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GENETIC RELATIONSHIPS AND POLLINATION STUDIES IN SWEET CHERRY (*Prunus avium* L)

Andrew Granger

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ABSTRACT

Methods were developed for isozyme analysis of sweet cherry (*Prunus avium*) and the best results were achieved using cellulose acetate as the matrix for electrophoretic separation of isozymes.

Isozyme analysis was carried out on protein extracts from cherry leaves which facilitated the unique identification of 70 cultivars from a collection of 78. Cultivars were compared individually using the number of isozyme differences as a measure of genetic distance. Cultivars were also grouped into country of origin and allele frequencies were used to determine genetic distance between groups. The average number of isozyme differences ranged from 5.97 ± 1.73 for St Margaret to 3.37 ± 1.54 for Merton Glory. This indicates that the sweet cherry cultivars studied were closely related, differing by no more than 2 isozyme genotypes. Allele frequencies of geographically distinct groups produced dissimilarity indexes in the range of $D=0.41$ to 0.54. This means that cultivars developed in different countries have diverged from the last common ancestor to a similar degree. There was only one statistically significant divergence for FDP which had a higher allelic frequency in the Swiss and Australian groups. Selection pressure associated with climate or breeding programmes was thought to be responsible for this result. Overall, cultivars from the U.S.A. showed the greatest divergence and this is probably because Bing dominates in the pedigree of those cultivars. The closest genetic distances were between the Australian and Canadian cultivar groups.

The examination of progeny from controlled hybridisations allowed genetic analysis of data to be carried out and the inheritance patterns of isozymes determined. Linkage between the isozymes and the self-incompatibility locus was also estimated. Glutamate oxaloacetate transaminase (GOT) was tightly linked to the S-locus ($r=0$) and this linkage was reflected in the segregation ratios for GOT. An unexpected result occurred when Stella was selfed, in that all of the progeny showed the bc genotype and there was no segregation for either the bb or cc homozygous genotypes. Stella

is heterozygous for self-fertility and carries the mutant self-fertile allele S_4'. Progeny were expected to segregate as self-fertile heterozygotes S_4S_4' and self-fertile homozygotes S_4'S_4'. The segregation of the closely linked GOT isozyme showed that this was not occurring and only S_4S_4' progeny had been produced. Fluorescent microscopy was used to observe pollen tube growth in hand pollinated cherry styles. After Stella was self-pollinated, inhibition of the S_4 pollen tubes was observed in the first third of the style. Those pollen tubes growing to the base of the style (S_4') were observed to penetrate the micropyle. Knowing that the self-fertile mutation was 'pollen reaction lost' and that the current model for gametophytic self-incompatibility based on work in *Nicotiana* depended on the pollen tube cell wall structure and uptake of S-RNases, it was concluded that S_4' pollen tubes were recognised at fertilisation when they discharged their contents into the ovule. Because there are two ovules in the sweet cherry ovary either an incompatible reaction (S_4'ovule) or compatible (S_4 ovule) mating would occur. Hence only S_4S_4' embryos were formed. The GOT isozyme acted as a marker for the S-alleles with all progeny showing the bc heterozygous genotype.

Isozymes were also used to determine gene flow in cherry orchards and to determine pollen donors of selected cultivars. Regarding the breeding behaviour of Stella, over a three year period 71% of embryos harvested from Stella trees at Lenswood Horticultural Centre were a result of outcrossing and 29% were generated by selfing. Sixty per cent of the outcrossing occurred with the nearest neighbouring trees flowering at the same time as Stella, in this study that was Venus, but all other sweet cherry cultivars are compatible with Stella and will cross pollinate if flowering times overlap. Interaction between honeybees (*Apis melifera*) and orchard design was thought to be having the greatest effect on gene flow in cherry orchards. A range of commercial recommendations for pollination in cherry orchards was derived from this work.

STATEMENT

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in text.

I give consent to this copy of my thesis, when deposited in the university library, being available for loan and photocopying.

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DATE. 5/10/95

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INTRODUCTION

Confused Varietal Identification

The names of many fruit tree varieties are surrounded by confusion,as exemplified by persimmons (Tao and Sugiura, 1987), apple (Bournival and Korban, 1987) and apple rootstocks (Menendez et al, 1986), Cherimoya (Ellstrad and Lee, 1987), peach (Carter and Brock, 1980) and probably none more so than sweet cherry (Bowman, 1937; Crane and Brown, 1937).

In most cases morphological characteristics such as mature tree habit, leaf shape and blossom characteristics are used to identify and differentiate between varieties. Observation of fruit characteristics is usually necessary for definite identification and in cherry fruiting may not occur for up to 7 years. These types of characteristics usually show continuous variability and only extremes are useful for identification purposes. Moreover, these characteristics are influenced by prevailing environmental conditions and, in general, this restricts their application to comparison between trees planted in close proximity.

Isozymes

Molecular and chemical basis of isozymes

Isozymes are multiple forms of proteins sharing the same enzymic function. Their primary structure consists of chains of amino acids and in some cases other molecules or cofactors may bind with them. The sequence of amino acids in the polypeptide chains is determined by the nucleotide base sequence comprising the deoxyribose nucleic acid (DNA) of the gene. The segment of DNA comprising each gene is made up of different functional sections. A series of exons and introns form the bulk of the gene. Transfer or transcript ribonucleic acid (RNA)

produces a copy of the DNA, the RNA undergoes a process of excision of the introns, the remaining exon fragments combine to produce messenger RNA (mRNA). The mRNA is transported out of the nucleus into the cytoplasm. The mRNA is translated by ribosomes into polypeptide chains. Each base triplet codes for a particular amino acid in the polypeptide chain. In this way the primary structure of a protein is formed. The covalently bound amino acids tend to spiral and produce a secondary structure. Eventually the long spirals may flop over to produce a globular tertiary structure.

Post-translational changes

On completion of the transcription process, enzymes may react with other chemicals in the cytoplasm. They may combine with other proteins. In some cases this must occur before an enzyme becomes functional (quaternary structure). Carbohydrates, nucleotides, peptides or coenzymes may also bind with the tertiary proteins. Subunits may bind or be lost through enzymic reactions or small molecules may bond with a polypeptide chain. The importance of these reactions in respect to electrophoresis is the effect they can have on the net charge of an enzyme and subsequently its relative mobility.

Expression of the genetic code in isozyme variants

A substitution or mutation of the nucleotide sequence on the DNA leading to a change in a single base of an mRNA triplet may not result in a corresponding change in the net charge of the translated enzyme. The altered nucleotide triplet may code for the same amino acid or an amino acid with the same net charge. Enzymes with similar charge cannot be differentiated on cellogel which has a large pore size and doesn't provide molecular sieving effects.

Alternatively the mutation or substitution may produce an amino acid which is ionically neutral or possesses an opposite charge to the one replaced. In both cases the net charge of the protein will be altered and detectable as separate bands on cellogel.

As mentioned previously mRNA is not complimentary to all parts of the DNA. Thus, isozymes translated from mRNA do not express the total genetic code. Consequently total genetic polymorphism can not be detected using isozyme analysis. However, this is actually an advantage because it reduces the amount of DNA being dealt with and results in more readily interpreted banding patterns.

Genotypes

Individual enzymes will migrate in an electric field under given pH conditions on cellulose acetate gel according to their net charge. As discussed above this is determined by the DNA sequence of genes involved and reactions occurring after transcription. Different molecular forms of the same enzyme are sometimes expressed as different bands or electromorphs. These differences are attributable to gene differences or differences between alleles at the same gene locus. In most studies polymorphism has been explained by multiple alleles and isozymes often referred to as allozymes. Typical banding patterns for heterozygotes express both alleles, indicating inheritance is on a codominant basis. To be fully confident of genotype assignment, parental and sibling zymograms must be compared to determine the basis of inheritance. Otherwise comparison to other closely related species can provide good support to the interpretation of genotypes. The underlying assumption in the study of any plant or animal population, with isozyme analysis is that it is in Hardy-Weinberg equilibrium and the laws of Mendelian inheritance are applicable.

Polyploidy or the multiplication of the basic chromosome number is a frequent occurrence in plant species. In *Prunus* species the basic chromosome number is 8, diploid tissue chromosome numbers vary from 8-176. In cherry 16 chromosomes appear in diploid tissue (Westwood, 1986). Thus polyploidy does not need consideration when interpreting electropheretic results from sweet cherry tissue.

Essentially isozyme electrophoresis is a comparative procedure. So if both homozygous and heterozygous forms are present and readily identifiable, genotypes can be assigned with some degree of confidence.

The form of banding patterns for a particular isozyme encoded by a single gene locus depends on the number of polypeptide chains in its structure, and the number of alleles at that locus. In diploid organisms only two alleles can be expressed simultaneously for a particular locus. Shannon (1968) provides a table which gives the number of isozymes produced by a certain number of similar or different subunits (polypeptide chains) assuming random association. A dimer (enzyme of two subunits) with only one type of subunit produces one isozyme while a dimer with two different subunits will generate 3 isozymes. A tetramer (enzyme of 4 subunits) with 2 different subunits will result in the expression of 5 isozymes. The different subunits are usually coded for by different alleles at a given locus and visualised as separate bands after electrophoresis. Only heterozygotes display all bands simultaneously. Differentiation between the effects of multiple alleles and multiple genes is difficult.

If isozymes are under the control of multiple genes the variation at different zones of banding is independent between zones. Whereas isozymes produced by alleles at a single locus show correlated variation between different zones of activity. In more practical terms the distance between two zones of activity is used to differentiate between loci. Richardson et al (1986) imply that a distance of 0.5cm or greater between zones of banding supports the assumption of separate loci.

Environmental effects could conceivably have an effect on polymorphism shown at isozyme loci. There are now many papers concerning isozyme analysis in plants and many of the isozymes affected by environmental influences have been identified (eg esterases). More importantly there are a range of isozymes which consistently prove to be useful and stable under a range of environments. In general these isozymes are involved in vital biological processes such as

glycolysis, the pentose cycle and citric acid cycle. Genes coding for isozymes involved in these biochemical pathways are active under most environmental conditions.

Current literature also characterises isozymes on the basis of number of subunits ie. monomer, dimer, trimer or tetramer and the tissue types carrying the isozyme. Kephart (1990) provides a summary of plant isozyme structures, tissue locality, and other characteristics.

With all the background information in place the aim is to describe observed isozyme polymorphism in terms of number of loci and alleles and then assign an appropriate genotype.

Relative mobility

Aruleskar and Parfitt (1986) refer to Rf values of different isozyme bands on starch gels and define them as the distance travelled by a band divided by the distance travelled by a bromophenol blue dye front. It is equally valid to call the most distant migrating band of a particular isozyme Rf or Rm 1 and divide the distance travelled by other bands accordingly. Alternatively, the most common band is assigned a mobility value of 100 and other bands a value in relation to their migratory distance be it greater or less than the reference band. (Richardson et al 1986).

Distance travelled by bands may vary between runs. Although in this study, using cellogel under standardised conditions, migration distances were found to be constant between gel runs. In this study the distance of bands from the origin was used as a direct measure of mobility.

Band Intensity

Some workers use band intensity to determine whether a single or double dose of an allele is present (Kephart, 1990). Differences in band intensity were not used to interpret genotypes here because the amount of protein extracted can vary between samples and extraction times and result in varying degrees of band intensity.

Isozyme Analysis

Isoenzyme analysis is the combination of the two techniques, electrophoresis and enzyme activity staining. The process of electrophoresis takes advantage of the existence of multiple forms of molecules having the same enzymic function. The multiple forms consist of small molecular differences resulting in slightly different net charges. To separate the isoenzymes electrophoresis is used. It can be described as a process of forced diffusion within an electrical field (Pierce and Brewbaker, 1973) a gel matrix is attached at either end to an electrode, one positive (anode) and the other negative (cathode), a source of power passes an electrical current through the gel. For a given pH isoenzymes assume a certain charge and this determines their direction and rate of migration within an electric field.

Following electrophoresis, the second process takes place; enzyme activity staining is applied to gels to reveal discrete bands of enzyme activity by chemical or physical means (Vallejos, 1983). Differences in band migration can be assigned different genotypes and varieties distinguished on this basis. The isozyme constitution of a plant is little affected by its external environment. However, they may be synthesised or depressed in the presence of insect or disease attack, be specific to certain tissues or change with developmental stage.

Given this, they still offer the most reliable single gene markers (Arulsekar and Parfitt, 1986) and often show close to expected Mendelian segregation ratios (Pierce and Brewbaker, 1973). Previously it was envisaged that isozymes alone could be used to provide 'fingerprints' of different tree crops. This would be useful for differentiating between clonally propagated trees particularly those protected by Plant Breeders Rights.

Studies involving isozyme analysis of fruit trees

Peach (*Prunus persica* (L) Batsch)

Contrary to expectations of isozyme analysis, being able to facilitate cultivar analysis Messequer, Arus and Carrera (1987) studying isozymes in peach pollen concluded that isoenzymes were only

effective for peach cultivar identification when employed as a complement to other characteristics. This they attribute to the efficiency of the method relying on the variability of the isoenzyme phenotypes. This philosophy is supported by Arulsekar et al (1986) who compared the isoenzyme variability in peach and almond cultivars of the United States. They found almond varieties had much more variability at the isozyme loci studied as compared to peach varieties and attributed this to the outcrossing nature of almonds. Never-the-less differences in banding patterns among cultivars were observed for 4 enzymes, namely esterase, isocitrate dehydrogenase, acid phosphatase and malate dehydrogenase, and used to classify 13 of 81 cultivars collected. Ten cultivars were uniquely classified based on morphological characters alone. Combination of the two classification systems enabled 48 of the 81 cultivars to be individually distinguished.

They concluded that the general lack of variability in peach cultivars for the enzyme systems studied was due to the narrow genetic base of domesticated peaches caused by the self pollinating characteristic of peaches.

