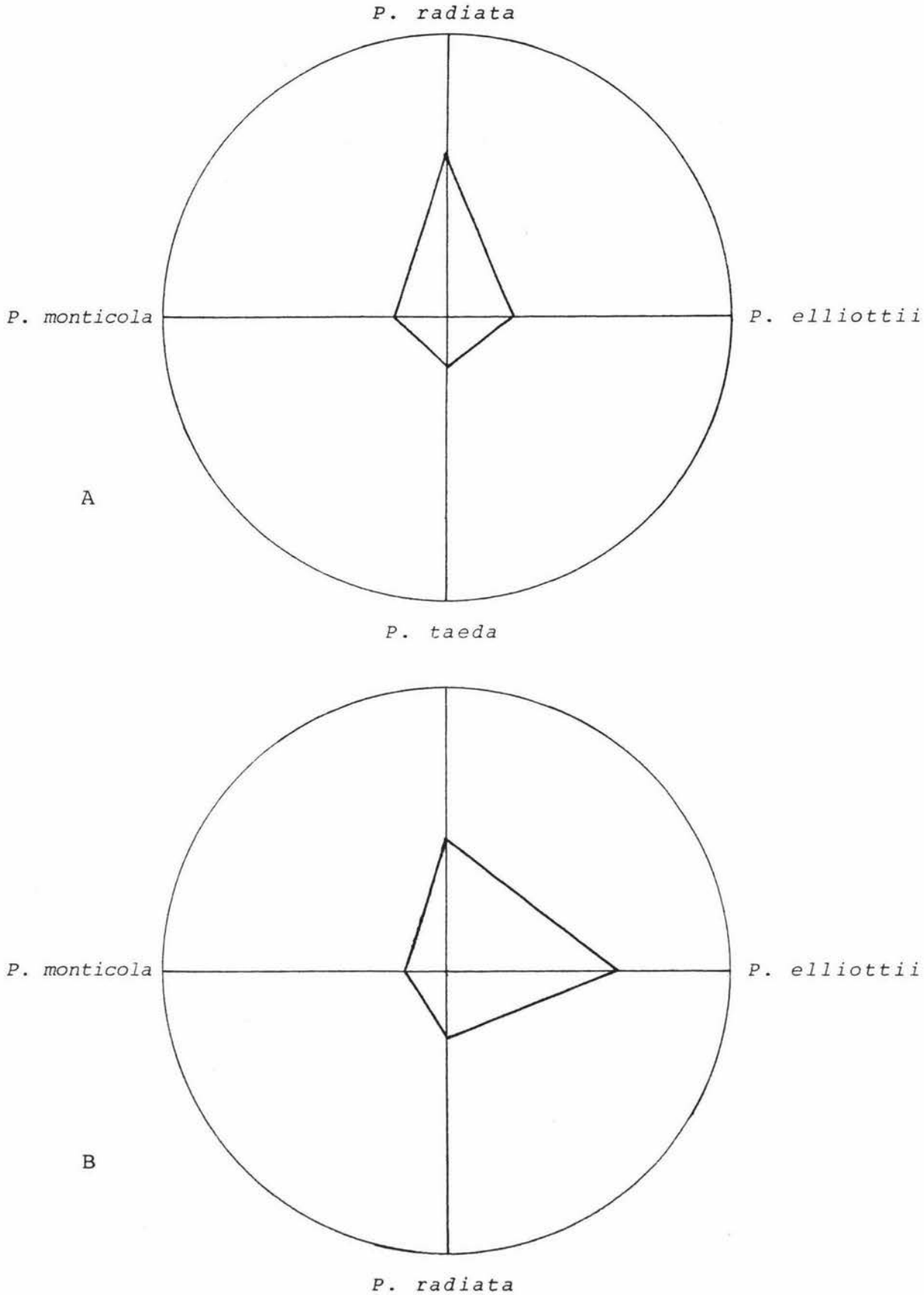
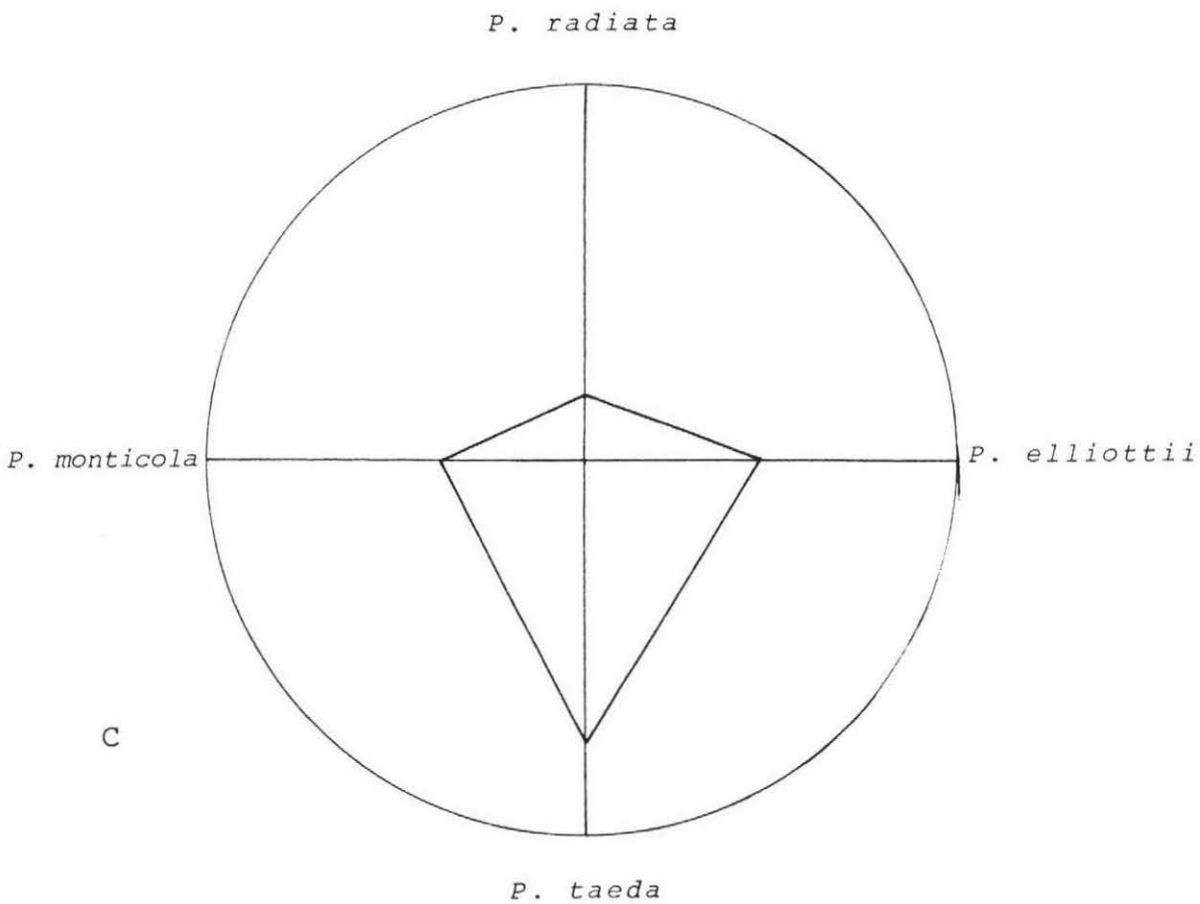


Fig 47 Polygonal representations of relationships between four *Pinus* species determined by isoelectric focussing.



C

Fig 47

The technique is also useful in determining intrageneric relationships in *Pinus* but may not be as useful in the determination of intergeneric relationships as the similarity indices for apparently distantly related species is relatively low (for example *P. elliottii* x *P. monticola* = 14.99).

The data on Table 20 indicate a close relationship between *P. elliottii* and *P. taeda* and a distant relationship between *P. monticola* and the other species. These data also suggest that although *P. radiata* is distantly related to the other species, it is more closely related to *P. elliottii* (and *P. taeda*) than to *P. monticola*. However, the difference in similarity indices may not be significant at this low level. The isoelectric focussing data therefore support, with greater confidence than the electrophoretic data, the division of the genus *Pinus* into two subgenera, and a close relationship between *P. elliottii* and *P. taeda*.