Carter and Brock (1980) were able to distinguish five peach cultivars by isoelectric focusing of protein from woody tissue of peach, each cultivar having a unique protein banding pattern. This result seemed to contradict the idea that inbreeding in the peach has led to limited polymorphism. However, it should be noted that the staining procedures used revealed all major proteins. That is, this procedure would reveal the majority of isozymes residing in the tissue and with the use of ampholines to produce a pH gradient, the separation of isozymes on the basis of molecular weight would be much more accurate and sensitive. Thus small changes in molecular structure, such as a single molecule substitution, which cause changes in molecular weight would be much more readily resolved. Further, a general protein stain would allow the examination of many proteins simultaneously further increasing the probability of detecting polymorphic phenotypes.

Starch gel electrophoretic analysis of isozymes has been used to positively identify interspecific hybrids (Parfitt et al 1985 and Chaparro et al, 1987). Parfitt et al (1985) demonstrated that

specific combinations of peach and plum genotypes produce unique patterns of enzyme banding for glucose phosphate isomerase and phosphoglucomutase in leaves. In a similar study, Chaparro et al (1987) used starch gel electrophoresis of two enzyme systems, phosphoglucomutase (PGM) and 6-phosphogluconate dehydrogenase (6-PGD), to confirm peach x almond hybrids. In fact, 6-PGD used alone could identify all of the hybrids. These two studies show that increased variability in isoenzyme phenotypes in peach brought about by hybridisation with plum or almond, leads to greater efficacy in the method of isozyme analysis for cultivar identification.

Persimmon (*Diospyros kaki* L.)

Leaf isozymes and morphological characters have also been used to discriminate between Japanese Persimmon cultivars (Tao and Sugiura 1987). Two enzyme systems were assayed using starch gel electrophoresis, glucose phosphate isomerase (GPI) and malate dehydrogenase (MDH). Although intracultivar polymorphism was absent for both enzymes GPI showed a variety of banding patterns between cultivars. Six cultivars were identified by unique GPI banding patterns. The MDH system showed less variation and none of the varieties could be uniquely classified. However, when both enzyme systems were combined a further 12 cultivars could be uniquely identified. Identification of all cultivars was facilitated by including fruit type (astringent or non-astringent) and pollination characteristic (constant or variable) in the classification categories.

Tao and Sugiura concluded that isoenzyme analysis would not be useful in distinguishing mutations from a common original cultivar. Similar or identical persimmon cultivars with different names showed identical isozyme phenotypes, indicating they had the same origins.

They indicated a need for experimentation with more enzyme systems to enable more cultivars to be identified and genetically characterised.

Apple (*Malus domestica* Borkh)

Using four polymorphic systems of the apple phosphoglucose isomerase, phosphoglucomutase, glutamate oxaloacetate transaminase and peroxidase, the cultivars Jonafree, Redfree, Delicious, Jonathan, Golden Delicious, Ben Davis, Turley, and McIntosh could all be differentiated from one another (Bournival and Korban, 1987). In addition, these workers used starch gel analysis of isozymes from seedlings with parents homozygous for a separate allele to determine the sub-unit structure of isozymes. A two banded pattern indicated that the enzyme was functional as a monomer, 3 bands implied a dimeric enzyme, and 5 bands a tetramer.

In another study, fifty-four apple cultivars were characterised using starch gel electrophoresis of six isozyme systems (Weeden and Lamb 1985). The two most useful isozyme systems for cultivar identification were 6 phosphogluconate dehydrogenase and aspartate aminotransferase. There was no polymorphism in any of the isozyme systems examined within clones of a cultivar.

Cherry (*Prunus avium* L.)

Arulsekar and Parfitt (1986) used starch gel electrophoresis to determine the banding patterns of 13 different isozyme systems in cherry. However, this information was not used to distinguish between cultivars. In France, Santi and Lemoine (1990) sampled dormant buds of wild cherry (*Prunus avium*) seedlings and polyacrylamide gel electrophoresis to analyse amylase, glutamate oxaloacetate transaminase and malic enzyme. Isoelectric focussing was used for acid phosphatase, isocitrate dehydrogenase, leucine amino pepsidase, malate dehydrogenase and thiol oxidase. Forty-one loci were distinguished and 13 displayed polymorphism. Segregation analyses revealed that leucine amino pepsidase was linked to glutamate oxaloacetate transaminase and malic enzyme.

Self-incompatibility

Self-incompatibility is the failure of the gametes to function normally in the pollination and fertilisation process. It causes self-sterility because of the failure of gametes from the same plant to combine and form a viable embryo. Crane and Brown (1937) first described the self-

incompatibility reaction in sweet cherry and generated the 'oppositional factor hypothesis' and assigned the controlling gene the S genotype.

In cherry pollen carrying an S-allele in common with one of those in the style initially alights and germinates on the stigma producing a pollen tube which is recognised as being the same and its growth is halted in the first third of the style. This is referred to as gametophytic self-incompatibility. In contrast, sporophytic incompatibility is caused by the diploid stigmatic tissue. Self-fertile cherry cultivars have been produced using gamma irradiation to induce a mutation at the S-locus whereby the pollen reaction was thought to be lost.

Later Lewis developed a theory of the basis of this mechanism which is outlined by de Nettancourt (1977). A Polypeptide is coded for in pollen and styles by a specificity segment of each S-allele. Every different S-allele produces a different polypeptide. After self-pollination, polypeptides on the surface of the pollen tube dimerise with identical polypeptides in the style to form a dimer repressor. In this model the dimer repressor switches off one or several loci responsible for pollen metabolism and pollen tube growth. Similarly, Pandey (1967) showed that each S-allele in *Nicotiana* (another species displaying gametophytic incompatibility) had a specific peroxidase band, regardless of whether or not plants were pure species or of hybrid origin. Pandey concluded that peroxidase isozymes determine S gene specificity and the basis of allelism for the S gene lay in the particular combinations of the peroxidase isozymes.

Like cherry, most apple cultivars are also self-incompatible, and under similar genetic control in the form of a multi-allelic S-gene. Manganaris and Alston (1987) found glutamate oxaloacetate and isocitrate dehydrogenase to be closely linked to the S-incompatibility locus using starch and polyacrylamide gel electrophoresis. Both isozymes were established as markers for the S locus and the combined incompatibility/isozyme genotypes of four cultivars were proposed. These results and those of Pandey (1967) implicate proteins and, in particular, enzymes as a functional part of the incompatibility reaction.

Mau et al (1982) extracted glycoproteins from the styles of Lambert sweet cherries and determined molecular weights using polyacrylamide gel electrophoresis. Two low molecular weight glycoproteins were called antigen P and antigen S and were associated with the S-gene. Kamboj and Jackson (1986) established that glycoproteins found in the pistils of *Petunia hybrida* were under the control of S-alleles. Later, McClure et al (1990) and Gray et al (1991) working with *N. alata* generated a model for the self-incompatibility reaction whereby ribonucleases, as specified by the S-alleles, are secreted into the transmitting tissue of the style. When a pollen tube is recognised as carrying the same S-allele as one of those in the style an incompatibility reaction takes place, S-RNase is taken up by the pollen tube where RNA translation, and in turn, protein synthesis is inhibited resulting in the cessation of pollen tube growth. The mechanism for recognition of the same genotype is unknown although it is thought to reside in the structure of the pollen tube cell wall and its differential uptake of the different S-RNase molecules.

Objectives of this work

1. To determine extraction, electrophoretic and isozyme procedures suitable for use with sweet cherry.
2. To determine isozyme genotypes for cultivars of sweet cherries held in South Australia and to use isozyme profiles to differentiate between cultivars.
3. To establish the linkage relationships between different isozymes found in sweet cherry and also between isozymes and the S-incompatibility gene in sweet cherry.
4. To examine the recognition of pollen tubes in the pistil of sweet cherry as part of the self-incompatibility reaction, to determine where and how the process is occurring.
5. To characterise gene flow by pollen in cherry orchards and derive recommendations for commercial planting plans and use of honeybees.

CHAPTER 1. DEVELOPMENT OF SAMPLING AND EXTRACTION METHODS

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1.1 Development of Sampling and Extraction Methods for Sweet Cherry Leaf Tissue for Starch and Polyacrylamide Gels

According to Shannon (1968), early evidence for isozymes or different forms of proteins with the same enzyme specificity was reported in 1952. Electrophoresis, described as a process of forced diffusion within an electric field (Pierce and Brewbaker 1973), is considered the most powerful technique for the separation of isozymes. Smithies (1955) first described starch gel electrophoresis while Chrambach and Rodbard (1971) describe polyacrylamide gel electrophoresis in detail. Both methods have been used with many plant and animal species. Few reports exist for isozymes in cherries so it was necessary to determine electrophoretic procedures best suited to use with cherries.

A high phenolic to protein ratio is often found in perennial angiosperms. (Kelley and Adams 1977). Protein extraction from plants usually involves cellular destruction. Phenols are liberated at this stage and oxidise to form quinones which inactivate enzymes (Torres 1984). They also tend to polymerise, and condense readily with reactive groups of protein. This is the basis of browning reactions in plant tissues and extracts (Loomis 1974). Thus, it was also important to determine the most suitable method of protein extraction for sweet cherry.

1.2 Materials and Methods

1.2.1 Extraction methodology for starch gels

Direct Physical Extraction

Fully expanded leaves were picked from various varieties of cherry trees and handled in either of 3 ways;

(i) Snap frozen in the field using liquid nitrogen, stored at -20°C until used, usually a maximum of 5-7 days after sampling.

(ii) Transported from the field on ice in plastic bags, placed in -20°C storage overnight, extracted the following day.

(iii) Leaves transported from the field in plastic bags on ice and used immediately.

Leaves were folded around a 5 x 7 mm wick of Whatman No. 3 filter paper. Pressure was applied with pliers to the folded leaf at the point of contact with the filter paper, the extraction was complete when the wick was saturated with green extract. Aluminium foil was used to cover the jaws of the pliers and changed between samples to prevent contamination.

Homogenisation

The first method used involved field sampling of cherry leaves and placement in liquid nitrogen. Samples were transported from the field in liquid nitrogen and placed in frozen storage; -20°C for 5-6 days. Extracts were ground in liquid nitrogen and 15% PVP. Two extraction buffers Tris-Citrate (Aruleskar and Parfitt, 1986) and Tris-HCl pH 7.45 were used. Tris-citrate extracts were frozen at -20°C, Tris-HCl pH 7.45 extracts were centrifuged at 5000 rpm for 20 minutes, and the supernatant pipetted off and frozen at -20°C. After electrophoresis gels were stained for ACP and EST.

As an alternative, instead of placing field sampled leaves in liquid nitrogen they were kept cool on ice for transport and extracted on the same day. Three tenths gram of a leaf sample was placed in a pre-cooled mortar and covered with liquid nitrogen then ground into a fine powder. Seven millilitres of 0.01M Tris-HCl pH 7.45 buffer and 0.3g of Polyvinylpolypyrillodone were added. The frozen mix was allowed to thaw, then decanted into centrifuge tubes and spun at 4000 rpm for 30 minutes. Supernatant was poured off and stored at -20°C. Prior to use the supernatant was thawed and paper wicks dipped into each sample then shaken to remove excess supernatant and loaded into the gel. A variation to this was used later whereby 20% glycerol, 20 mM Beta-mercaptoethanol (B-ME) and 0.5% Triton-X-100 were added to the extraction buffer as well as 0.3g PVPP. B-ME and Triton-X-100 were added immediately before use and centrifugation was

shortened to 60 seconds. Alternatively, extracts were allowed to stand in ice for 1-2 hours. After this time solids had settled and a clear extract remained above this. Filter paper wicks were dipped into the extract for the gel run. Supernatant from centrifugation was also stored at -20°C for later use.

Frozen homogenised samples were compared to pliers extracted samples.

Comparison between three different extraction methods was also made. Citrus and cherry leaves were sampled, held on ice and treated in the following ways :-

(i) Leaves ground in Tris-HCl pH 6.85 with mortar and pestle, extract absorbed onto wicks through tissue paper.

(ii) Leaves ground in 0.16M potassium phosphate, pH 7.5 buffer (Torres, 1984) with mortar and pestle absorbed extract onto wicks through tissue paper.

(iii) Leaves squashed onto wicks with pliers.

All extractions were made at 4°C. and gels were stained for AAT and ACP.

1.2.2 Electrophoretic Procedure for Starch Gels

Starch gel preparation

Hydrolysed potato starch from Sigma Chemical Co. was suspended in buffer to make a 12% solution. The mixture was constantly swirled in a vacuum flask while heating over a bunsen burner. This was continued until the mixture thickened, at this point boiling was evident. A vacuum was applied immediately and degassing carried out until all air was removed, usually about 30 seconds. The starch solution was then poured onto a glass plate boarded by 6mm thick x 10mm wide perspex held in place with vacuum grease and bulldog paper clips. The surface area being 17cm x 20cm. The gel was allowed to cool overnight at room temperature.

The next morning the top cover plate was carefully pried off and the gel covered in plastic wrap, to prevent drying, and refrigerated until required for loading (1-2 hours).

Buffers

The buffer system consisted of a gel buffer used in preparing the gel and an electrode buffer used to conduct current to the gel via buffer soaked sponges. A discontinuous system, which involves using buffers of different chemical composition for gel and electrode was used because this tends to compact bands and sharpen resolution (Kephart, 1990). Buffers used varied depending on the isoenzymes under study (Appendix I).

Run Conditions

Gels were prepared for loading by cutting a line, parallel to its width, about 4cm from the bottom edge. The gel was separated at the cut. Each wick was then soaked in a single sample, excess sample blotted off and the wick placed on the cut surface. When all wicks were loaded 2-3mm apart the gel pieces were pushed back together and a glass rod inserted between the gel and the edge of the mould to ensure good contact at the origin during the run.