The average similarity indices for interspecific comparisons from isoelectric focussing (Table 20) are substantially different from those obtained from electrophoresis (Table 16). The majority of the interspecific comparisons from the electrophoretic data are approximately three times larger than those obtained from isoelectric focussing data. The values for the intraspecific comparisons, and the interspecific comparison between *P. elliottii* and *P. taeda* are very similar, although the *P. elliottii* x *P. taeda* datum is still higher from electrophoretic analysis.

The increased resolution and lower possibility for errors in the analysis of isoelectric focussing gel results allows greater confidence to be applied to these data, suggesting that the analysis of electrophoretic results has greatly overestimated the relationship between apparently distantly related species. A comparison of Tables 16 and 20 suggest that polyacrylamide rod gel electrophoresis is useful for determining differences between closely related species, as discussed previously, but the errors involved in analysing the data, and the increase in chance pairing of bands from extracts of distantly related species, produces unreliable results when interspecific comparisons are made in this way.

Reliability in assessment of band similarity using electrophoretic techniques would be expected to be increased using isozyme analysis, as this method uses the added dimension of specific protein function recognition. Thus if two protein bands occupy position within 0.02 Rm units in gels after electrophoresis (that is, have the same size/charge attributes) and the proteins are shown to have the same enzymatic function, there is a high probability that the proteins are identical or very similar.

Isozyme analysis has been used extensively to study the genetics of different populations of organisms, including conifers. The genetics of plant isozymes can often be analysed by using seed haploid tissues, for example, the megagametophyte tissue in conifers. The components of the extracts from the seed material are usually separated on starch gels and therefore this technique is particularly suitable for conifer tissues, as the complex problems involved in protein extraction from mature tissues are avoided.

Bergmann (1978) found a geographic variation in the pattern of acid phosphatase alleles in *Picea abies* possibly determined by temperature. Similarly, Bonnet-Masimbert and Bikay-Bikay (1978) analysed the intraspecific variations of glutamate-oxaloacetate-transaminase in four subspecies of *Pinus nigra*. O'Malley *et al.* (1979) analysed the inheritance pattern of several isozymes in ten stands of *P. ponderosa*, and Adams and Joly (1980 a, b) analysed the genetics of ten allozyme variants visualised in *P. taeda*.

Polyacrylamide gel electrophoresis has also been used to study isozyme variation in conifers, although more complex extraction systems are generally required. Feret (1971) analysed isozymes of peroxidase and esterase in *Picea glauca* seedlings while Hamaker and Snyder (1973) screened fifty-three enzymes from *Pinus taeda* and *P. palustris*.

In this study, the advantages inherent in the use of specific enzyme stains in polyacrylamide gel electrophoresis have been used to study the isozyme variation in five clones of *P. radiata* and individuals of *P. elliottii*, *P. taeda*, and *P. monticola*. In comparison with the isozyme results in starch gels, the isozyme bands detected in polyacrylamide gels (Figure 15) appear to be sharp and more distinct. Copes and Beckwith (1977) using starch gels, found that acid phosphatase, esterase, and peroxidase bands were inconsistent in stain development, or "the bands were not distinct enough for accurate measurement" and the data were not recorded in their report. The increased resolution and definition of peroxidase bands using polyacrylamide gels allows greater accuracy in the determination of band position compared with starch gel results.

The components of the extracts of *P. radiata* 517 were separated by electrophoresis and stained for the presence of peroxidase enzyme activity at different times over a fifteen month period. The relative mobility of each band in three of these runs (Table 21) indicates the degree of variation (expressed as an "error") in band position. This "error" was used to determine the limits of band similarity in electrophoresis. The electrophoregram for these three runs (Figure 16) indicates that the variation is not consistent, that is, is not always in one direction. For example, the top band in the gel (4.6.81) has a slightly higher relative mobility than the comparable band in gel (10.11.80), while the third band in gel (4.6.81) has a slightly lower relative mobility than the comparable band in gel (10.11.80). Thus, band positions are not merely altered in relation to the dye front, but also with respect to each other.

The isozyme patterns detected in the five clones of *P. radiata* (Figure 17), and the designation of classes to groups of bands with similar relative mobilities is consistent with allozyme patterns found in other *Pinus* enzyme systems by other workers. Rudin (1977) found two areas of banding for leucine amino peptidase in *Pinus sylvestris* with similar banding patterns to the isozymes detected here, and demonstrated segregation of these bands in megagametophyte tissue. Guries and Ledig (1978) also demonstrated the segregation of bands (alleles) in several enzyme systems detected in embryo and megagametophyte tissue of *P. rigida*. As three of the four classes of peroxidase activity detected here (A, B, and D) contained either one or two bands, it seems likely that the patterns present monomeric allozymes with the single bands representing homozygous parents.

The number of peroxidase bands detected in four species of *Pinus* are similar to the number of bands found by other workers. Van Lear and Smith (1970) detected up to three peroxidase bands in *P. elliottii* under different nutritional regimes using discontinuous polyacrylamide rod gel electrophoresis. Conkle (1971, b) found an increase in the number of peroxidase bands in germinating *P. attenuata* seeds, detecting up to five anodally migrating bands in seedling tops, and up to fifteen in the roots. Ramaiah *et al.* (1971) found a similar increase in the number of peroxidase bands during germination of *P. banksiana* seeds, detecting up to nine bands at eleven days after germination. Hamaker and Snyder (1973) detected up to seven peroxidase bands in hybrids of *P. taeda* and *P. palustris* while Juo and Stotzky (1973) detected four bands in *P. radiata*, five in *P. taeda*, and nine in *P. elliottii* (see Table 24).

The peroxidase bands detected in extracts in this study show a wide variation in relative mobilities, from 0.02 (*P. elliottii* extract 2) to 0.70 (*P. taeda* extract 2). Bands within 0.02 Rm units detected in extracts from the same species were generally considered to represent the same protein, except for bands A1 and A2 (*P. elliottii*) which showed differential staining, and bands D1, D2, and D3 in *P. taeda*, of which two were present in the one extract. These bands may represent different alleles as discussed for *P. radiata* but, in all cases, this can be confirmed only by segregation analysis in megagametophyte or progeny tissues.

Peroxidase patterns here, and as found by other workers, show a high degree of intraspecific, and interspecific variation.

Juo and Stotzky (1973) found no common trends or homologies between any species they examined for peroxidase activity, (including *P. radiata*, and *P. elliottii* and *P. taeda*), and observed considerable intraspecific variation in seeds of the same "batch". These workers used "hybrid gel" analysis but detected no homologies between any of the species examined.

The peroxidase isozymes here are probably not identical proteins, as suggested by the work of Juo and Stotzky, but it would be unlikely that highly variable proteins such as peroxidase enzymes, should remain identical between species. Indeed, it may be expected that two proteins originating from the same genetic ancestor would not retain an identical amino acid sequence when the enzyme apparently has several different forms (as shown by the variation in relative mobilities) but still retains activity. Thus two isozymes with similar relative mobilities (for example, bands A1 and A2 in *P. elliottii*, and A in *P. taeda*) may represent the same proteins (or alleles) but with slightly altered amino acid composition. This point is discussed further in relation to the immunoelectrophoretic data (page 171).

The peroxidase isozyme data supports the division of the genus *Pinus* into two subgenera, and the close relationship suggested between *P. elliottii* and *P. taeda*. The high degree of variability exhibited by the enzyme suggests that this enzyme system would be of limited use in wide taxonomic studies, but useful for differentiating between closely related species and in the identification of intraspecific groups.

Acid phosphatase isozymes have been used by several workers to study the inheritance and allelic variation in populations of conifers. Lundkvist (1975) studied the inheritance patterns in parental trees and progeny of *Picea abies* and demonstrated the apparent dimeric structure of the enzyme. Lundkvist and Rudin (1977) analysed eleven populations of *Picea abies* and showed major genetic differences occurred at acid phosphatase and esterase loci.

Isozymes have generally been separated using electrophoretic techniques on starch, polyacrylamide rod, and polyacrylamide slab gels. Copes (1975) demonstrated isozyme (including acid phosphatase) differences between normal and dwarf Douglas fir trees using starch gels. Hamaker and Snyder (1973) detected between four and nine acid phosphatase bands in *Pinus taeda* and *P. palustris* species and hybrids using polyacrylamide rod gels, while Lundkvist (1975) and Lundkvist and Rudin (1977) used acid phosphatase isozymes separated in polyacrylamide slab gels to demonstrate the inheritance, dimeric structure and genetic variation between populations in *Picea abies*.

Recently, Adams and Joly (1980a), using starch gels, detected two zones of activity in *Pinus taeda*; a lower zone with three single banded variants, and a low resolution but also polymorphic upper zone. The increase in the number and spread of acid phosphatase isozymes detected in this study suggests that the genetic variability of this enzyme system may be more complex then previously demonstrated.

The increase in band resolution obtained by isoelectric focussing combined with the advantages of isozyme analysis and the direct comparison of band position, suggests that data obtained using this combined technique are less prone to error and more reliable than data obtained using electrophoretic techniques, particularly when discrete gels are used.

A high degree of intraspecific similarity was detected using isoelectric focussing. Four bands were common to all *Pinus radiata* clones (Figures 19 and 20) and all the bands detected in *P. elliottii* extract 2 and *P. monticola* extract 1 were detected in the respective conspecific extracts. At the same time, substantial intraspecific variation was observed, for example, two bands detected in *P. radiata* 511 were absent from the other two *P. radiata* clones although an unresolved area of staining close to these bands was found in *P. radiata* 517. This staining appeared to be due to sample smearing rather than the presence of a band(s). Differences were also detected between the *P. taeda* extracts with apparently unique bands in each extract. The apparently unique bands detected in *P. elliottii* extract 1 and *P. monticola* extract 2 may be due to increased sample loadings of these extracts compared to their respective conspecific extracts (Figure 19). However, the major bands present in the lower part of *P. elliottii* extract 1 appear to be absent from *P. elliottii* extract 2, while less densely stained bands are present in both.

As expected, fewer bands were found to be common to extracts of different species.

Three bands were found to be common to *P. radiata* and *P. elliottii* and two of these were also found in one extract of *P. taeda*. The detection of bands common to the three species may suggest the existence of subgeneric acid phosphatase bands but the absence of these bands from *P. taeda* extract 2 is confusing and analysis of a number of individuals from each species would be required to determine the extent of the differences before taxonomic comparisons could be made. This point is further discussed with reference to the immunoelectrophoretic results. Bands common to *P. elliottii* and *P. taeda* were also detected although they were not always detected in both extracts of one species. The number, pattern, and position of bands suggest that *P. elliottii* and *P. taeda* are closely related species. No common bands were detected between *P. monticola* and the other species suggesting this species is distantly related.

The absence of bands common to all species analysed suggests that acid phosphatase is not useful for the analysis of taxonomic relationships at the generic level but may be useful at the subgeneric, specific, and subspecific levels. At these levels, these data support the division of the genus *Pinus* into two subgenera, and also suggest a close relationship between *P. elliottii* and *P. taeda*. Thus these data support hypothesis one illustrated in Figure 1A.

The use of proteins has reached 'pre-eminence' in classification studies due to the recognition that proteins form an apparent 'third copy' of the D.N.A. Thus the sequence of amino acids reflects the sequence of nucleotides in the D.N.A., the "blueprint" of an organism (Smith 1976).

Serological techniques offer a further refinement over general protein separation and isozyme techniques by allowing a direct comparison of proteins of different species (Vaughan and Gordon 1969) and utilising protein recognition at a basic level. The basis of the antibody/antigen reaction is a "complementarity of surface sites on antibodies with others on the antigens"(Smith, 1976). Several combining sites (2-5) are present on each antibody molecule and these 'recognise' the corresponding sites on the surface of the antigens.

If antibody/antigen recognition is established, the antibody binds to the antigen forming an antibody/antigen complex. As several binding sites are present on the molecule, cross-linking between the antibody/antigen complexes can occur, leading to a 'precipitin formation', which can then be stained with normal protein stains. Thus serological techniques utilise the recognition (or partial recognition) by specific antibodies of the sequences of amino acids and, to some extent therefore, the sequence of nucleotides in the D.N.A.

The sequence recognition by antibodies is very specific and this specificity is one of the features of the immune response system. Changes or mutations in the sequence of amino acids on the surface of the protein are, in general, limited to a few amino acid substitution groups (for example, a hydrophobic amino acid is probably successfully replaced only with another hydrophobic amino acid), and to mutations which will not cause major conformational changes. Therefore, the probability that two <u>completely different</u> proteins will have, by change alone, identical or very similar amino acid sequences in the correct position on the protein is very small.

The results of serological analysis can, in general therefore, be received more confidently than other techniques used in this study.

The use of serological techniques for the analysis of proteins in conifers has been limited. Prager *et al.* (1976) produced antiserum to seed proteins of twenty-three different conifer species and analysed the intra- and intergeneric relationships using spur analysis. To the best of the author's knowledge, the use of serological techniques in this thesis is first serological analysis of proteins extracted from mature conifer tissue.

The development of multiple sharp bands in double diffusion analysis (Figures 21 to 28) demonstrate the usefulness of the method presented in this study for extracting protein from mature *Pinus* tissue suitable for serological analysis. The number of precipitin bands formed is similar to the number of bands detected in other published results using various plant species. For example, El-Lakany *et al.* (1977) developed up to nine precipitin bands in extracts of *Casuarina* species challenged with antiserum prepared against the same species. El-Tinay *et al.* (1979) developed up to three bands in immunodiffusion analysis of some *Acacia* species and varieties when challenged with antiserum prepared against the same species.

In the results presented in this study (Figures 21 to 28) interspecific differences in the 'quality' of band formation can be seen. Sharp bands are evident when some extracts are challenged with antiserum prepared against *Pinus radiata* 511, for example, extracts of *P. radiata* (Figure 21), *P. elliottii* and *P. taeda* (Figure 22).

However, some bands are more diffuse indicating a lower level of recognition, for example, when *P. monticola* extract 2 is challenged with antiserum prepared against *P. elliottii* extract 1 (Figure 17).

Interspecific differences were also detected in the number of bands formed when the extracts were challenged with each antiserum (Table 25). The greatest number of bands were formed when the "parent extract", that is, the extract used to produce the antiserum, was challenged with that antiserum. High numbers of bands were also formed when extracts were challenged with antiserum prepared against extracts from other clones or individuals of the same species.

These trends are reflected in the average number of bands produced for each species when challenged with antiserum prepared against either *P. radiata* 511 or *P. elliottii* extract 1 (Table 25, A, B). In each case, the highest average values were obtained for the intraspecific comparisons. A high average value was also obtained when the *P. elliottii* extracts were challenged with antiserum prepared against *P. radiata* 511, suggesting a close relationship between these two species. A relatively high value was also obtained for the *P. taeda* extracts challenged with antiserum prepared against *P. radiata* 511, while the lowest value was obtained when *P. monticola* was challenged with the same antiserum. This pattern is not repeated when extracts were challenged with antiserum prepared against *P. elliottii* extract 1. Here, very little difference was found between the average number of bands produced in interspecific comparisons.

As previously noted band number analysis is not a good indicator of the relationships between extracts. The bands formed in double-diffusion are composite bands, that is, are formed from several component antigen/antibody complexes, and some bands can be seen to "run together", as in Figure 23 for the extract of *P. radiata* 511 and *P. monticola* extract 2. Similarly, some bands may be masked or absent due to large differences in the relative concentrations of antigen and antibody. If the antigen is in large excess, the precipitin line will be 'pushed' into the central well. The analysis of band numbers and position in double diffusion analysis may therefore be unreliable. A second analysis of the double diffusion results can be performed by taking into account spur formation. Spur analysis, only 'considers' the antigens which are visualised and does not assume that the absence of a band indicates the absence of an antigen. Spur analysis, while subjective, is probably more reliable than band number and position analysis.

A spur will be produced in double diffusion analysis when the components of two extracts are related but are not identical, hence the degree of recognition of each extract by the particular antiserum will also differ. This is illustrated in Appendix V. The precipitin line which forms for both extracts indicates the presence of immunologically identical (or very similar) components while the spur indicates the presence of components present in the 'parent extract' and one of the test extracts, but not both. The spur so formed will 'point' to the non-identical or less similar extract. Thus spur analysis indirectly compares the antigen similarly of two samples and also allows an estimate of the relationship between the samples to be made.