'Wettex' sponges were used to carry electrode buffer and charge from each electrode well to the gel.

Wicks were removed from the gel after 30 minutes of sample elution. They were cut to a length which protruded above the gel to aid in easy removal. Loading was carried out at 2-4°C in a walk-in coolroom. Gels were run at either constant voltage or constant amperage, depending on time constraints. A constant 250 volts was used with a usual runtime of 4 hours, and sometimes as long as 7 hours. Constant currents ranging from 20-32mA were used with a corresponding range of run times depending on buffer systems used. For example, proteins on a starch gel consisting of electrode buffer:water in a 1:10 ratio and run with 0.1M tris, 0.1M malate, 0.01M Na_2EDTA, 0.01M $MgCl_2$ pH 7.4 electrode buffer migrated 10cm after six hours at constant current of 32mA.

In comparison proteins on a starch gel made using electrode buffer:water pH 7.2 (1:28), and run with 0.228M tris, 0.069M citric acid pH 7.2 covered the same distance in 17.5 hours. Run times were determined empirically by observing the migration of the brown front created by borate ions contained in certain buffers or by including 1ml of a 0.1% bromophenol blue solution in the starch gel mix or on a single wick as a tracking dye where buffers used did not contain borate ions.

1.2.3 Staining starch gels

In most cases one gel was stained for one enzyme. Sometimes, however, the gel was sliced in 2 or 3 segments to allow 2-3 enzymes to be studied. Gels were sliced using monofilament line and strips of perspex about 1.5mm thick. The cut surface of the gel was placed face up in the staining tray. Staining was carried out using enzyme activity staining. In this way the formation of discreet bands of specific electrophoresed enzymes could be detected by the addition of substrate alone or with the addition of reagents and other enzymes which react or complex with one of the products to form a chromatic precipitate, observed by the naked eye as a coloured band. This is known as positive staining. Negative staining involves inhibiting or destruction of the chromatic precipitate and staining the remainder of the gel, areas of enzyme activity being indicated by the absence of staining. (Vallejos, 1983).

Each enzyme system has a specific stain recipe to locate the relative position of each isozyme (Appendix II).

In general 100ml of stain solution was prepared and the gel or gel slice immersed in it and incubated in the dark at 37°C. All stain solutions were made immediately before staining and covered with aluminium foil to prevent the entry of light, because some of the reagents were light sensitive.

1.2.4 Extraction methodology for polyacrylamide gel electrophoresis(PAGE) and isoelectric focusing(IEF)

Pistils from flowers of the cultivars Williams Favourite, Clements Pride, Bedford Prolific, Black Douglas, and Stella were removed from flowers at the balloon stage (just prior to opening) to avoid contamination. Williams Favourite and Stella flowers were collected and snap frozen in liquid nitrogen. All the other cultivars were collected and held in an insulated container, after which they were stored at -20°C. Samples were removed from storage, placed on ice to thaw, then ground in liquid nitrogen (unless previously treated) with 10ml Tris-HCl pH 7.5 (in the ratio 100mg tissue:1ml buffer). To 200ul of crude homogenate was added 200ul Sodium dodecyl Sulphate buffer, which had the following components; 0.125M Tris, 20% Glycerol and 0.1% SDS, 10% mercaptoethanol and 0.01% bromophenol blue. The mixture was heated for 3 minutes in a boiling water bath, cooled on ice and centrifuged. Ten and 25ul aliquots of supernatant were loaded onto a 12% polyacrylamide gel. (Kamboj and Jackson, 1986).

For Isoelectric focusing (IEF) the remainder of the crude homogenates from the SDS-PAGE had been stored at -15°C. These were thawed and 0.5ml aliquots taken. To each was added 0.3g Urea, 10ul Triton X-100, 25ul ampholines and 25ul β-mercaptoethanol.

Samples were mixed and centrifuged at 4000 x g for 10 minutes. Supernatants were then used for the IEF gel.

1.2.5 Electrophoresis and staining for SDS-PAGE and IEF

The SDS-PAGE running gel consisted of 12% acrylamide, 0.3% bisacrylamide, 0.75M Tris pH 8.8 and ammonium persulphate. This solution was degassed and 10% SDS and 50ul of N,N,N,N-tetramethylethylene diamine (TEMED) added. A stacking gel was used and consisted of 4.5% acrylamide, 0.12% bisacrylamide, 0.25M Tris pH 6.8, $(NH_4)_2S_2O_8$ 0.1% SDS and 0.1% TEMED. Vertical gel supports were used and the running buffer was 0.025M Tris, 0.19M Glycine and 0.1% SDS. Electrophoresis was conducted at 4°C, and 250V variable current, until the

bromophenol blue front passed the boundary between the stacking and running gels. At which time the voltage was increased to 350V until the end of the gel was reached by the blue front.

IEF was conducted using methods as described by Kamboj and Jackson (1986), gels contained 4.5% acrylamide, 8.4M Urea and ampholines (pH range 3.5 to 10.0). The gel was cooled to about 6°C before the run, and a set voltage of 1000 and current of 20 watts applied for 1 hour before sample loading. After sample loading the same voltage and current were applied for 2 hours.

After electrophoresis gels were placed into a staining solution: 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories), 45% ethanol and 10% acetic acid. Staining was carried out followed by destaining with a solution of 45% ethanol and 10% acetic acid.

Molecular weight markers used for SDS-PAGE were purchased from Pharmacia as low molecular weight kit proteins. After staining direct comparisons were made for molecular weight determinations of the extracted proteins.

1.3 Results

1.3.1 Results for starch gels

In general isozyme bands did not appear. When they did appear, resolution was poor for any of the grinding buffers used. Initially pliers extraction produced feint bands for GPI and some activity for LAP. Subsequently, using both cherry and citrus leaves for pliers extraction, strong banding occurred for both species after staining for GOT (plate 1). This was repeated using a number of cherry varieties and an additional isozyme system in ACP. While there was good activity, there was little variation for either system. Over the following 5 month period, using the same methods, cherry leaf extracts did not yield banding patterns for PER, SOD, PGM, CAT, GDH, FDP or LAP. Good results were obtained for parallel samples of citrus and *P. mahaleb* leaves.

When homogenisation and physical extraction were directly compared, bands appeared on gels stained for AAT from samples of citrus leaves extracted with pliers. ACP bands were resolved where extractions had been made by homogenisation with Tris-HCl pH 6.85 and also with pliers. Both homogenisation procedures produced green extracts. Pliers extraction generated a browning reaction from sweet cherry leaves, but this did not occur with leaves of *P.mahaleb*.

1.3.2 Results for SDS-PAGE and IEF

The general protein stain Coomassie Blue used with both of these procedures did not reveal any differences in banding patterns between the cultivars tested (plates 2 and 3).

1.3.3 Discussion

The sporadic results encountered with starch gel electrophoresis were caused by the high phenol content in cherry leaves as evidenced by the strong and immediate browning reaction when extracting leaves and also in many cases the absence of isozyme bands. It was also suspected that the amount of protein extracted was often below the critical amount required for enzyme activity staining. This was peculiar to *P.avium* because bands were resolved for extracts from *P. mahaleb* and citrus leaves on every gel run.

The failure of PAGE to show any polymorphism was unexpected because Mau et al (1982) using PAGE had determined different molecular weights of glycoproteins extracted from styles of Lambert and two minor components were labelled antigens P and S with molecular weights of 32,000 and 37-39,000, respectively.

Furthermore, Carter and Brock (1980) used IEF to distinguish between five peach cultivars, each having a unique banding pattern for protein extracted from woody tissue. Peach is a self-fertile species and is naturally self pollinating. Consequently commercial peach cultivars have a narrow genetic base (Mowrey et al 1990). Cherry is self-incompatible and an obligate outbreeder and as such would be expected to show more variation than peach.

1.4 Conclusions

Further investigations were required to determine the best method(s) for extracting protein from a range of cherry tissue which supported consistent and resolute isozyme banding patterns.

A change of support medium was thought to be advantageous. Cellulose acetate gels were chosen for the following advantages over and above starch and polyacrylamide as detailed by Richardson et al (1986): less stain required, less sample required (0.5-2 μl compared to 10-50 μl with the other methods), less preparation time, shorter run times, shorter staining time, lower voltage requirements, and ease of handling.

Plate 1

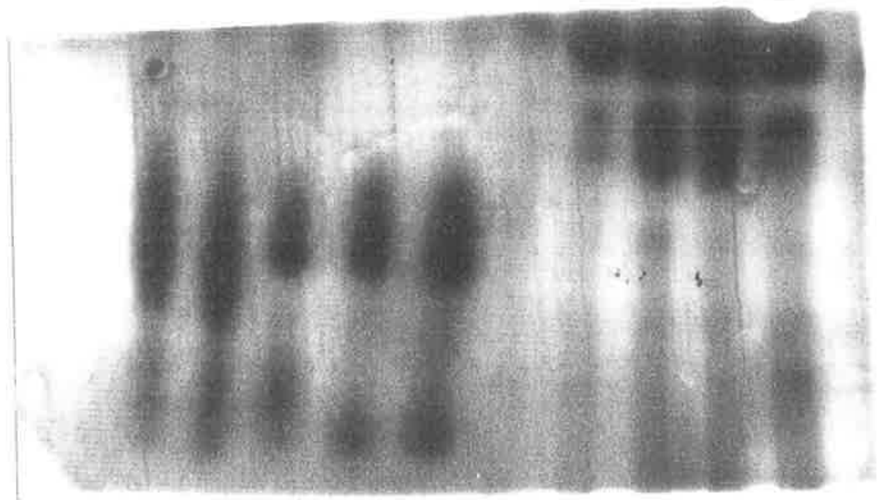


Plate 1 Starch gel stained for GOT. The first five lanes are extracts from cherry leaves and the last four lanes on the gel are extracts from citrus leaves.

Plate 2 SDS Polyacrylamide gel stained with Coomassie blue. Molecular weight markers were loaded in lanes a, j and q. Weights are indicated on the right hand side of the figure. The remaining lanes contain protein extracts from sweet cherry pistils. Each cultivar was loaded twice in consecutive lanes. The first lane was a 10 microlitre aliquot and the second 25 microlitres. Lanes were loaded as follows: Lanes b&c, Williams Favourite; d&e, Clements Pride; f&g, Bedford Prolific; h&i, Black Douglas; k&l, St Margaret; m&n, Williams Favourite; o&p, Stella.

Plate 3 Polyacrylamide gel showing the results of isoelectric focussing. Lanes were loaded with protein extracts from sweet cherry pistils as follows: Lane a, Williams Favourite; b, Clements Pride; c, Bedfords Prolific; d, Black Douglas; e, St Margaret.

Plate 2

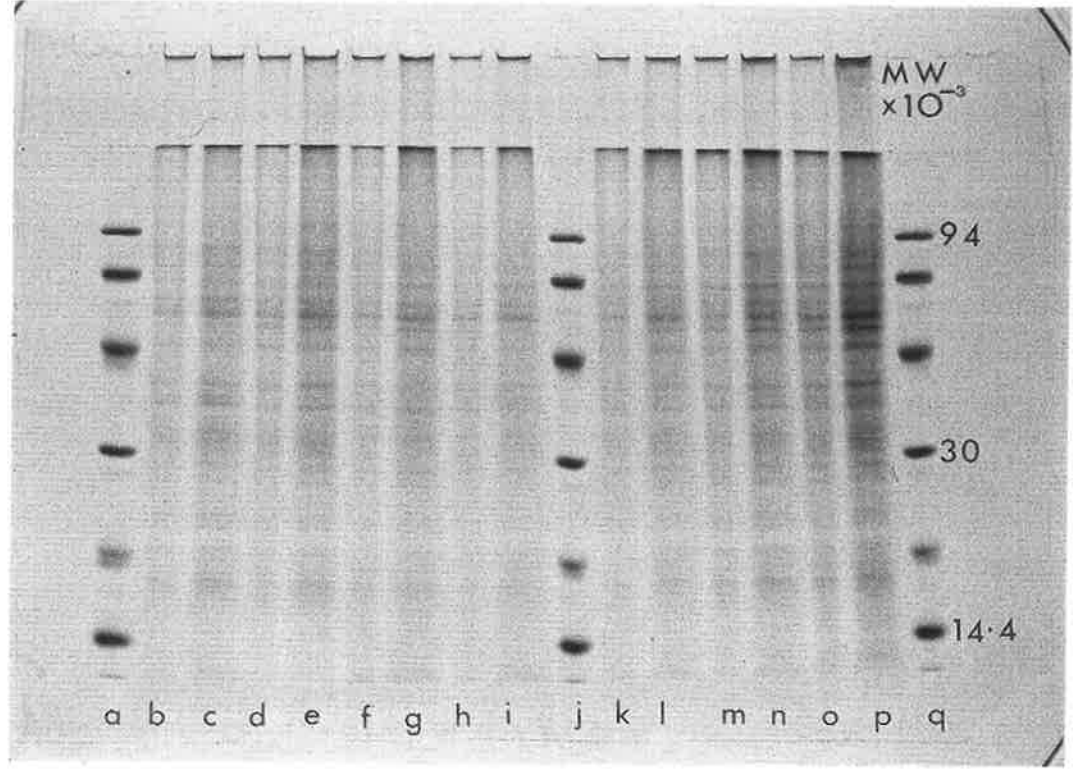
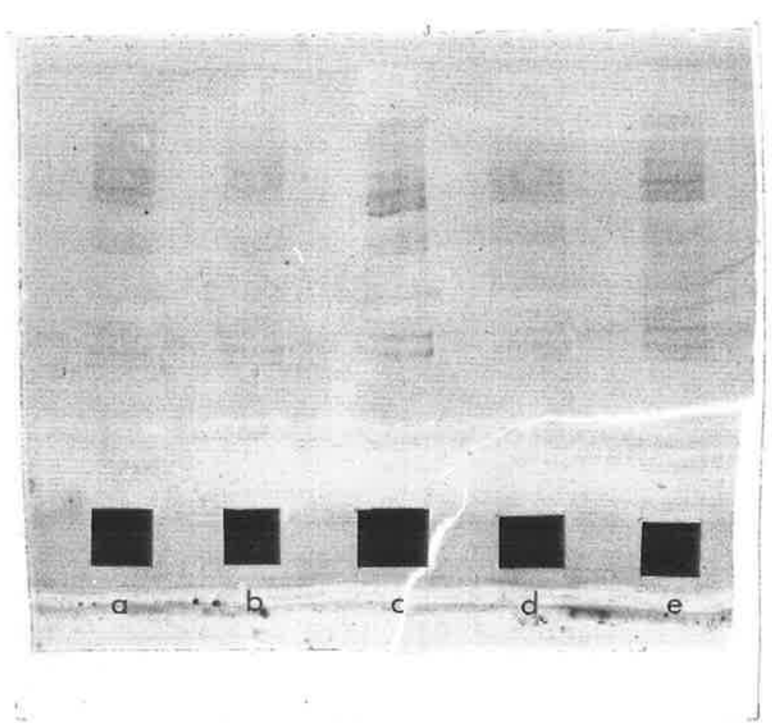


Plate 3



1.5 Development of Extraction and Sampling methods of Sweet Cherry Tissue for Cellulose Acetate Gel Electrophoresis.

1.5.1 Background

Homogenisation is the accepted extraction method for use with cellogel electrophoresis (Richardson et al 1986). The decision to use cellogel for further experimentation brought with it the need to determine suitable extraction buffers for cherry tissue. Timing the collection of different cherry tissues was often restricted by their function in the plant. For example, pollen and styles were only available in spring for a 2-4 week period. While leaves were available over a longer time, the effect of growth stage on leaf proteins was unknown. A series of experiments was devised to establish the most efficient procedure for extraction of protein from cherry tissue for the purpose of isozyme analysis. Determination of the optimal timing in terms of plant growth stage and development was also studied.