This estimation of relationship is demonstrated in Figure 27 where a large spur (arrowed) is assigned a size score of '5'. In this case, the result suggests that some components of the parent extract (*P. elliottii* extract 1) and *P. radiata* are similar and form a sharp precipitin line. However, some components of *P. monticola* extract 2 are distantly related to components of the parent extract, thus forming a diffuse line. Furthermore, some components in *P. radiata* 511 which are similar to components in the parent extract are absent (or immunologically dissimilar) from *P. monticola* extract 2 forming a spur.

Data on Table 26A suggests that a close relationship exists between the clones of *P. radiata*; between the individuals of *P. elliottii*; and between *P. elliottii* and *P. taeda*. A more distant relationship is suggested between *P. radiata* and *P. elliottii* extracts, with a slightly closer relationship suggested between *P. radiata* and *P. taeda* extracts. Spur size scores for comparisons with *P. monticola* suggest this species is distantly related to all the other species.

The intraspecific and interspecific trends on Table 26A are repeated on Table 26B except that a score size of 'two' is obtained for the intraspecific comparison of *P. elliottii*. This probably reflects intraspecific differences between the extracts which were recognised by the antiserum prepared against *P. elliottii* extract 1 but would not be expected to be recognised by the antiserum prepared against *P. radiata* 511. Thus the data on Table 26B support the data presented on Table 26A.

The average spur size scores (Table 27) suggest a low intraspecific variation, and a close relationship between the extracts of *P. elliottii* and *P. taeda*. *P. radiata* is apparently distantly related to *P. elliottii* and *P. taeda* while *P. monticola* is apparently distantly related to the other species.

Immunoelectrophoresis is a further refinement of the double diffusion technique where the antigen components of the extracts are electrophoretically separated before the double diffusion step. In effect, this technique spreads out the antigens so that a greater area for the formation of precipitin arcs is produced. In this way, the problem of the masking of small precipitin lines in the narrow space available in Ouchterlony double diffusion, is largely avoided. This is illustrated in Figures 29A and B where the extract of *P. radiata* 511 was electrophoretically separated before being challenged with antiserum prepared against the same extract. Here fifteen precipitin arcs were detected (Table 28) compared to the five bands detected in double diffusion analysis (Figure 21, Table 25A).

Electrophoresis in agarose gels, as performed in the first stage of immunoelectrophoresis, is a relatively low resolution technique when compared to polyacrylamide gel electrophoresis, and "hidden heterogeneity" may be expected to be a problem. However, the problem is lessened because the precipitin arcs of two electrophoretically similar components will probably form in a different position in the gel (relative to the trough) if the relative concentrations of antigen and antibody are different.

This is illustrated in Figure 33, where two components of the extract of *P. elliottii* extract 2 have migrated approximately the same distance (c. 6.5 cm) but the precipitin arcs are formed separately because of difference in the ratio of antigen to antibody. Thus, in effect, immunoelectrophoresis is a two dimensional technique which increases the chances for the separation of extract components, and therefore increases resolution.

The results of the immunoelectrophoretic analysis of three *P. radiata* clones and individuals of *P. elliottii*, *P. taeda*, and *P. monticola* challenged with antiserum prepared against *P. radiata* 511 (Figures 29B to 37) demonstrate intraspecific and interspecific similarities and differences. The pattern of distribution of precipitin arcs for the *P. radiata* clones (Figures 29B, 30 and 31) are similar with (for example) three closely positioned arcs detected in all clones. Individual differences were also detected with an arc present in *P. radiata* 522 and 511 (C. -3.0 cm) but absent from *P. radiata* 517. Similarly, another four precipitin arcs were detected in *P. radiata* 511 which were not detected in *P. radiata* 517 or 522 (Table 28).

Differences and similarities were also detected between each pair of extracts of the other species. The antigens responsible for precipitin arcs in other species, must be present in *P. radiata* 511 but they are not always detected in both extracts of that species. For example, a precipitin arc present in *P. elliottii* extract 1 (c. -2.5) appears to be absent from *P. elliottii* extract 2. Similarly, a precipitin arc at c. 10.6 in *P. elliottii* extract 1 appears to be absent from *P. elliottii* extract 2 while two precipitin arcs were detected in *P. elliottii* extract 2 (at c. 6.5) while only one was detected in *P. elliottii* extract 1.

A similar situation is found in the *P. taeda* extracts (Figures 34, 35) where two precipitin arcs present in *P. taeda* extract 1 were apparently absent from *P. taeda* extract 2. No differences were detected between the extracts of *P. monticola* (Figures 36, 37) where the number of precipitin arcs and the patterns were almost identical.

Similar patterns were observed when the extracts were challenged with antiserum prepared against *P. elliottii* extract 1 (Figures 38-45). The patterns and numbers of precipitin arcs for each pair of extracts are similar, for example, in *P. radiata* 511 and 517 (Figures 38 and 39). Individual differences were detected between extracts challenged with this antiserum. While the patterns of precipitin arcs detected in the extracts of *P. taeda* were similar (Figures 42, 43), two extra precipitin arcs were detected in *P. taeda* extract 1. A similar situation was found for the extracts of *P. monticola*, where an extra band was detected in the extract of *P. monticola* extract 2.

The presence of components detected in only one extract of a particular species, but found in extracts of other species, was discussed previously with respect to the analysis of acid phosphatase isozymes and is supported by the results of immunoelectrophoresis. The situation is made more complex in immunoelectrophoresis by the possibility that antigens may be undetected due to large differences in the concentrations of antigens and antibodies. For example, a precipitin arc detected in *P. elliottii* extract 2 (c. 11.0 cm) was not detected in *P. elliottii* extract 1 yet must be present to have produced the respective antibody.

If this antigen was present in large excess over the antibody, the 'precipitin arc' will be pushed into the antiserum trough and thus be undetected. This is unlikely to have occurred in all such cases however, as it would be expected that the tips of at least some of the precipitin arcs would be detected, whereas none were detected in this analysis.

The apparent absence of bands in particular extracts may also be due to very low concentrations of antigens producing precipitin arcs too faint or diffuse to be detected. This is illustrated in the double diffusion results, for example in Figure 24 where the "parent extract" is *P. radiata* 511. Here, sharp precipitin bands are formed against the extracts of *P. elliottii* and *P. taeda* while the equivalent band formed against the extracts of *P. radiata* 522 and 525 can only just be detected. A further possibility for the apparent lack of antigens in the parent extract would occur when the parent extract was homozygous for a particular allele, while the conspecific extracts were heterozygous for the same allele. In this case, the parent extract would have one antigen present while the conspecific extract may have two related (antigenically identical or similar) but electrophoretically different antigens.

The presence of similar proteins with different electrophoretic mobilities was discussed previously in relation to the peroxidase isozyme results. This phenomenon is graphically illustrated in Figure 46 and appears to be widespread. The relative mobility data for the immunoelectrophoresis results (Table 30, 31) can be used to produce electrophoregrams so that direct comparisons of precipitin arc positions can be made, (Figures 48-50).

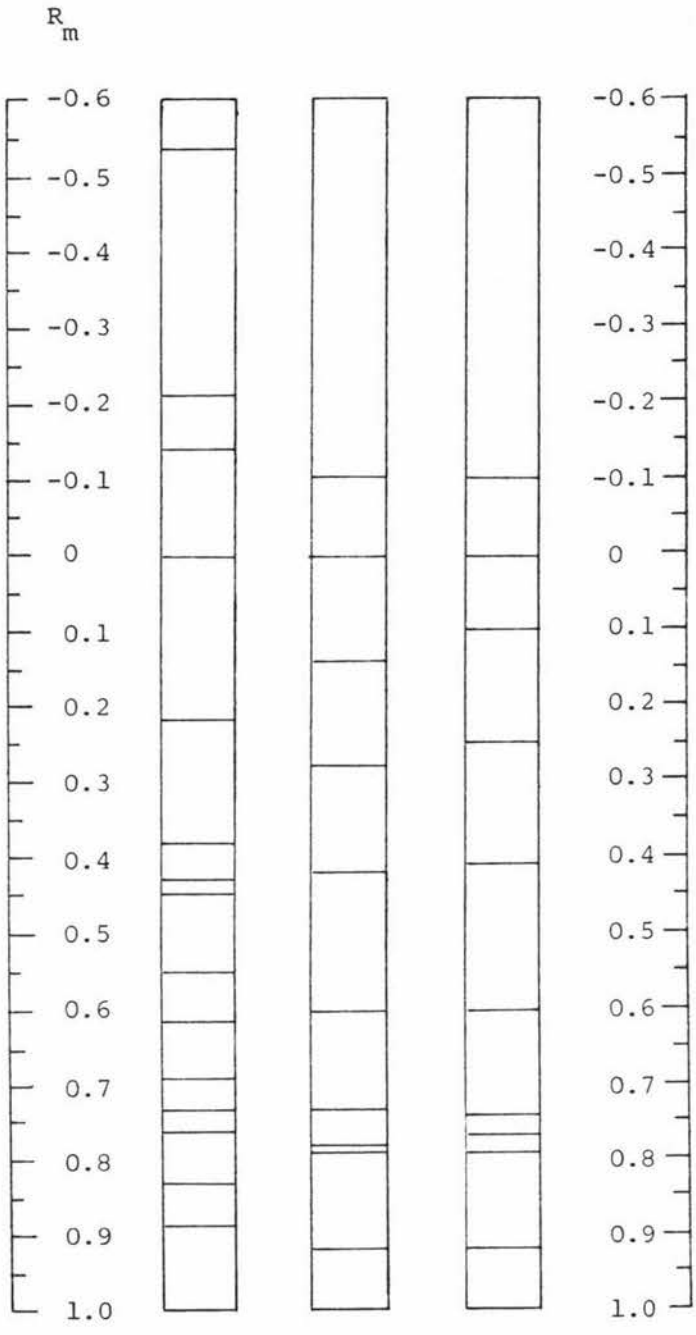


Fig 48 Representation of the relative mobilities of protein bands for extracts of three *P. radiata* clones. Samples were separated by immunoelectrophoresis and challenged with antiserum prepared against *P. radiata* 511.

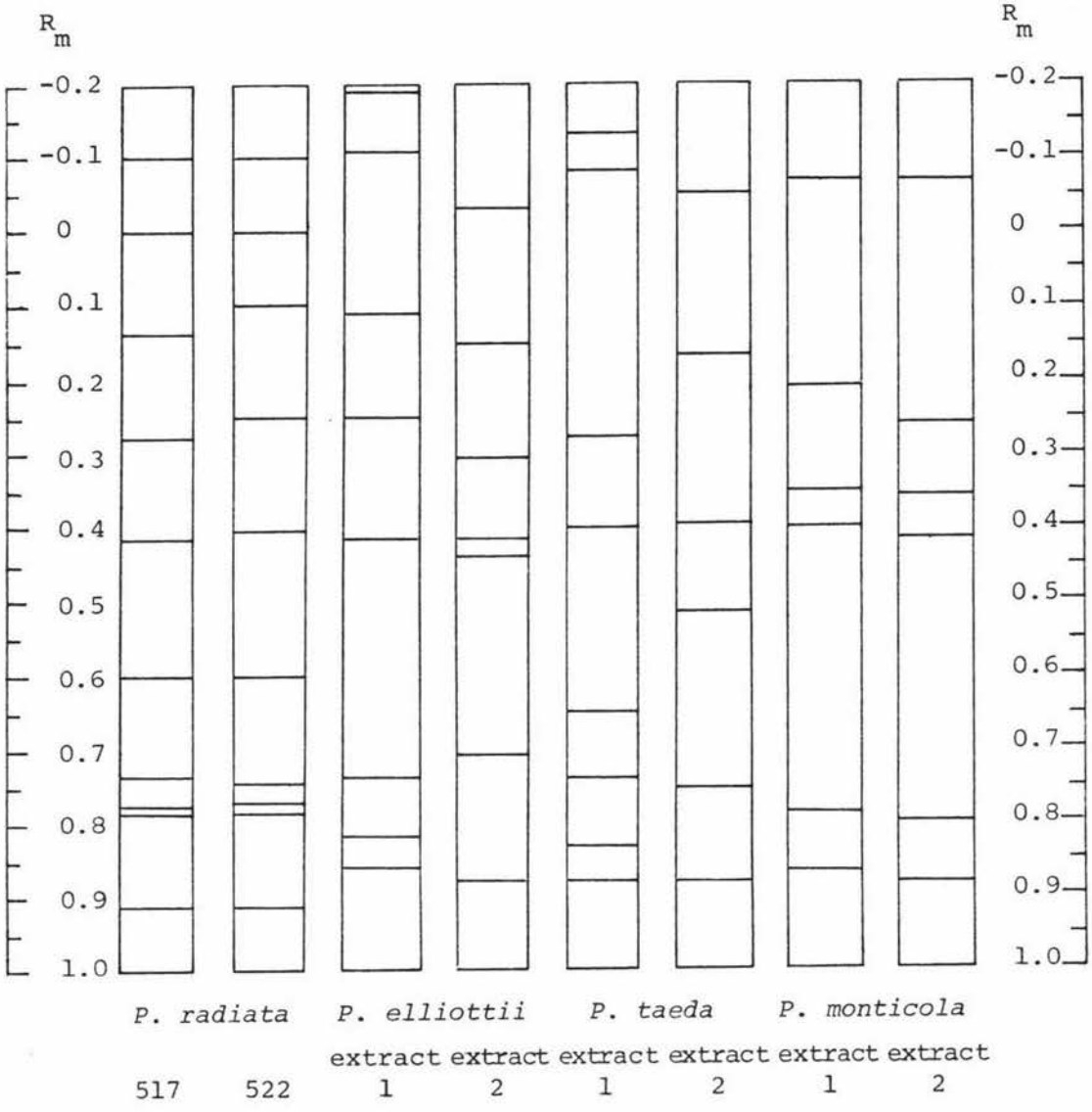


Fig 49 Representation of the relative mobilities of the protein bands for the protein extracts shown when challenged with antisera prepared against *P. radiata* 511. Samples were separated by immunoelectrophoresis.