1.6 Materials and Methods

1.6.1 Evaluation of extraction procedures for cherry leaves

Cherry leaves grow on trees from August to April each year in South Australia. When available, leaves were picked in the cool temperatures of the morning or evening. Leaves were placed immediately into labelled plastic bags, sealed and held on ice. Samples were then transported directly to the laboratory for extraction. If necessary, leaves were stored overnight or up to 7 days at 2-4 ° before extraction.

Arulsekar and Parfitt (1986) had developed an extraction buffer for cherry leaves which supported good isozyme banding on starch gel electrophoresis. It consisted of 0.05M Tris-Citrate pH 8.0 and included beta-mercaptoethanol and polyethyleneglycol. Jackson and Clarke (1991) reported a similar extraction of enzymes from almond embryos for the purposes of cellogel electrophoresis.

It was referred to as normal buffer and consisted of 0.05M Tris, 0.15% citric acid, 0.12% cysteine hydrochloride and 0.1% ascorbic acid with a final pH 8.0.

Experiments began in spring, sampling the first available leaf tissue at budburst, and continuing through the growing season until autumn, comparing the amount of protein extracted from leaves by homogenisation with either Parfitt's or normal buffer.

Protein content was determined using cherry leaf extracts (100 μl) which were each mixed with 3 ml of Bradfords reagent (Bradford, 1986) and allowed to equilibrate for 15 minutes. Samples were then placed in a spectrophotometer and optical density measurements made at wavelength 595 nm. A standard curve was established using bovine serum albumin to make up known concentrations of protein which were mixed with Bradfords reagent and the corresponding optical densities recorded to produce a curve with the variable protein (μg) on the x-axis versus optical density on the y-axis. In this way ug of protein in leaf extracts could be determined by the optical density of a sample and its corresponding value on the standard curve.

Cellogel electrophoresis of isozymes was carried out after each extraction to assess their suitability in producing consistent resolute banding patterns. Almond leaf or embryo extracts using the same methods were run as a check on each gel.

In an initial experiment sampling at different times of the season and sampling leaves from different parts of the growing shoot were evaluated in relation to their effect on subsequent extracts. This was followed by an experiment comparing the effects of various leaf ages, centrifugation and detergents added to Parfitt and normal buffers on the protein content of extracts.

Later experiments included comparison of extracts using Parfitts buffer on recently emerged leaves from the terminal of actively growing shoots, young leaves from just below the shoot tip, unfolded but not fully expanded leaves and mature fully expanded leaves.

Mature leaves were also extracted using normal extraction buffer and centrifugation of 4000 x g for 15 minutes substituted by 50,000 x g for 15 minutes.

Mature leaves from the varieties Vista and Stella were extracted using Parfitt and normal buffers with the addition of EDTA and Triton-X-100, alone or in combination. "Polymin-P" was another stand-alone addition to the buffers.

These compounds were selected for their detergent properties which could aid in separating enzymes from other adhering molecules, which may effect migration on the gel.

During 1991, 216 leaf protein determinations were made between budburst and late November, when fruit begins changing colour from green to red. Average protein content was calculated and plotted over time to determine seasonal protein variations.

A review of similar studies involving seventeen publications were examined and all chemical constituents of extraction buffers noted. The compounds most often used were identified and used as a guide for generating new extraction buffers (Appendix III).

New extraction buffer mixtures were derived and existing buffers modified for further use and comparison. The base recipe for the new buffer (Buffer A) was 2% PVP, 0.1M Tris, 20mM Sodium metabisulphite and 0.1M ascorbic acid. The pH was acidic and adjusted up to 6.85 using 0.1M NaOH. To this was added singularly or in combination Sodium Tetraborate - 0.03M and 1mM EDTA. The addition of both of these compounds to buffer A produced a final pH of 8.2.

Initially Buffer A and Buffer A plus Sodium Tetraborate and EDTA were compared to Parfitt buffer and modified Parfitt buffer (Parfitt buffer with PEG excluded) for protein extraction. PVPP was added just prior to grinding with both Parfitt buffers. Three samples included PVP with modified Parfitt buffer. Parfitt modified with PVP or PVPP and buffer A were added to

Bradford's solution to check if any reactions occurred and produced a colour change which would be interpreted as protein; this did not occur. Comparison between these two extraction buffers was repeated and also carried out with Parfitt and normal buffer. The normal buffer was ground with PVP, extractions made with normal buffer and PVPP were not measured for protein content or run on gels, because no colour change occurred at all when added to Bradford's reagent, indicating no protein was present.

Following this, twelve different extraction procedures were evaluated for extract characteristics and protein content. Normal, modified Parfitt, Parfitt, Buffer A, Buffer A + Sodium Tetraborate + EDTA and water were crushed with PVP or PVPP and leaves from the cultivar Stella.

1.6.2 Extraction of isozymes from tissue other than leaves

Extractions were also made on pollen, embryo, fruit flesh and fruit stalk tissue. Pollen in particular is advantageous because this tissue has little or no polyphenol content and protein profiles are highly stable (Sugiura et al, 1988). Embryos are also thought to have little polyphenol content and share the same advantages with pollen.

Pollen can be collected from flowers over a 1-2 week period during flowering which occurs during August and September in South Australia. To ensure that pollen was uncontaminated, flowers were harvested at the 'popcorn' or balloon stage equivalent. At this stage the flower petals are closed preventing pollen transfer by wind, gravity or insects. Pollen, however, is viable at this stage. Flowers were rubbed over flywire mesh to separate anthers from other flower parts. Collected anthers were dried overnight in an oven at 25°C, after which a fine sieve was used to separate pollen from anthers.

Pistils were also collected from flowers at the popcorn stage to ensure that no pollen had contaminated the sampled tissue.

Embryos of cherries are fully mature at the time of fruit maturation, in South Australia depending on variety and location, this occurs during the months November through to January. Fruit was harvested at maturity and seeds removed from the flesh by hand. Pliers were used to crack the seed coat and remove the embryo. In sweet cherry part of the endosperm may remain in the mature seed (Ryugo 1988). To avoid sampling this triploid tissue a section of the internal seed opposite the micropyle end was sliced off using a razor blade. A thin, dry, covering layer thought to be associated with the seed coat was also peeled from each embryo prior to extraction. Often the embryos of early ripening varieties cannot be collected due to abortion. Apparently abortion occurs because nutrients and energy supplied by the endosperm, which develops while the embryo is still microscopic, are depleted and the young embryo cannot develop (Ryugo 1988).

In the first experiment stigmas, styles and embryos were extracted with normal buffer and PVPP, in anticipation of low polyphenol contents.

In follow up experiments extracts were made from embryos, fruit stalks and fruit flesh using normal buffer and PVPP. Staining was carried out for eleven isozyme systems.
Experiments were conducted on 5-11-90 and 7-11-90 to determine protein contents for extracts made with different amounts of pollen and buffer (Table 1).

Stella pistils were extracted on 12-11-90 with the ratios of tissue : PVPP : buffer shown in Table 2.

Embryos were extracted using 100mg of tissue and PVPP and normal buffer in the same ratio as for leaves.

Table 1. Amounts of pollen (mg) and normal buffer (μl) used for isozyme extraction and the corresponding protein content (mg/ml) as determined using Bradfords reagent, 5 and 7-11-90.

Cherry pollen (mg)	Normal buffer (μl)	Protein (mg/ml)
2	250	0.210
2	250	0.182
2	250	0.166
2	250	0.142
4	250	0.164
4	250	0.195
4	250	0.143
4	250	0.122
2	250	0.862
40	500	0.539
20	250	2.256

Table 2. Amounts of pistillate material (mg) sampled from Stella, polyvinylpolypyrillidone (PVPP) and extraction buffer and the corresponding protein content in extracts as determined using Bradfords reagent on 12-11-90.

Pistil (mg)	PVPP (mg)	Buffer	Amount of buffer (ul)	Protein mg/ml
40	15	normal	200	5.853
40	0	normal	200	2.448
40	0	Parfitt	200	3.981
20	7.5	normal	100	3.230
20	7.5	normal	100	1.523
20	7.5	Parfitt	100	2.841

1.7 Electrophoretic Procedure for Cellulose Acetate

1.7.1 Cellogel

Sheets of cellulose acetate 30cm x 30cm and 200μm thick manufactured by Chemetron (via Gustavo Modena, 24-Milan, Italy), were used and cut down to 15cm x 30cm and up to 62 samples loaded. Smaller test strips were also cut when required. While not in use gels were stored in a 30% methanol solution.

1.7.2 Cellulose acetate gel support and electrode compartments

Perspex boxes were constructed following the design described by Richardson et al (1986). The boxes were 350mm wide by 190mm wide by 45mm deep. Each box was divided into two compartments furnished with platinum wire electrodes, one positive (anode), and the other negative (cathode). Each electrode compartment contained an upright piece of perspex glued edge on to the base of the box. These uprights carried the cellogel which was held in place with magnetic strips.

1.7.3 Buffers for cellulose acetate gel electrophoresis

A continuous buffer system was used with cellogel. Gels were bathed in the desired running buffer for 10 - 15 minutes then removed and blotted dry with commercially available white blotting paper. Gels were immediately placed in an electrophoretic box in preparation for a run.

1.7.4 Loading samples on cellulose acetate gels.

Supernatant from extractions was stored in 1.5ml Eppendorf 5414S centrifuge tubes and the tip of a draughtsman's pen dipped into the sample. Alternatively, when dealing with small sample volumes, a pasteur pipette was used to load the draughtsman's pen. Approximately 1 microlitre of each sample was loaded onto the gel along the origin. A ruler was placed at 1cm above the bottom edge of the gel and bulldog paper clips placed on the edge of the electropheretic box to keep it in place, providing a straight edge to guide the loading pen. Up to 62 samples could be loaded onto a 30cm wide gel. Usually 48 samples were preferred to ensure no mixing of samples, and to avoid warping effects on samples close to the edge of the gel. When only a few samples were being used and investigations being made on a range of isozymes, gels were divided into equal sections so the loading sequence could be repeated in each section. The gel was cut after electrophoresis and each section separated for staining.

1.7.5 Run conditions for cellulose acetate gels

All gels were run in a walk-in cold-room at 2-4°C. A constant voltage of 200 volts DC was applied for 2 hours, as standard practice. If a shorter run-time was required voltage was increased up to 250 volts for 1 - 1.5 hours.

1.8 Staining Cellulose Acetate Gels

Specific enzyme recipes found suitable for cherry are given in Chapter 2. Herein follows a description of the base methodology used for each isozyme. Preparations were made for staining during the gel run. A stiff cardboard square 35cm x 35cm covered with a disposable plastic sheet, attached with clips, formed the staining base. Small plastic vials with about 10ml capacity were used to carry staining solutions. Enzyme activity staining was used for cellogel. Specific recipes varied; all included a substrate usually as a dry component, and most involved the reduction of tetrazolium salts such as Phenazine methosulphate (PMS) and methyl-thiazolyl blue (MTT) to a coloured insoluble formazan, by reducing agents like NADH or NADPH generated from NAD and

NADP in reactions, catalysed by oxidoreductases (Vallejos, 1983). In addition some stain recipes require the addition of a linking enzyme to couple with another reaction which then forms a coloured precipitate (Richardson et al, 1986). Some of the reagents such as PMS, MTT, NAD and NADP were made up into stock solutions and stored until needed. Richardson et al (1986) describes stock solutions for reagents and staining buffers. Immediately before the end of the gel run the substrate was weighed and added to labelled plastic vials. This was carried out close to the completion of electrophoresis because many of the substrates used in staining were light sensitive or breakdown at room temperature. At the end of the electrophoresis, power was turned off and gels carried to the staining area. Each staining solution was made up in turn. Solutions were spread evenly over the prepared plastic sheet. The gels were passed backwards and forwards over the solution for 30 seconds. Gels were then blotted to remove excess solution and placed in a plastic folder labelled with the date, sample details, isozyme and buffer used, and incubated at 37°C until bands appeared.

1.8.1 Recording of results

As bands appeared the position of bands was recorded and their distance from the origin measured. A photocopy of each gel was taken, providing a permanent record. Gels were then placed into storage at -20°C for the duration of the project. Frozen gels were often used to recheck or clarify results and then replaced in -20°C storage.

1.9 Results

The comparison of normal and Parfitt buffers for extraction of leaves beginning in spring showed best clarity of isozyme banding patterns with the normal buffer system. It appeared that the beta-mercaptoethanol in the Parfitt buffer may have been interfering with banding. Extractions continued and sharp interpretable banding patterns were produced for GPI, LAP, GOT, IDH and PGM. Again, clarity of banding was superior for those samples extracted using normal buffer.

By late October bands did not appear for many samples after staining for GOT, LAP, GPI, IDH, MDH, 6PGD and G6PD.

Protein determinations of leaf extracts showed variable levels of protein. Samples with higher protein contents corresponded with the presence of bands after staining for PGM (Table 3). Values from Table 5 indicate that approximately 0.2mg/ml of protein was required to detect isozyme activity. Interestingly, older leaves generally had higher protein contents (Table 4).

Table 3. Banding and protein contents (mg protein/ml) of extracts from cherry leaves made on 25-10-89

Sample no.	Banding evident	Protein mg/ml	Sample no.	Banding evident	Protein mg/ml
1	no	0.08	25	no	0.15
2	no	0.13	26	no	0.09
3	no	0.1	27	no	0.09
4	no	0.11	28	no	0.11
5	no	0.11	29	no	0.1
6	no	0.9	30	no	0.08
7	no	0.1	31	no	0.1
8	no	0.085	32	no	0.04
9	yes	0.295	33	no	0.14
10	no	0.045	34	no	0.15
11	no	0.085	35	no	0.09
12	no	0.1	36	yes	0.45
13	no	0.08	37	no	0.1
14	no	0.07	38	yes	0.22
15	no	0.07	39	no	0.135
16	no	0.07	40	no	0.11
17	yes	0.49	41	yes	0.23
18	no	0.1	42	no	0.09
19	no	0.19	43	no	0.11
20	no	0.09	44	no	0.14
21	no	0.1	45	yes	0.215
22	no	0.07	46	no	0.1
23	no	0.1	47	yes	0.23
24	no	-	48	no	0.12

Furthermore, the use of Parfitt's buffer for extractions made during the October-November period was yielding up to five times more protein than Normal buffer (Table 5). Zymograms showed strong activity evidenced by dark staining but were uninterpretable due to extensive smearing. Discrete banding did not occur.

The addition of detergents and increased centrifugation for samples of young and mature leaves extracted with either Parfitt or Normal buffer did not produce improved zymograms for GOT, GPI, IDH and 6PGD. Samples 29 and 30 (Table 2) included Triton X-100, and could not be loaded because of excessive spreading on the gel. Samples 13, 14, 15, 17, 18, 27 and 28 (Table 3) had insufficient protein content, and were all extracted with normal buffer. Samples 27 and 28 were extracts of mature leaves with the addition of detergents, indicating no effect on increasing extracted protein by EDTA alone or in combination with Triton X-100. Extra centrifugation was given to samples 13, 14, 15, 17 and 18 and this in combination with Normal extraction buffer and mature leaves did not increase extracted protein contents.

Table 5 gives results for the continued comparison between Parfitt and Normal buffers. Almost all varieties excepting Williams Favourite, extracted with Normal buffer yielded low or undetectable amounts of protein. Extracts made with Parfitt buffer spread out when loaded. Gels stained for GPI, LAP, GOT, IDH, 6PGD and PGM displayed good activity, but once again were smeared.

Extraction with Parfitt buffer of leaves from 48 different cherry varieties produced resolvable banding patterns after staining for PGM, GOT, G6PD, GPI, IDH and LAP. All gels appeared to be overloaded, as stain activity was too strong.

Successes became sporadic as the growing season advanced. Protein levels where highest in extracts made using modified Parfitt with PVPP and Buffer A with sodium tetraborate and EDTA added (Table 6). Interestingly, normal buffer + PVP-40 yielded much higher protein contents in

extracts as compared to Normal buffer + PVPP. Extracts made using PVP were noticeably more viscous and less practical to handle and load.

Electrophoresis and enzyme activity staining was carried out as a check. Zymograms again displayed much activity for GOT, GPI, IDH, LAP and PGM which was smeared and uninterpretable. For the remainder of the season Parfitt buffer was used for the extraction of isozymes from leaves. While protein content was always satisfactory giving good activity on gels, resolution was almost always poor. Exceptions did occur but would vary from one day to the next. For example, in April 1990 when young cherry leaves where extracted with Parfitts buffer and PVPP, protein levels ranged from 5.5 to 5.79 mg/ml. Activity was detected for seven isozymes 6PGD, ACP, SKDH, G6PD, XLDH, FDP and GPI. Only 6PGD and FDP showed good resolution. The following day extractions were made with Normal buffer producing very low protein levels of 0.079 to 0.162 mg/ml while extractions with Parfitt buffer had levels ranging from 3.4 to 3.68 mg/ml protein. Extracts from the previous day had been frozen at -18°C and thawed protein measurements were about 1.02 mg/ml less than the fresh extract ranging from 3.53 to 4.56 mg/ml. Thawed extracts were variable in their isozyme performance.
Table 2 provides details of protein contents in extracts made with various amounts of pistil material, PVPP and extraction buffer. Highest protein contents were associated with the use of normal buffer and PVPP. Protein contents of embryo extracts from certain cultivars are given in Table 7. The first two records in the table are for stylar extracts, and when compared to extracts from embryos, suggests that embryos yield more protein in extracts compared to styles. The results also demonstrate the range in protein contents within and between cultivars. Protein content can effect the intensity and clarity of banding on gels subsequently stained for the presence of specific enzymes and in turn influence the interpretation of banding patterns.

Table 4. Protein content (mg/ml) of leaf extracts as described in Appendix I.

Sample no.	Leaf age	Aliquot size used in determination (μl)	Protein (mg/ml)
1	new	50	0.1
1	new	100	0.08
2	old	50	0.2
2	old	100	0.21
3	old	50	0.28
3	old	100	0.195
4	new	50	0.59
4	new	100	0.5
5	old	50	1.06
5	old	100	0.695
6	old	50	0.93
6	old	100	0.635

Table 5 Protein content (mg/ml) of leaves from selected cherry varieties extracted with different buffers

Sample no.	Variety	Extraction buffer	Protein mg/ml
1	Vista	Normal	-
2	Merton Bigarreau	Normal	-
3	Vittoria	Normal	-
4	Williams Favourite (M)	Normal	0.333
5	Stella	Normal	-
6	Williams Favourite (L)	Normal	0.37
7	Vista	Parfitt	5.593
8	Merton Bigarreau	Parfitt	5.251
9	Vittoria	Parfitt	5.251
10	Williams Favourite (M)	Parfitt	5.877
11	Stella	Parfitt	5.469
12	Williams Favourite (L)	Parfitt	5.484
13	Vista	New Normal	-
14	Vista	Old Normal	0.013
15	Williams Favourite (L)	New Normal	0.442
16	Williams Favourite (L)	Old Normal	0.319
17	Vittoria	Parfitt	4.684
18	Williams Favourite (M)	Parfitt	5.346

Table 6. Protein content of cherry leaf extracts made using different buffers

No.	Variety	Buffer	Protein mg/ml
1	Vista	Normal + PVPP	0.000
2	Williams Favourite	Normal + PVPP	0.000
3	Stella	Normal + PVPP	0.020
4	Vista	Normal + PVP.40	2.362
5	Williams Favourite	Normal + PVP.40	2.658
6	Stella	Normal + PVP.40	3.085
7	Vista	Parfitt + PVPP	4.650
8	Williams Favourite	Parfitt + PVPP	4.911
9	Stella	Parfitt + PVPP	4.259
10	Vista	Buffer A + NaTB + EDTA	3.867
11	Williams Favourite	" " " "	3.731
12	Stella	" " " "	3.856
13	Vista	T.B. Buffer A	3.488
14	Williams Favourite	T.B. Buffer A	3.144
15	Stella	T.B. Buffer A	3.873

Table 7. Protein content (mg/ml) of extracts from cherry styles and embryos 12/12/89

Variety	Tissue	Protein (mg/ml)
Black Tartarian	Style	1.840
Stella	Style	3.136
Stella	Embryo	5.564
Vista	Embryo	5.987
Vista	Embryo	4.512
Vista	Embryo	4.902
Vista	Embryo	5.275
Vista	Embryo	6.127
Vista	Embryo	exceeded upper limit of standard curve
Stella	Embryo	6.335
Stella	Embryo	6.416
Stella	Embryo	4.491
Stella	Embryo	exceeded upper limit of standard curve
Stella	Embryo	5.382
Seedling	Embryo	4.555
Seedling	Embryo	3.931
Seedling	Embryo	4.296
Seedling	Embryo	3.054
Seedling	Embryo	2.775

1.10 Discussion

Extraction procedures were developed which yielded very high levels of proteins and correspondingly strong staining of isozyme activity on gels. Unfortunately the resolution of bands was usually unsatisfactory due to excessive smearing, particularly in leaf extracts taken after fruit began to accumulate sugar. Initially it was thought that polyphenols and their oxidative products, quinones, had been absorbed or bonded with other molecules. This was further supported when it was established that PVP-40 was most efficient in generating higher protein levels in extracts presumably by absorbing phenols. While this reduced smearing, discrete bands could not be resolved. Thus migration of the enzymes, rather than the activity was being inhibited. It was presumed that something, possibly polysaccharides or pectins, were binding to the enzymes and effecting their migration through the molecular matrix of the cellogel, or maybe lignins from cell walls could also have formed cross-linkages across the pores of the gel. Dye marker studies at Cornell University, Geneva, New York have shown that xylar embolisms occur in sweet cherry during the fruit growth stage and correspond with the appearance of high concentrations of sugar in the fruit (Lakso, pers comm 1990). These blockages of the xylem are caused by high molecular weight polysaccharides that prevent excessive water movement toward the high osmotic potential of the fruit. The polysaccharides are produced in the leaves when sugar accumulation begins in the fruit and are transported through the xylem. Thus, for best, results leaves should be sampled from budburst until sugar accumulation begins in fruit.

In summary, the procedure considered best for sweet cherries is as follows:

leaves were sampled from the terminal of actively growing shoots at green tip/leaf emergence stage. Four hundred milligrams of leaf material was placed in a cooled pestle (2.4°C.) PVPP (0.15g) and 2 ml of Parfitts' buffer were added. The mixture was ground vigorously with a pre-cooled mortar (2.4°C) into a smooth slurry. The slurry should show no browning reaction and the consistency should not be too watery or fibrous. The crude extract was then scraped into a 5414S Eppendorf centrifuge tube held on ice. When all samples were complete they were centrifuged at

3000 x g for 15 minutes. Supernatant was pipetted off with a glass pipette and stored in an Eppendorf centrifuge tube (5414S) on ice until loaded.

1.11 Conclusions

For pollen, pistils and embryos extraction of isozymes by grinding with normal buffer and PVPP was found to be satisfactory. Quantities used were as follows:

Pollen; 40mg pollen : 500ul normal buffer : 0mg PVPP.

Pistils; 40mg style and stigma : 200ul normal buffer : 15mg PVPP (the ovary was removed from pistillate material to avoid triploid endosperm tissue.)

Embryos; 100mg embryo : 500ul normal buffer : 15mg PVPP.

CHAPTER 2 ISOZYME POLYMORPHISM IN SWEET CHERRY

2.1 Introduction

This section deals with the evaluation of staining and buffer systems for resolution of isozymes showing activity and variation in sweet cherry tissue. To describe the observed variation in banding patterns genotypes have been assigned to suitable isozymes.

Following electrophoresis resolution of isozymes is achieved by using procedures which generate a reaction or set of reactions that will reveal a discreet band of enzyme activity by any chemical or physical means (Vallejos 1983).

A search of the literature revealed many isozyme staining/buffer systems for a range of plant species. (Soltis et al, 1983); (Shields et al, 1983); (Vallejos, C.E., 1983); (Arulsekar and Parfitt, 1986); (Richardson et al, 1986); (Ashari et al, 1989). Some plant species were reported to show activity and variation for certain isozymes while others did not. While those showing activity for the same isozymes may exhibit different genetic control or require different methodology to reveal bands of activity. At the beginning of this study very little work had been published regarding isozymes in cherries. Apparently the only work completed was that of Aruleskar and Parfitt (1986) at University of California, Davis.

The approach adopted here was to survey leaf, and to a lesser extent other tissue extracts, for the presence of isozyme activity using stain and buffer solutions from the literature, and, if necessary, modifications of them were made or new techniques developed to visualise isozymes.

2.2 Materials and Methods

In surveying for isozyme activity, extractions were made using Parfitt buffer and gels divided into test strips. This enabled the evaluation of up to 28 different buffer/staining protocol combinations.

In some instances different tissue types were extracted. For example leaf, embryo and pollen were run on the same day to determine isozyme expression in different tissues (Appendix IV).

Once several isozymes were found to be present, resolute and polymorphic in leaf extracts it remained to determine the stability of these isozymes under a range of environmental conditions. Isozyme banding patterns of extracts from leaves of trees grown in pots in the glasshouse were compared to field grown trees.

The performance of extracts from a virus free Williams Favourite (M) tree was compared to extracts from an adjacent tree of the same variety infected with Prunus Necrotic Ringspot Virus to evaluate the effect of the presence of virus and its possible interference with DNA replication within the plant.

Isozyme genotypes of functionally different diploid tissue, leaves and pistils were compared within varieties. Leaf genotypes were also compared to the haploid pollen genotypes by running leaf and pollen extracts on the same gel for each isozyme.