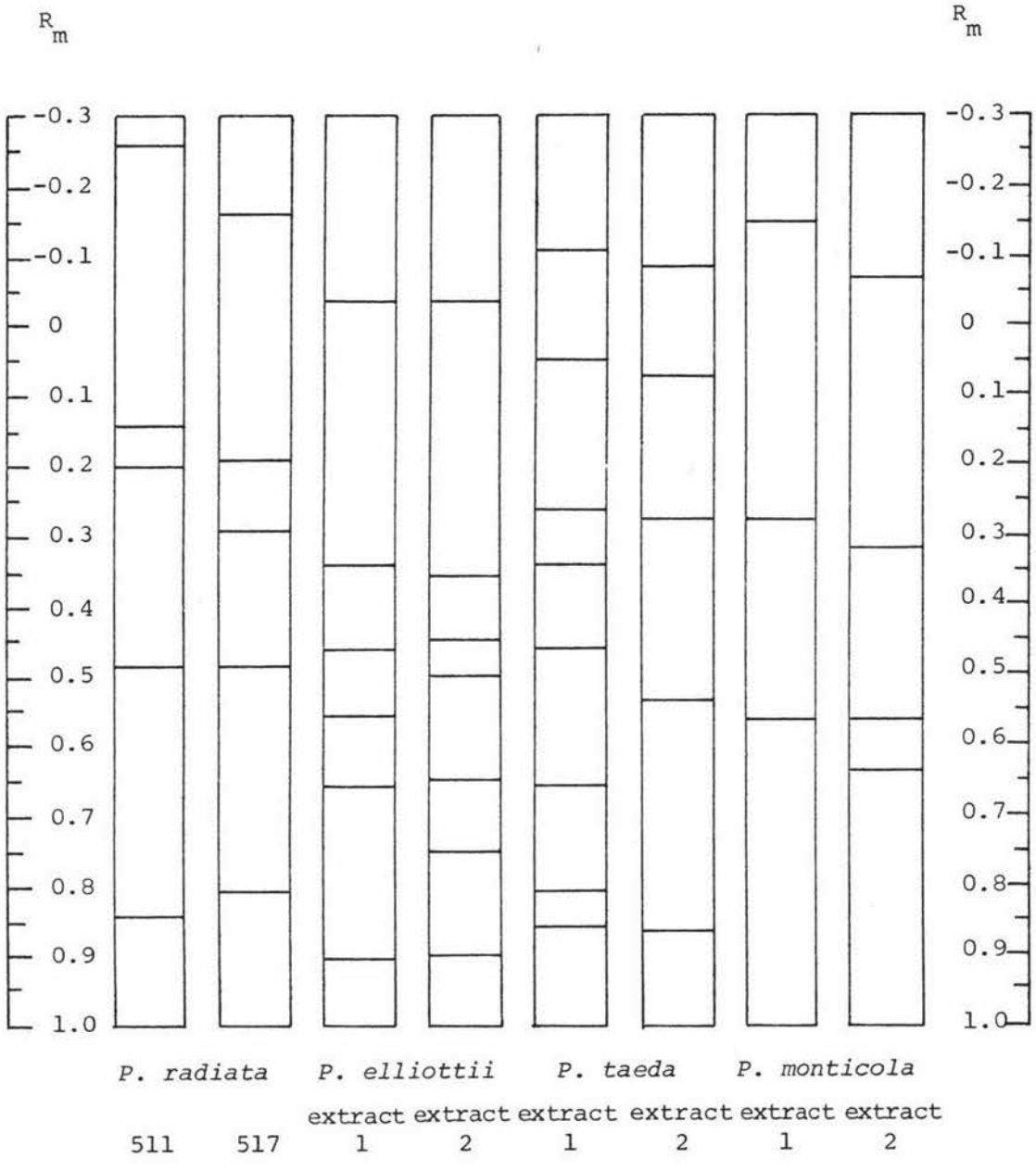


Fig 50 Representation of the relative mobilities of the protein bands for the protein extracts shown when challenged with antisera prepared against *P. elliottii* extract 1. Samples were separated by immunoelectrophoresis.

The antigens responsible for the "bands" in Figures 47 and 48 must be related to antigens present in *P. radiata* 511 but the electrophoregram shows considerable variation in their position. For example, only four "bands" appear to be common (using the electrophoretic "error" of 0.02 Rm units) between the three *P. radiata* clones (Figure 47, Table 30). The errors involved in the technique of immunoelectrophoresis are compounded by the low resolution of the electrophoretic technique and the subjective estimation of the centre of each precipitin arc. However, these errors are not great enough to account for all the variation observed. A similar variation in the positions of the "bands" can be seen in Figure 49.

Only the results from comparisons with a particular antiserum can be compared directly, thus the actual number of precipitin arcs presented on Tables 28 and 29 cannot be directly compared. These data do show, however, a high degree of intraspecific similarity in that the number of precipitin arcs detected in one extract of a particular species is generally similar to the number of precipitin arcs detected in the other extracts when challenged with the same antiserum. Interspecific differences are also apparent, in that a different number of precipitin arcs were detected in extracts of different species when challenged with the same antiserum. For example, five precipitin arcs were detected in both *P. radiata* clones but six and eight precipitin arcs were detected in *P. taeda* extracts when challenged with antiserum prepared against *P. elliottii* extract 1 (Table 29).

The similarity in the number of precipitin arcs detected in extracts of *P. elliottii* and *P. taeda* when challenged with either antiserum suggest that these species have similar antigenic components, that is, are closely related. The low numbers of precipitin arcs detected in extracts of *P. monticola* suggest this species is distantly related to the other species examined.

The detection of antigens common to all species examined and the increased number of bands detected in *P. radiata, P. elliottii,* and *P. taeda,* compared to *P. monticola,* suggests that immunoelectrophoresis may be useful for analysis of the taxonomic relationships of conifers at the generic and subgeneric levels. The specificity of the technique also enables differences between apparently closely related species, and between individuals or clones of a particular species, to be detected. The results presented here support the division of the genus *Pinus* into two subgenera, and also support hypothesis 1 (Figure 1A), that is, the suggested close relationship between *P. elliottii* and *P. taeda*.

Previously, results of electrophoretic analysis of protein and isozyme components of conifer species have, in general, supported the classical taxonomic relationships suggested by morphological analysis. However, the results presented here suggest that the electrophoretic techniques as applied to a wide variety of plant genera and species, may be underestimating the intraspecific variation and overestimating the interspecific relationships.

The results of isoelectric focussing, for example, suggest that the results of electrophoresis in this study have underestimated the interspecific differences between most of the species examined, resulting in higher similarity indices than obtained by isoelectric focussing. In emphasising the differences, however, the results of isoelectric focussing may be of limited use in general taxonomic studies as the technique does not provide information on how different two extract components are. In the extreme case, if the technique was able to distinquish between proteins with single amino acid substitutions, it may be possible to have two closely related species, that is, with all proteins differing by one amino acid, yet have a similarity index of zero. The lower resolution of electrophoresis, on the other hand, would not detect these subtle changes, and would produce a high similarity index.

The serological techniques of spur analysis in immunodiffusion, and immunoelectrophoresis enable an estimation of the degree of the differences between two extracts to be made. If a particular protein is present in two extracts but some amino acid substitutions have occurred, isoelectric focussing may differentiate between them (similarity index = '0') whereas electrophoresis will not detect any difference (similarity index = '100'). The serological techniques, however, may recognise one protein as being the specific antigen, and partially recognise the other protein forming a spur in double diffusion, and two precipitin arcs with slightly different electrophoretic mobilities or positions relative to the trough in immunoelectrophoresis.

Taken together, it is apparent that the various analyses shown here support the division of the genus *Pinus* into the two subgenera Haploxylon and Diploxylon as first suggested by Koehne (1893). Furthermore, evidence was obtained in support of the relationship suggested in the Shaw/Duffield classification as depicted in Figure 1A. Only one analysis (band analysis of double diffusion results) suggested a relationship at variance with that shown in this figure.

The classification of the pines according to Pilger, which excessively relies on the needle number of the short shoot (Duffield 1952) was not supported by evidence obtained from any of the analyses performed here. This classification system, while apparently widely used in Europe, is considered a backward step by Mirov (1967), a view supported by the results presented in this study.

The protein extraction method and results described in this thesis meet the aims outlined in the Introduction. The various high resolution techniques described here have been shown to be useful in analysing the relationships within and between the four species examined. Mirov (1967) suggests that "a species should not be established on the basis of a single character ... (but all) ... feasible appraoches should be used". This study demonstrates the feasibility of protein analysis techniques at all levels of taxonomic study in pines.