Embryos were examined to see if they had equivalent banding patterns, to that of leaves. Genotypes of individual embryos were nor expected to be the same as those of parents because only half of each parents genome is donated to each embryo.

2.3 Results

Isozymes in leaves having good, scorable resolution with variation evident were PGM, GPI, GOT, IDH, G6PD, MDH, 6PGD, SKDH, FDP and PER. Isozymes found in embryos with similar characteristics were 6PGD, GPI, G6PD, GOT, SKDH, FDP, PGM, IDH and MDH. LAP appeared to be showing variation initially. However, this was discounted after many runs and seemed to be related to protein content rather than any genetic control. Pollen contained the following isozymes, G6PD, 6PGD, XO-2, GOT, MDH, PGM and AK as active resolute and

showing evidence of variation. Suitable isozymes in pistils were G6PD, 6PGD, GOT, GPI, SKDH, PGM and MDH. (Appendix IV).

Appendix V details reagents used in staining for activity of the best isozymes for leaves, pollen and embryos as determined by results from Appendix IV for cellulose acetate gels.

2.3.1 Isozyme stability

Glasshouse grown and field grown trees produced identical isozyme patterns for GOT, G6PD, 6PGD, IDH, MDH, GPI, PGM, FDP, SKDH and PER. Plate 4 displays the result for 6PGD. There are 33 lanes, representing 33 different samples. Eleven varieties; Williams Favourite (M), Vista, Victor, Napoleon, Sunburst, Vega, Lyons, Summit, Stella, Van and Compact Stella were loaded on each gel in the same order. Each variety made up a block of three lanes, ie. Lane 1 is Williams Favourite (M) from the glasshouse extracted with Parfitt buffer. Lane 2 is field grown extracted with Parfitt buffer and Lane 3 is field grown extracted with Normal buffer. Lanes 4-6 comprise Vista treated in the same way and so on. Lanes 12, 26 and 33 show no activity. Lanes 12 and 33 were extracts made with Normal buffer while Lane 26 was Stella, field grown extracted with Parfitt buffer. Lanes 9, 21 and 27 are all extracts made with Normal buffer and show reduced band activity. Lane 9 is difficult to interpret and while the other samples of Victor suggest it is an ab, it could be mistaken as an aa.

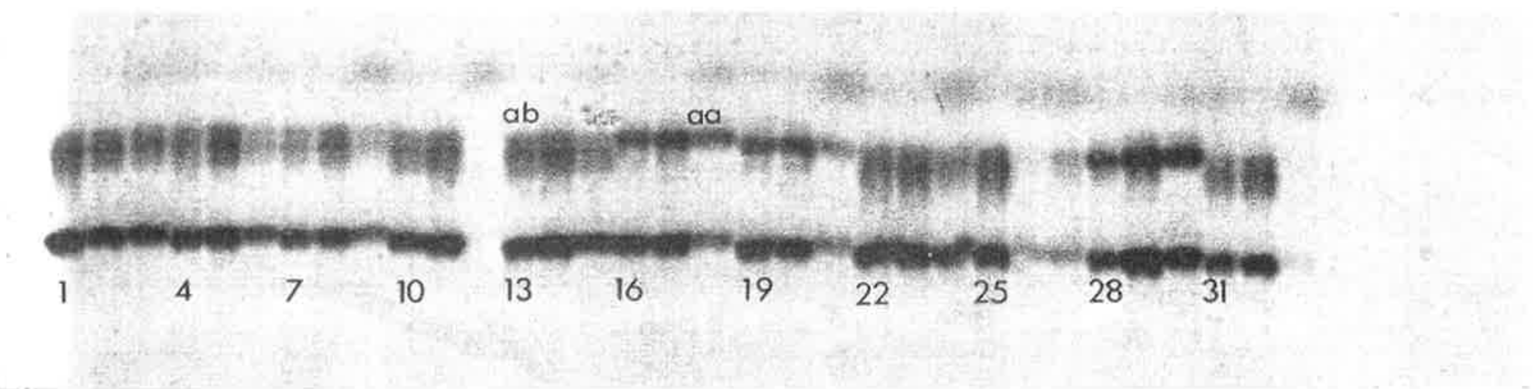
Virus infected Williams Favourite (M) and uninfected produced identical banding patterns for G6PD, 6PGD, GOT, PER, PGM, FDP, GPI, MDH, SKDH and IDH.

Leaf and pollen zymograms have been compared in almonds (Jackson and Clarke, 1991) and banding patterns correspond for each cultivar and isozyme. The main difference being that pollen banding patterns usually only showed two banded heterozygotes whereas leaf patterns would show three. This is thought to be because of pollen is haploid and each allele present only being capable of producing a single band. This is in contrast to the diploid situation where post-transcriptional

events in cells can lead to combinations of peptide chains produced by different alleles. This is visualised as a third or hybrid band in heterozygotes. Diploid tissue such as pistils and leaves of the same cultivar share the same genotypes for isozymes common to both tissues.

Plate 4 Results for 6-Phosphoglucose Dehydrogenase (6-PGD) as an example of isozyme stability in extracts taken from trees grown under glasshouse and field conditions using different extraction buffers. Lanes 1-3 are Williams Favourite (M); 4-6 Vista; 7-9 Victor; 10-12 Napoleon; 13-15 Sunburst; 16-18 Vega; 19-21 Lyons; 22-24 Summit; 25-27 Stella; 28-30 Van; 31-33 Compact Stella. For each cultivar the first lane contained glasshouse grown leaves extracted with Parfitt buffer, the second lane field grown leaves extracted with Parfitt buffer and the third lane field grown leaves extracted with Normal buffer for each cultivar.

Plate 4



2.3.2 Isozyme interpretation for *Prunus avium*

The survey of a range of cherry varieties and tissues has been described. Once activity, resolution and variability had been ascertained it remained to interpret banding patterns and assign loci and genotypes.

Leaves

Isozyme: Fructose-1,6-Diphosphate (E.C. 3.1.3.11) Tetramer.

Loci: Four loci were apparent at regular spacings the zone with the greatest stain intensity and variation was scored. This system was always feint which made genotype scoring more difficult.

Genotypes: FDP-2 1. aa 2. ab 3. bb (Plate 5).

Isozyme: Glutamate Oxaloacetate Transaminase (E.C. 2.6.1.1) Dimer.

Loci: Kephart (1990) reports GOT to have a dimeric structure. In cherry there are 5 zones of brown banding on orange background probably corresponding to 4-5 loci. Only one loci shows variation all other electromorphs are monomorphic.

Genotypes: GOT-3 1. ab 2. ac 3. bc

Isozyme: Glucose Phosphate Isomerase (E.C. 5.3.1.9) Dimer

Loci: Kephart (1990) lists GPI as usually having 2 loci in most plants. In cherry leaves there is only one discreet zone of activity. The activity is very strong and makes it difficult to confidently separate out 2 loci. For sweet cherry leaves one loci appears to be showing activity and resolution.

Genotypes: GPI-1 1. aa 2. ab.

Isozyme: Glucose-6-Phosphate Dehydrogenase (E.C. 1.1.1.49) Dimer.

Loci: G6PD showed less intense and smeared staining activity. Two zones of activity were detected probably corresponding to two different loci. G6PD-2 was less resolute and not scored.

Genotypes: G6PD-1 1. aa 2. ab c. bb

Isozyme: Isocitrate Dehydrogenase (E.C. 1.1.1.42) Dimer.

Loci: One zone of intense activity was well resolved for IDH probably representing a single locus. The banding patterns suggest that 3 alleles are present at this locus. Homozygous bands appear to be two very closely spaced bands. Other bands appear at times (particularly in embryo analysis) and are much less intense, and are thought to be ghost bands or artifacts. If the ghost bands are in fact real, band positions may be best explained as comigration of 2 loci. Phenotypes correspond with those found in the closely related almond species (*P. amygdalus* Batsch) as described by Jackson and Clarke (1991). In contrast to this work conventional genotype assignment has been used for cherries, so the aa genotype has been assigned to the fastest migrating band rather than the slowest.

Genotypes: IDH 1. aa 2. ab 3. bb 4. bc

Isozyme: Malate Dehydrogenase (E.C. 1.1.1.37) Dimer

Loci: There are five zones of activity associated with MDH. Two zones occur close to the origin and may be stain artifacts. Two or three loci appear to be operating in the anodal zone of activity. MDH-1 and MDH-3 show much variability while MDH-2 is monomorphic. MDH-3 shows the most intense staining, to the extent that interpretation is difficult. Hancock and Iezzoni (1988) found two loci operating in sweet cherry leaves, producing monomorphic three banded patterns which correspond to the 2-3 anodal loci identified in this study. Pollen extracts studied by Hancock and Iezzoni displayed an additional 4 bands, probably

controlled by one locus, and cathodal to the same three banded pattern found in leaves. This cathodal zone in pollen appears to correspond to the MDH-3 locus scored for leaves in this study.

Genotypes: MDH-3 1. aa 2. ab 3. bb

Isozyme: Phosphoglucomutase (E.C. 2.7.5.1) Monomer.

Loci: For this isozyme there are three anodal zones of activity, the least anodal zone shows interpretable variation. The other two may be the product of a single locus as banding is in relatively close proximity (< 0.5cm). According to Kephart (1990) it is usual for plants to have two loci for this isozyme. Thus, adhering to convention, 2 loci have been assigned for PGM in cherries. This isozyme shows high activity which makes differentiation of the slow homozygote impossible and it may be that many individuals scored as a heterozygote are homozygous bb.

Genotypes: PGM-2 1. aa 2. ab

Isozyme: 6-Phosphogluconate Dehydrogenase (E.C. 1.1.1.44) Dimer.

Loci: Two loci show coincidental variation. The cathodal activity may be stain artifacts or closely linked to the anodal zone of activity. In any case, only the anodal locus was scored.

Genotypes: 6PGD-1 1. ab 2. aa 3. bb

Isozyme: Shikimic Dehydrogenase (E.C. 1.1.1.25) Monomer.

Loci: Only one zone of activity exists for SKDH. Activity is good. Although in the majority of runs two bands for heterozygotes are not detectable. In a few instances they are readily visualised and provide evidence for genotypes in other runs.

Genotypes: SKDH-1 1. aa 2. ab

Isozyme: Peroxidase (E.C. 1.11.1.7) Monomer.

Loci: Peroxidase displays very strong stain activity at one locus. Three variably intense bands appear at the anodal side of the locus and are essentially monomorphic. Occurring below these bands are up to 3 closely spaced bands. These bands show variation in the form of fast and slow homozygotes and a three banded heterozygote. Bands are ephemeral and disappear from gels after time even if stored at -18°C. No cathodal banding below the origin was detected for PER in any of the tissues analysed.

Genotypes: PER-1 1. aa 2. ab 3. ac

Plate 5 Banding patterns for the ten isozymes which produced best results after electrophoresis of protein extracts of sweet cherry tissue on cellulose acetate gel. The abbreviated name of each isozyme appears in the left hand column. Lower case lettering in the right hand side of the figure denotes the genotypes observed for each isozyme.

Plate 5

FDP	aa ab bb
GOT	aa ab
GPI	aa ab
G6PD	aa ab
IDH	aa ac bb ab
MDH	ab aa
PGM	ab aa
6PGD	aa bb ab
SKDH	aa ab ac
PER	aa ab

2.4 Discussion

The differential presence and absence of isozymes in leaf, pistil and pollen tissue may reflect the different functions of each tissue. Different cell types may express different loci for a particular enzyme (Richardson et al, 1986). All cell types were extracted by homogenisation of each tissue, and while the relative intensity of bands may have varied between the tissue types, the genotypes were the same for those enzymes common to the three tissue types compared. At times, depending on the conditions of each run, banding patterns could appear to vary from tissue to tissue and this is probably caused by subcellular compartmentalisation of isozyme loci into the cytosol, plastids, mitochondria and microbodies (Kephart, 1990). The relative abundance of these in different tissue types would correlate to the amount of activity of each enzyme. Richardson et al (1986) note that "relative strengths of each band can occur between samples and in fact bands can be present or absent in a fashion mimicking allelic variation".

PGM usually has two loci in plants (Kephart, 1990), Jackson and Clarke (1991) observed polymorphism at two, well separated loci in almonds. In cherry, the loci were very close and the banding patterns resemble those typical of two loci comigrating. This would also explain why only aa and ab phenotypes were distinguished for a single locus, bands of the other locus being so close that they cannot be uniquely differentiated.

Ghost or shadow bands are thought to represent degradation products of primary isozymes. Kephart (1991) listed those isozymes with which ghost bands were readily associated and they included SKDH, PGM, GPI, MDH, IDH and 6PGD. As mentioned in the results IDH often produced ghost bands. The other enzymes listed produced ghost bands infrequently and may have been artifacts associated with a particular analysis.

In comparing MDH banding patterns after starch gel electrophoresis of cherry species including *P.avium* Hancock and Iezzoni (1986) reported different patterns for leaf and pollen extracts. Pollen was shown to have four additional cathodal bands which seem to correspond to the isozyme patterns observed for leaves in this study.

Hancock and Iezzoni did not record polymorphism for MDH in sweet cherry. MDH was particularly difficult to score because of the strong activity at the MDH locus, and the close spacing of bands. There may also have been overlap or comigration of bands encoded by other loci. This often makes phenotype recognition difficult (Kephart, 1990).

In this study GOT appeared to be under the control of 3 alleles each encoding a different sub-unit of the dimeric enzyme. This should result in six banded heterozygotes according to Pasteur et al (1988). However both leaf and pollen ac heterozygotes showed 5 banded patterns and other heterozygotes showed 3 and 4 band patterns for leaf isozymes. Santi and Lemoine (1990) reported 5 banded heterozygotes and homozygotes with 3 or 4 bands for cherry bud extracts assayed on polyacrylamide gels. This evidence tends to suggest that GOT may be a tetramer rather than a dimer. Again though, close spatial distribution and comigration of bands encoded by different alleles may have resulted in fewer than expected bands, especially considering that GOT bands are brown on a yellow/brown background. While the banding form of heterozygotes provides some evidence it is only indicative, and it does not necessarily mean that an enzyme under consideration has a two or three chain quaternary structure. Furthermore, all possible heteropolymers are not necessarily formed, in particular when the structure is asymmetric. (Pasteur et al 1988).

Santi and Lemoine (1990) described only two zones of activity for GOT for cherry. GOT-1, described in their work, showed similar banding patterns to GOT-3 isozymes in this study. Santi and Lemoine suggested duplication of the GOT locus was responsible for the presence or absence of a single band closely associated with GOT-1. A similar band was not found in this study nor were any aa phenotypes observed at the equivalent locus. Comparison of the two studies was

inconclusive due to procedural differences between the two studies. Furthermore, to adopt the genotype assignment of Santi and Lemoine (1990) would require the exclusion of the cathodal section of GOT-3, and because this did not vary independently from the rest of the GOT-3 locus, it is not a separate locus. Rather it was thought that the variation at the GOT-3 locus was controlled by three different alleles each coding for a different subunit of the dimeric enzyme. So genotypes were assigned on this basis.

CHAPTER 3: CULTIVAR IDENTIFICATION AND GENETIC RELATIONSHIPS USING CHERRY LEAF ISOZYMES

3.1 Introduction

Identification of tree crop species is important for commercial reasons ie. orchard layout for correct and efficient pollination; and for breeders to ensure that new selections are uniquely identified, particularly where plant breeders rights or patents are to be sought. Isozymes have been used to help identify individual cultivars of almond (Jackson and Clarke 1991; Hauagge et al 1987), apple (Weeden and Lamb 1985; Menendez et al 1986), cherimoya (Ellstrand and Lee 1987), citrus (Ashari et al 1989), macadamia (Vithanage and Winks 1992), mango (Degani et al 1990), peach (Messenguer et al 1987) and persimmon (Sugiura et al 1988). Isozyme phenotypes have also been used to group cultivars of apricot (Byrne and Littleton 1989; Battistini and Sansavini 1991). And they have been used to distinguish interspecific hybrids of plum x peach (Parfitt et al 1985) and peach x almond (Chaparro et al 1987). Zygotic and nucellar seedlings of citrus have also been discriminated by Ashari et al (1988) and Moore and Castle (1988) using isozyme phenotypes.

Isozyme phenotypes have been shown to be simply inherited in apple (Manganaris and Alston 1992), apricot (Byrne 1989), guayule (Hashemi and Estilai 1992), blueberry (van Heemstra et al 1991) and peach (Maurey et al 1990). The most acceptable evidence for this is results of analysis of segregation ratios in progeny from controlled hybridisations. However, comparison with related species and parental propagules of a single cultivar also provide good support. Genotypes can be assigned and, in the case where distinct identification is required, the combination of single locus genotypes into a multilocus profile can label each cultivar with a unique genotype, in this way they can be differentiated.

Genotypes are a direct measure of genetic distance. Ashari et al (1989) used the number of loci at which the isozyme pattern differed as the basis of describing genetic relationships between

mandarin types. Population genetics involves the estimation of genetic distances between populations of different species and often includes the generation of a phylogenetic tree. This approach could be applied to the situation existing during this study in which a collection of a single species - *Prunus avium* was comprised of cultivars collected from geographically isolated populations. It is thought that these populations have common origins which in theory should result in genetic divergence in each isolated population, the speed of which would be dependent on the number of individuals isolated and the selection pressure applied to certain characteristics.

For the purpose of comparing between populations of cultivars, we can use allele frequency estimates and estimates of genetic distance adapted from species based studies. Nei's similarity index (Nei, 1972) is one of the most frequently cited in the literature. Nei defines the best measure of genetic distance as the number of nucleotide or codon differences per unit length of DNA. This can be determined directly by using DNA hybridisation techniques or estimated from protein electrophoresis data using Nei's formula. This similarity index measures accumulated allele differences per locus for diploid populations. Mating system considerations are embedded in the formula and if the rate of gene substitution each year is constant it is linearly related to geographical distance in some migration models (Nei 1972).

3.2 Materials and Methods

Leaf samples were taken from a collection held at Lenswood Horticultural Centre (L.H.C.) (34° 57'S; 138°48'E; altitude 480m) and nearby orchards. Cherry cultivars in the L.H.C. collection have been sourced as budwood from overseas germplasm repositories, breeding programs or other government research station collections. After Australian quarantine procedures for virus testing of each budwood entry were complete, budwood was obtained directly from quarantine or after multiplication by other State Agricultural Departments. Cultivars held on commercial orchards in close proximity to L.H.C. and sampled for isozyme analysis had doubtful authenticity. Sources of budwood were unknown. Granger et al (1992) describes the origin of each cultivar. The cultivars Beauchamps Black, Bedford Prolific, Early Rivers, Lustre, Napoleon, Waterloo and Williams

Favourite were sampled from more than one location. Different locations are indicated by a letter in parentheses following the cultivar name as follows; (B) Mr W Bishop, Basket Range, SA, (G) Mr K Green, Lenswood, SA, (L) L.H.C., Lenswood, SA, (M) Mr R Meiglich, Lenswood, SA, (P) Mr David Pike, Neerim Junction, Victoria. The word Mother following a cultivar name indicates that the tree sampled was the original seedling selection of that particular cultivar.

Waterloo in South Australia is a white cherry with colourless juice. Whereas Grubb (1949) describes Waterloo as a dark cherry with coloured juice. It is thought that the white cherry cultivar referred to as Waterloo in South Australia is in fact Florence (W.N. Bishop Pers. Comm.). Coincidently, Florence and Waterloo budwood was brought to South Australia at the same time and incorrect labelling probably occurred. It should also be noted that in no other Australian cherry growing State is there a white cherry cultivar known as Waterloo, Florence on the other hand is widely grown.

Extraction of leaf samples and electrophoresis using cellulose acetate support media was carried out as detailed in Chapter 1. Isozyme patterns were interpreted as described in Chapter 2 and genotypes assigned for each isozyme for each variety. The ten isozymes assayed were 6PGD, GOT, G6PD, GPI, IDH, PGM, FDP, SKDH, PER and MDH. The cultivars sampled are listed in Appendix VI. Those cultivars with recorded parentage were examined to verify parentage and support the assignment of genotypes. The number of loci at which the isozyme pattern differed was determined by making pairwise comparisons between all the cultivars. Statistical analysis of the pairwise comparisons was not possible because there was no replication. So a significant number of isozyme differences could not be established. Ashari et al showed that citrus cultivars derived from interspecial hybridisation averaged 6.0 - 2.5 (50 - 21%) isozyme loci differences at 12 polymorphic loci. In contrast, cultivars within the Tangelo species which shared the same parent, the average number of loci differences was 2.01 - 1.0 (17 - 8%). Some Tangor cultivars were also compared which had the same reported parentage or were thought to be vegetative or seedling selections from progeny of the same parentage. In this situation the mean number of locus

differences was 3.2 - 1.9 (27 - 16%). These results reflected the expected genetic differences for the reported phylogeny of each group of cultivars.

An average number of isozyme loci differences was determined for each cherry cultivar with a standard deviation. The averages were subject to the Wilk - Shapiro/Rankit's procedure to examine whether or not data conform to a normal distribution.

Following Ashari et al (1989), a general rule of thumb was adopted in determining genetic relatedness based on differences at isozyme loci. That is, any two cultivars separated by more than 2 isozyme differences (20%) were considered to be less likely to be related, in particular this was applied to trace the parentage of the local varieties Black Douglas, Clements Pride, Lustre, Opal and Up-to-Date and to elucidate the inter-relationships within Beauchamps Black, Lustre, Waterloo and Williams Favourite sampled from different sites (see Table 2 in Granger et al 1992).

Finally, cultivars were grouped according to country of origin ie. Australia, England, Canada, Switzerland, USA, Italy and Germany. Frequencies for each allele of the 10 isozymes were determined from the data in Appendix VI. The rootstocks Mazzard F12-1 and Colt were excluded as were the Italian and German groups which included only 2 cultivars in each. A chi-squared test for homogeneity between allele frequencies was carried out on contingency tables comparing each group and the total sample for each isozyme.

Allele frequencies were pooled for each group and Nei's similarity index calculated the Naperian cologarithm of this was then used to calculate Nei's index of dissimilarity or genetic distance as described by Pasteur et al (1988).

3.3 Results

The ten isozymes analysed resulted in seventy different cherry genotypes from the seventy eight cultivars samples (Appendix VI). Genotypes have been computer sorted in descending order (a to z) one isozyme at a time. Thus for 6PGD there are 3 main groups with cultivars falling into aa, ab or bb genotypes. Next each group was sorted for GOT genotypes and so on. The overall result from this data manipulation in terms of separate and identifiable cultivar groups was 6PGD = 3 groups; + GOT = 8 groups; + G6PD = 14 groups; + GPI = 21 groups; + IDH = 29 groups; + PGM = 41 groups; + FDP = 58 groups; + SKDH = 65 groups; + PER = 69 groups; + MDH = 70 groups.

Those cultivars which shared the same isozyme genotype were JI 10981 and Schneiders Spate Knorpelkirsch; JI 11247 and Vernon; Beauchamps Black (G) and Lustre (B); JI 14007, Waterloo (L) and Vega; Beauchamps Black (B) and Williams Favourite (G); Waterloo (B) and Williams Favourite (M) Mother; Burgsdorf and Merton Crane. These cultivars could not be separated from one another within each pair or triplet, on the basis of isozyme genotype, but each group could be discriminated from all other observed cherry genotypes.

Examination of trees at fruit harvest revealed that incorrect sampling of Waterloo (B), a white cherry in South Australia, had occurred and a nearby dark red cultivar was sampled in its place. Isozyme analysis also revealed it as a different genotype to that of Waterloo (L), and identical to Williams Favourite (M) Mother.

Those cultivars sampled from more than one source and having more than one genotype as determined from banding patterns were Williams Favourite, Lustre, Beauchamps Black, Bedford Prolific and Early Rivers. Within the Williams Favourite cultivar close relations exist between samples (G) and (M), differing at only 1 - 2 isozyme loci (Appendix VI). Three differences occurred between Williams favourite (M) Mother and Williams Favourite (M). Also closely related are Williams Favourite (B) and (L) separated by only two differences at isozyme loci. However, 3

to 5 differences occur between the later 2 Williams Favourite and samples (G), (M) and (M) Mother. Lustre (L) and (B) were 5 and 4 differences apart from Lustre (P), respectively and were separated by 3 differences themselves. Early Rivers (B) and (L) were separated by 6 differences. Samples of Bedford Prolific and Beauchamps Black were both separated by 3 differences.

Table 8 shows isozyme genotypes for cultivars with documented parentage where the parents have also had isozyme genotypes determined in this study. Isozyme genotypes for each parent are also given in the table. All the documented crosses can produce the cultivar genotypes shown. For example, Lapins was a result of a cross between Stella and Van, following are each of the genotypes for 6PGD, GOT, G6PD, GPI, IDH, PGM, FDP, SKDH, PER and MDH involved in the cross, then the corresponding Lapins genotypes in parentheses: ab x aa (ab); bc x ac (ac); ab x ab (ab); ab x aa (aa); ab x ab (ab); ab x aa (ab); aa x ab (ab); aa x ab (ab); ab x ab (ab).
Note here that while Vista has been reported as a cross between Hedelfingen and Victor (Dickson, 1958) and genotypes for both parents are in Appendix VI, it has not been included in Table 8 because of the existence of genetically different clones of Hedelfingen (Tehrani and Lay, 1991).

Table 9 summarises pairwise comparisons of cultivars giving a total number of differences for each cultivar, the average number of differences for each cultivar and the standard deviation for each mean. The average number of differences at each isozyme loci for each cherry cultivar as shown in Table 13 were plotted against Wilk-Shapiro rankits and gave an approximate value of 0.9802. This indicates that the data has a normal distribution and implies that the sample is representative of the worlds' population of domesticated cherries.

Table 8. Cultivars and reported parents with isozyme genotypes determined.

Cultivar	Parents	Reference	Isozymes									
			6PGD	GOT	G6PD	GPI	IDH	PGM	FDP	SKDH	PER	MDH
Lapins	Stella x Van	Lane & Schmid, 1984	ab	ac	ab	ab	aa	ab	ab	aa	ab	ab
Rainier	Bing x Van	Knight, 1969	aa	bc	ab	aa	aa	ab	aa	aa	ab	ab
Salmo	Lambert x Van	Brooks & Olmo, 1971	ab	ac	ab	ab	aa	ab	aa	aa	aa	ab
Summit	Van x Sam	Lapins, 1974	ab	bc	ab	aa	aa	ab	bb	aa	aa	ab
Sunburst	Stella x Van	Lane & Schmid, 1984	ab	ac	aa	ab	aa	ab	ab	ab	aa	ab
Vega	Bing x Victor	Tehrani & Dickson, 1967	aa	bc	ab	aa	aa	ab	ab	aa	ab	ab
V69061	Stella x Van	Button, 1982	aa	bc	ab	aa	aa	aa	ab	aa	ab	aa
	Bing		aa	bc	ab	ab	aa	ab	aa	aa	ab	ab
	Lambert		bb	bc	ab	ab	aa	ab	aa	ab	ab	ab
	Sam		bb	bc	ab	aa	aa	ab	ab	ab	aa	ab
	Stella		ab	bc	ab	ab	ab	ab	ab	aa	aa	ab
	Van		aa	ac	ab	aa	aa	ab	aa	ab	ab	ab
	Victor		ab	ac	ab	aa	aa	aa	ab	aa	ab	ab

Table 9. Total number of isozyme differences, average number of isozyme differences and standard deviation for each cherry cultivar studied.