APPENDIX I

Computer Program for calculating similarity indices for Sel data or random numbers

```
 10    UTCLEAR
20    DIM X#(30) : R#(30) : Z1#(30) : W1#(25)
30    OPEN "PRT" AS FILE 1 MODE 3
40    PRINT CLEAR
     :PRINT CURSOR(0,23)
50    INPUT "DO YOU WANT TO ENTER YOUR OWN DATA?
          (Y/N)....." , F1$
60    INPUT "DO YOU WANT THE INDIVIDUAL VALUES PRINTED
           OUT?(Y/N)....." , F6$
70    IF F1$ = "Y" THEN GOTO  1370  ELSE GOTO  80
80    INPUT "WHAT IS THE UPPER LIMIT OF THE VALUES
          REQUIRED?....." , A2#
90    PRINT #1 USING "----------------------------------
          ----------------------------------------"
100   PRINT #1 USING "**********************************
          ****************************************"
110   PRINT #1 USING "                    RANDOM NUMBERS
          BETWEEN 0 AND ####       " , A2#
120   INPUT "HOW MANY GROUPS OF VALUES DO YOU
          WANT?....." , A1
130   PRINT #1 USING "NUMBER OF GROUPS =####" , A1
140   INPUT "HOW MANY VALUES IN EACH GROUP OF THE
          FIRST SERIES DO YOU WANT?....." , N2
150   INPUT "HOW MANY VALUES IN GROUP OF THE SECOND
          SERIES DO YOU WANT?....." , N1
160   PRINT
170   PRINT
180   PRINT #1 USING "NUMBER OF VALUES IN EACH GROUP
          OF THE FIRST SERIES = ####" , N2
190   PRINT #1 USING "NUMBER OF VALUES IN EACH GROUP
          OF THE SECOND SERIES = ####" , N1
200   PRINT "Wait - I'm thinking"
210   LET A9 = A1*2
220   IF N2 > N1 THEN LET N3 = N2 ELSE LET N3 = N1
230   DIM B#(N3,A9) : U#(A1)
240   LET N = N2
250   LET B5 = 1
260   LET B6 = 1
270   LET C = A1
280   FOR A = B5 TO C
290     LET B7 = B7+1
300     LET Z# = 0#
310     LET Z2# = 0#
320     RANDOMIZE
```

```
330     FOR I = 1 TO N
340       LET Z# = Z#+1#
350       LET Z1#(I) = Z#
360       LET Z2# = Z2#+Z1#(I)
370       LET X1# = INT(A2#*RND)
380       IF X1# = 0# THEN GOTO  320
390       LET N6 = I-1
400       FOR J = 1 TO N6
410         IF X#(J) = X1# THEN GOTO  320
420       NEXT J
430       LET X#(I) = X1#
440     NEXT I
450     FOR I = 2 TO N
460       IF X#(I) < X#(I-1) THEN GOTO  480
470       GOTO  530
480       LET X1# = X#(I-1)
490       LET X#(I-1) = X#(I)
500       LET X#(I) = X1#
510       LET C1 = 1
520       GOTO  540
530       IF I = N AND C1 = 0 THEN GOTO  570
540     NEXT I
550     LET C1 = 0
560     GOTO  450
570     LET R1# = 0#
580     LET C2# = 1#
590     LET J = 0
600     FOR I = 1 TO N
610       IF I = N THEN GOTO  630
620       IF X#(I) = X#(I+1) THEN LET J = J+1
630       IF X#(I) = X#(I+1) THEN GOTO  650
640       GOTO  680
650       LET C2# = C2#+1#
660       LET R1# = R1#+Z1#(I)
670       GOTO  780
680       LET R#(I) = Z1#(I)
690       IF C2# = 1# THEN GOTO  770
700       LET R1# = (R1#+Z1#(I))/C2#
710       LET L = I-J
720       FOR K = L TO I
730         LET R#(K) = R1#
740       NEXT K
750       LET C2# = 1#
760       LET J = 0
770       LET R1# = 0#
780     NEXT I
790     LET C3 = 0
800     FOR I = 1 TO N
810       IF X#(I) = X#(1) THEN LET C3 = C3+1
820     NEXT I
830     PRINT  USING "DATUM          RANK"
```

```
840       FOR I = 1 TO N
850         PRINT  , X#(I) , I
860         LET B#(I,A) = X#(I)
870       NEXT I
880       PRINT  USING "SERIES  ##,GROUP  ## " , B6 , B7
890       PRINT "Wait - I'm thinking"
900       PRINT
910     NEXT A
920     IF C6# = 1# THEN GOTO  1010
930     LET C6# = 1#
940     PRINT #1
950     LET N = N1
960     LET B5 = A1+1
970     LET B6 = B6+1
980     LET B7 = 0
990     LET C = 2*A1
1000    GOTO  280
1010    IF F6$ = "Y" THEN GOTO  1020  ELSE GOTO  1170
1020    FOR I = 1 TO A1
1030      FOR J = 1 TO N2
1040        PRINT #1 USING "## " , B#(J,I) ;
1050      NEXT J
1060      PRINT #1
1070    NEXT I
1080    PRINT #1
1090    LET K = A1+1
1100    FOR I = K TO A9
1110      FOR J = 1 TO N1
1120        PRINT #1 USING "## " , B#(J,I) ;
1130      NEXT J
1140      PRINT #1
1150    NEXT I
1160    PRINT #1
1170    LET N = N1
1180    PRINT "Wait - I'm thinking"
1190    FOR I = 1 TO A1
1200      LET W# = 0#
1210      LET K = I+A1
1220      FOR J = 1 TO N2
1230        FOR L = 1 TO N1
1240          IF B#(L,K) = 0# THEN GOTO  1300
1250          IF B#(J,I) = B#(L,K) THEN GOTO  1270
1260          GOTO  1300
1270          LET W# = W#+1#
1280          LET B#(L,K) = 0#
1290          GOTO  1630
1300        NEXT L
1310        FOR L = 1 TO N1
1320          IF B#(L,K) = 0# THEN GOTO  1380
1330          IF B#(J,I) = B#(L,K)+1# THEN GOTO  1350
1340          GOTO  1380
1350          LET W# = W#+1#
1360          LET B#(L,K) = 0#
1370          GOTO  1630
```

```
 1380  NEXT L
1390  FOR L = 1 TO N1
1400    IF B#(L,K) = 0# THEN GOTO  1460
1410    IF B#(J,I) = B#(L,K)+2# THEN GOTO  1430
1420    GOTO  1460
1430    LET W# = W#+1#
1440    LET B#(L,K) = 0#
1450    GOTO  1630
1460  NEXT L
1470  FOR L = 1 TO N1
1480    IF B#(L,K) = 0# THEN GOTO  1540
1490    IF B#(J,I) = B#(L,K)-1# THEN GOTO  1510
1500    GOTO  1540
1510    LET W# = W#+1#
1520    LET B#(L,K) = 0#
1530    GOTO  1630
1540  NEXT L
1550  FOR L = 1 TO N1
1560    IF B#(L,K) = 0# THEN GOTO  1620
1570    IF B#(J,I) = B#(L,K)-2# THEN GOTO  1590
1580    GOTO  1620
1590    LET W# = W#+1#
1600    LET B#(L,K) = 0#
1610    GOTO  1630
1620  NEXT L
1630  NEXT J
1640  LET W1#(I) = W#
1650  PRINT #1 USING "NUMBER OF PAIRS OF SIMILAR BANDS
        FOR PAIR ### = ###" , I , W1#(I)
1660  NEXT I
1670  FOR I = 1 TO A1
1680    LET V# = W1#(I)
1690    LET V1# = FLOT(N1)-V#
1700    LET V2# = FLOT(N2)-V#
1710    LET V3# = V1#+V2#
1720    LET V4# = V3#+V#
1730    LET V5# = V#/V4#
1740    LET V6# = V5#*100#
1750    LET U#(I) = V6#
1760  NEXT I
1770  FOR I = 1 TO A1
1780    PRINT #1 USING "SIMILARITY INDEX FOR PAIR
       ## = ###.##" , I , U#(I)
1790  NEXT I
1800  FOR I = 1 TO A1
1810    LET U1# = U1#+U#(I)
1820  NEXT I
1830  IF F1$ = "Y" THEN GOTO  2290
1840  LET U2# = U1#/FLOT(A1)
1850  PRINT #1 USING "AVERAGE S.I. FOR ## PAIRS
       = ###.##" , I , U2#
1860  GOTO  2290
1870  LET A1 = 1
1880  INPUT "ENTER NAME FOR FIRST GEL....." , F7$
```

```
 1890  INPUT "ENTER NAME FOR SECOND GEL......" , F8$
1900  INPUT "HOW MANY BANDS APPEAR IN THE FIRST
        GEL?......" , N2
1910  INPUT "HOW MANY BANDS APPEAR IN THE SECOND
        GEL?......" , N1
1920  PRINT #1
1930  LET A9 = A1*2
1940  IF N2 > N1 THEN LET N3 = N2 ELSE LET N3 = N1
1950  DIM B#(N3,A9) ; U#(A1)
1960  LET K = N2
1970  FOR I = 1 TO 2
1980    PRINT  USING "ENTER DATUM FOR GEL #  AS THE
     QUESTION MARK APPEARS" , I
1990    FOR J = 1 TO K
2000      INPUT B#(J,I)
2010    NEXT J
2020    INPUT " ARE ALL THE VALUES CORRECT?(Y/N)...." , C$
2030    IF C$ = "Y" THEN GOTO  2080
2040    INPUT "ENTER DATUM NUMBER FOR INCORRECT VALUE...." , C1
2050    INPUT "ENTER CORRECT VALUE...." , C2#
2060    LET B#(C1,I) = C2#
2070    GOTO  2020
2080    LET K = N1
2090  NEXT I
2100  LET I = 1
2110  PRINT #1 USING "-----------------------------------
     ----------------------------------"
2120  PRINT #1 USING "**********************************
     ****************************************"
2130  PRINT #1 USING "                    DATA FOR GEL
     <<<<<<<<<<<<                    " , F7$
2140  PRINT #1 USING "NUMBER OF BANDS = ###" , N2
2150  IF F6$ = "N" THEN GOTO  2200
2160  FOR J = 1 TO N2
2170    PRINT #1 USING " ##" , B#(J,I) ;
2180  NEXT J
2190  PRINT #1
2200  LET I = 2
2210  PRINT #1 USING "                    DATA FOR GEL
     <<<<<<<<<<<<<                   " , F8$
2220  PRINT #1 USING "NUMBER OF BANDS = ###" , N1
2230  IF F6$ = "N" THEN GOTO  2280
2240  FOR J = 1 TO N1
2250    PRINT #1 USING " ##" , B#(J,I) ;
2260  NEXT J
2270  PRINT #1
2280  GOTO  1180
2290  END
```

APPENDIX II

Example of the computer printout after calculating the average similarity index for gel data

---

****

DATA FOR GEL RADIATA 525

NUMBER OF BANDS = 22
16 20 25 27 32 34 37 42 45 51 59 62 68 71 74 78 82 85 87 90 95 97

DATA FOR GEL ELLIOTTII 1

NUMBER OF BANDS = 18
14 22 29 36 43 52 57 59 66 69 71 74 76 78 82 87 93 97
NUMBER OF PAIRS OF SIMILAR BANDS FOR PAIR 1 = 15
SIMILARITY INDEX FOR PAIR 1 = 60.00

****

DATA FOR GEL RADIATA 525

NUMBER OF BANDS = 22
16 20 25 27 32 34 37 42 45 51 59 62 68 71 74 78 82 85 87 90 95 97

DATA FOR GEL THEDA 0

NUMBER OF BANDS = 15
10 20 29 41 45 51 55 57 62 65 71 73 83 86 94
NUMBER OF PAIRS OF SIMILAR BANDS FOR PAIR 1 = 12
SIMILARITY INDEX FOR PAIR 1 = 48.00

APPENDIX III

```
**********************************************************************
                RANDOM NUMBERS BETWEEN 0 AND  100
NUMBER OF GROUPS =  10
NUMBER OF VALUES IN EACH GROUP OF THE FIRST SERIES =   18
NUMBER OF VALUES IN EACH GROUP OF THE SECOND SERIES =   15

 9 10 19 20 25 27 30 35 39 42 52 57 64 65 66 78 85 91
13 15 18 36 43 49 51 53 59 63 67 68 71 73 80 84 89 98
 6 11 34 36 40 48 50 53 64 66 68 70 71 76 78 83 84 97
 6 21 24 25 32 38 42 59 60 62 65 70 72 74 85 93 96 98
 2  4  7  8 14 16 21 23 25 46 49 50 61 68 71 81 90 98
 3  5  8 10 18 19 25 29 30 32 37 38 39 56 60 78 86 91
 6 10 11 12 13 26 31 32 41 57 58 67 71 86 87 89 93 94
 1  6 16 26 31 34 39 49 52 61 66 75 78 82 86 93 96 99
 5  9 11 27 28 50 52 55 57 58 70 74 78 83 86 87 96 97
 6 16 18 19 20 21 25 34 36 42 44 48 57 65 67 81 91 97

 3  9 20 21 33 34 50 52 56 63 68 74 82 86 95
 6 13 24 29 39 45 47 50 55 60 69 78 82 84 94
12 13 19 27 32 35 41 42 45 66 70 73 75 91 94
 1  8 11 16 19 30 39 44 48 54 69 71 72 89 94
10 13 17 24 25 29 33 40 45 52 56 67 80 90 97
 6 11 14 15 18 19 22 32 33 35 52 61 66 77 83
10 13 17 22 24 31 37 39 40 42 52 62 63 75 83
 3 10 19 24 28 30 32 67 69 73 74 76 81 91 94
10 19 22 30 31 36 53 54 56 58 64 72 90 93 95
 5 10 16 23 28 29 30 31 34 44 68 77 81 92 95

NUMBER OF PAIRS OF SIMILAR BANDS FOR PAIR   1 =   9
NUMBER OF PAIRS OF SIMILAR BANDS FOR PAIR   2 =   9
NUMBER OF PAIRS OF SIMILAR BANDS FOR PAIR   3 =   8
NUMBER OF PAIRS OF SIMILAR BANDS FOR PAIR   4 =   8
NUMBER OF PAIRS OF SIMILAR BANDS FOR PAIR   5 =  11
NUMBER OF PAIRS OF SIMILAR BANDS FOR PAIR   6 =   9
NUMBER OF PAIRS OF SIMILAR BANDS FOR PAIR   7 =   5
NUMBER OF PAIRS OF SIMILAR BANDS FOR PAIR   8 =  10
NUMBER OF PAIRS OF SIMILAR BANDS FOR PAIR   9 =   8
NUMBER OF PAIRS OF SIMILAR BANDS FOR PAIR  10 =   9
SIMILARITY INDEX FOR PAIR  1 =  37.50
SIMILARITY INDEX FOR PAIR  2 =  37.50
SIMILARITY INDEX FOR PAIR  3 =  32.00
SIMILARITY INDEX FOR PAIR  4 =  32.00
SIMILARITY INDEX FOR PAIR  5 =  50.00
SIMILARITY INDEX FOR PAIR  6 =  37.50
SIMILARITY INDEX FOR PAIR  7 =  17.85
SIMILARITY INDEX FOR PAIR  8 =  43.47
SIMILARITY INDEX FOR PAIR  9 =  32.00
SIMILARITY INDEX FOR PAIR 10 =  37.50
AVERAGE S.I. FOR 10 PAIRS =  35.73
```

APPENDIX IV
Chi-squared analysis on similarity index data

RADIATA/RADIATA

No.	observed values	expected values
1	70.370	41.020
2	70.370	41.020
3	60.710	39.070
4	76.920	41.020
5	66.660	39.070
6	76.920	39.070

CHI SQUARE = 141.55 FOR 5 DEGREES OF FREEDOM

RADIATA/ELLIOTTII

No.	observed values	expected values
1	51.050	42.150
2	66.660	39.070
3	64.000	42.150
4	45.160	39.070
5	46.420	42.150
6	55.170	39.070
7	60.000	38.630
8	62.960	44.120

CHI SQUARE = 60.56 FOR 7 DEGREES OF FREEDOM

RADIATA/TAEDA

No.	observed values	expected values
1	52.000	38.370
2	58.330	38.370
3	58.330	38.370
4	48.000	33.530

CHI SQUARE = 31.78 FOR 3 DEGREES OF FREEDOM

RADIATA/MONTICOLA

No.	observed values	expected values
1	42.300	36.630
2	48.000	36.630
3	54.160	36.630
4	50.000	34.780

CHI SQUARE = 19.45 FOR 3 DEGREES OF FREEDOM

ELLIOTTII/TAEDA

No.	observed values	expected values
1	49.500	35.730
2	67.680	33.590

CHI SQUARE = 39.90 FOR 1 DEGREES OF FREEDOM

ELLIOTTII/MONTICOLA

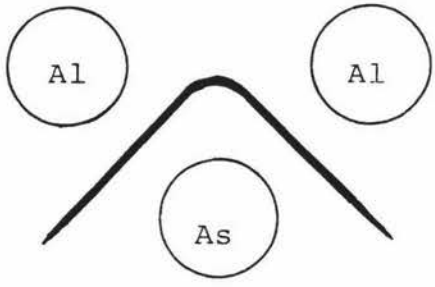
No.	observed values	expected values
1	45.450	36.530
2	50.000	34.780

CHI SQUARE = 8.83 FOR 1 DEGREES OF FREEDOM

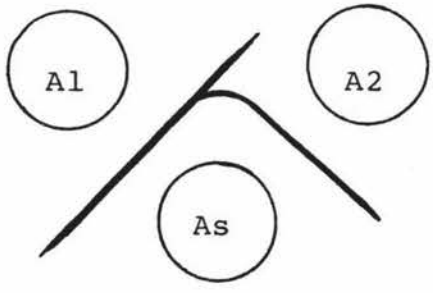
APPENDIX V Identification of Reaction Types in Double Diffusion Analysis

(Adapted from Goodman & Moore, 1971)

Identity reaction: Antiserum (As) is prepared against extract A1. If this extract is compared to an identical extract, an interconnecting precipitin line without any spur will form.



Partial identity (homologous) comparison: In this case, a second extract A2, not identical with, but related to A1, is compared using the same antiserum. A spur will form on the precipitin line facing the 'non-identical' extract.



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