No.	Cultivar	Total for cultivar	Average	Std deviation
1	13S-18-15	363	4.59	1.55
2	13S-24-28	332	4.20	1.42
3	Basler Longstieler	349	4.42	1.38
4	Beauchamps Black (B)	361	4.57	1.66
5	Beauchamps Black (G)	335	4.24	1.49
6	Bedford Prolific (B)	334	4.23	1.77
7	Bedford Prolific (P)	356	4.51	1.69
8	Bing OB260	327	4.14	1.50
9	Black Douglas Mother	378	4.78	1.67
10	Black Tartarian	384	4.86	1.40
11	Black Boy	471	5.96	1.43
12	Burgsdorf	271	3.43	1.53
13	Clements Pride	343	4.34	1.52
14	Colt	421	5.33	1.39
15	Compact Lambert	413	5.23	1.46
16	Compact Stella	403	5.10	1.48
17	Delta	331	4.19	1.63
18	Early Purple Guinge	398	5.04	1.53
19	Early Rivers (B)	388	4.91	1.50
20	Early Rivers (L)	389	4.92	1.69
21	Hedelfingen	458	5.80	1.85
22	Hendersons Bedford	351	4.44	1.60
23	JI 10981	276	3.49	1.66
24	JI 11247	295	3.73	1.58
25	JI 11376	350	4.43	1.50
26	JI 11610	439	5.56	1.72
27	JI 12526	407	5.15	1.83
28	JI 14007	283	3.58	1.79
29	JI 11253	301	3.81	1.58
30	Krasarica	434	5.49	1.58
31	Lambert	409	5.18	1.67
32	Lapins	298	3.77	1.50
33	Larian	321	4.06	1.44
34	Lustre (B)	335	4.24	1.46
35	Lustre (L)	392	4.96	1.47
36	Lustre (P)	342	4.33	1.82
37	Lyons	414	5.24	1.59
38	Magda	437	5.53	1.45
39	Mazzard F12-1	364	4.61	1.70

No.	Cultivar	Total for cultivar	Average	Std deviation
40	Merton Bigarreau	310	3.92	1.71
41	Merton Crane	266	3.37	1.54
42	Merton Glory	326	4.13	1.44
43	Mora Di Vignola	327	4.14	1.73
44	Napoleon	309	3.91	1.57
45	Noir De Guben	379	4.80	1.39
46	Nordwunder	406	5.14	1.57
47	NY3308	361	4.57	1.71
48	Opal	358	4.53	1.43
49	Oregon	385	4.87	1.32
50	Rainier	297	3.76	1.61
51	Salmo	333	4.22	1.44
52	Sam	370	4.68	1.71
53	Schauenburger	356	4.51	1.47
54	Schnieders Spate Knorpelkirsche	283	3.58	1.69
55	Seneca	356	4.51	1.66
56	St Margaret	472	5.97	1.73
57	Star	363	4.59	1.34
58	Stella	386	4.89	1.52
59	Sue	353	4.47	1.45
60	Summit	313	3.96	1.36
61	Sunburst	412	5.22	1.61
62	Ulster	305	3.86	1.65
63	Up-To-Date	296	3.75	1.58
64	V690616	346	4.38	1.56
65	Van	325	4.11	1.46
66	Vega	285	3.61	1.74
67	Venus	419	5.30	1.43
68	Vernon	294	3.72	1.61
69	Vic	332	4.20	1.37
70	Victor	296	3.75	1.40
71	Vista	395	5.00	1.59
72	Vittoria	283	3.58	1.40
73	Waterloo (B)	349	4.42	1.56
74	Waterloo (L)	281	3.56	1.74
75	Williams Favourite (B)	413	5.23	1.57
76	Williams Favourite (G)	367	4.65	1.65
77	Williams Favourite (L)	335	4.24	1.55
78	Williams Favourite (M)	294	3.72	1.42
79	Williams Favourite (M) Mother	347	4.39	1.56

Besides comparing between individuals it is essential to compare offspring with reported parents on the basis of the number of differences at each of the 10 isozyme loci, for added insight into the inheritance of isozymes and also to quantify genetic distances between parents and offspring which can then be used when attempting to trace the parentage of cherry seedlings or cultivars. Table 10 lists cultivars sampled in this study with one or both parents also analysed. The number of isozyme differences between each parent and the offspring are given in the last column. The average number of differences at isozyme loci between cultivars listed in Table 10 and their parents was 3.6 ± 1.2.

Table 10. Number of isozyme differences between cultivars and recorded parents.

Cultivar	Documented parents[a]	Number of isozyme differences
13S-24-28	Van[b] x Newstar	5, -[c]
JI 11247	Merton Glory O.P.*	2, -
JI 11253	Merton Glory O.P.	4, -
JI 11376	Merton Glory O.P.	2, -
Lapins	Van x Stella	4, 3
Merton Bigarreau	Knights x Napoleon	-, 3
Merton Crane	Napoleon x Bigarreau de Schreken	2, -
Seneca	Early Purple Guigne x Unknown	4, -
Stella	Lambert x JI 2420	4, -
Sue	Bing x Schmidt	5, -
Ulster	Schmidt x Lambert	-, 4
Vista	Hedelfingen x Vista	3, -
Venus	Hedelfingen x Windsor	6, -

[a] Reference for parents of each cultivar in Granger et al (1993)

[b] Female parent listed first.

[c] indicates unknown or not analysed.

* O.P. abbreviation for open pollinated.

The local variety Black Douglas showed close relations with Lustre (B) and Opal, having one and two differences out of the 10 isozyme loci analysed with each, respectively (Appendix VI). This was expected because Opal, Lustre and Black Douglas are thought to be sister seedlings, all found

by chance beneath a Williams Favourite tree, the suspected maternal parent. (W.N. Bishop, pers comm.) There are 3 isozyme differences between Lustre (B) and Opal. Five Williams Favourite trees were sampled from five locations. None of them shared identical isozyme profiles (Appendix VI). Comparing Williams Favourite (B), sourced from the same property that Black Douglas, Lustre (B) and Opal were raised showed 6, 6 and 7 isozyme differences, respectively. Black Douglas has two differences with Williams Favourite (G) and four with Williams Favourite (M) and three with Williams Favourite (M) Mother. In contrast Opal has four differences with both Williams Favourite (G) and Williams Favourite (M) while Lustre (B) has three differences with both of these cultivars. Black Douglas shows a close relationship to both Beauchamps Black (B) and (G) with 2 and 1 differences, respectively. Lustre (B) was identical for the 10 isozyme genotypes to Beauchamps Black (G) and showed 3 differences between it and Beauchamps Black (B). Opal had 4 and 3 differences between it and Beauchamps Black (B) and (G).

Clements Pride showed closest links with Up-To-Date and Early Lyons of the local cultivars having 1 and 2 differences, respectively. Up-to-Date was also closely related to other local cultivars including Burgsdorf, Waterloo (L) and Williams Favourite (M), separated from each by two differences. Clements Pride and Williams Favourite (M) are separated by 3 differences. Bedford Prolific (P) was separated by 2 differences from Lustre (B).

It should be noted that the local cultivars had small differences between them and some overseas cultivars (Appendix VI), for example, Up-to-Date, had only one isozyme difference between it and JI11247, Schnieders Spate Knorpelkirsche and Vittoria. This can only be considered coincidental as these cultivars were developed overseas and were introduced to Australia in more recent times.

Table 11 displays allele frequencies for each isozyme in each group based on country or origin. Chi-squared tests for homogeneity produced one significant result, observed values for FDP showing significant divergence away from expected values at the 5% level ($X^2_{(5)} = 12.18$). In particular Swiss and to a lesser extent Australian cultivars showed higher frequencies for the a-allele of FDP as compared to other groups, and a correspondingly low frequency for the b-allele.

Other statistically non-significant results were the higher frequency of GOT-b alleles in the Canadian group, most of which are self-fertile cultivars. The high frequency of the b-allele for SKDH associated with the Swiss group is about double that of other groups. Similarly, Australian cultivars have a higher frequency of the GPI a-allele compared to cultivars from other countries. All cultivars in this sample appear to be approaching fixation for the IDH a-allele.

Table 11. Allele frequency at polymorphic loci in cultivars from different sources for 10 leaf isozymes.

Country of origin	Isoenzyme	Allele frequency		
		a	b	c
Australia	6PGD	0.74	0.26	-
Canada		0.53	0.47	-
England		0.80	0.20	-
Switzerland		0.78	0.22	-
U.S.A.		0.56	0.44	-
ALL		0.68	0.32	-
Australia	GOT	0.32	0.18	0.50
Canada		0.21	0.50	0.29
England		0.40	0.22	0.38
Switzerland		0.20	0.30	0.50
U.S.A.		0.19	0.37	0.44
ALL		0.28	0.26	0.46
Australia	G6PD	0.47	0.53	-
Canada		0.58	0.42	-
England		0.59	0.41	-
Switzerland		0.40	0.60	-
U.S.A.		0.50	0.50	-
ALL		0.54	0.46	-
Australia	GPI	0.97	0.03	-
Canada		0.40	0.6	-
England		0.88	0.13	-
Switzerland		0.71	0.29	-
U.S.A.		0.75	0.25	-
ALL		0.84	0.16	-
Australia	IDH	0.88	0.09	0.03
Canada		0.97	0.03	0.00
England		0.91	0.06	0.03
Switzerland		0.9	0.1	0.00
U.S.A.		0.94	0.06	0.00
ALL	IDH	0.91	0.06	0.03
Australia	PGM	0.68	0.32	-
Canada		0.66	0.34	-
England		0.63	0.38	-
Switzerland		0.70	0.30	-
U.S.A.		0.56	0.44	-
ALL		0.66	0.34	-
Australia	FDP	0.74	0.26	-
Canada		0.53	0.47	-

Country of origin	Isoenzyme	Allele frequency		
		a	b	c
England		0.44	0.56	-
Switzerland		0.9	0.1	-
U.S.A.		0.69	0.31	-
ALL		0.54	0.46	-
Australia	SKDH	0.94	0.06	-
Canada		0.84	0.16	-
England		0.84	0.16	-
Switzerland		0.50	0.50	-
U.S.A.		0.81	0.19	-
ALL		0.84	0.16	-
Australia	PER	0.68	0.29	0.03
Canada		0.59	0.41	0.00
England		0.70	0.30	0.00
Switzerland		0.63	0.37	0.00
U.S.A.		0.69	0.29	0.01
ALL		0.82	0.18	0.00
Australia	MDH	0.65	0.35	-
Canada		0.58	0.42	-
England		0.59	0.41	-
Switzerland		0.60	0.40	-
U.S.A.		0.5	0.5	-
ALL		0.6	0.4	-

Table 12 gives values for Neis index of dissimilarity or genetic distance, for each pairwise comparison of the geographical groups.

Table 12. Group comparisons and Neis' dissimilarity index.

Groups compared	Dissimilarity index (D)
Australia/Canada	0.41
Australia/England	0.47
Australia/Switzerland	0.42
Australia/U.S.A.	0.48
Canada/England	0.48
Canada/Switzerland	0.54
Canada/U.S.A.	0.54
England/Switzerland	0.44
England/U.S.A.	0.52
Switzerland/U.S.A.	0.49

From Pastuer (1988) D=0.41 is interpreted as 41% or an average of 41 electrophoretically detectable allelic substitutions will have taken place in the period since the two populations being compared diverged from the last common ancestor.

Of the populations compared, Australia and Canada were the closest while Canada and U.S.A. were most distant. In general, U.S.A. was associated with larger genetic distances, and Switzerland with the least.

3.4 Discussion

The combination of the genotypes of ten isozyme loci to form an isozyme profile is a powerful tool for distinguishing individual cultivars. A difference at a single locus can be discriminating. Where no differences exist, it cannot be known weather the two cultivars are the same or different. Thus, cultivar identification with isozymes relies on the detection of differences.

The number of isozyme differences is a direct measure of genetic distance. The results provide an average for each cultivar (Table 9). However, it is interesting to note that the average number of differences across all varieties is 4.48 per 10 isozyme loci or 45%. This is of particular note when

this is compared to the average genetic distance from Table 11 of 0.469, or about 47%. Both figures are similar and are mutually supportive. It is important to note that isozyme differences can be used to compare between individual cultivars while allelic frequencies are calculated from populations of cultivars or representative samples. The rule of thumb invoked for determining relatedness of no more than 2 differences at isozyme loci was based on work conducted by Ashari et al (1988) using self-fertile *Citrus* species. Comparing this with the self-incompatible sweet cherry *Prunus avium*, it is not surprising that on average a higher number of isozyme differences were recorded for cultivars from hybridisations within one species.

In tracing the parentage of the local cultivars, Williams Favourite (B) can be assumed to be the female parent, because seedlings of Black Douglas, Lustre and Opal were found under such a tree. In this case it is most likely that Beauchamps Black is the male parent of both Black Douglas and Lustre(B). It is less probable that Beauchamps Black is the male parent of Opal being separated by 3 and 4 isozyme differences from Beauchamps Black (B) and (G), respectively. In addition, Opal is a white cherry. Fogle (1958) has described skin colour inheritance as being controlled by two genes with incomplete dominant epistasis. This implies that Williams Favourite (M) and Beauchamps Black (B) and (G) are heterozygous for one or both genes. Alternatively, the male parent of Opal could have been either Napoleon or Waterloo (white cherries). If this were the case, the male parent would be Waterloo because it is different at only 3 isozyme loci compared to 5 for Napoleon.

None of the Williams Favourite types have 2 or less isozyme differences with Lustre or Opal, and while Williams Favourite (B) is 6 differences away from Black Douglas, other Williams Favourite types are within 2 and 3 differences. There appears to be no alternative possibilities for parentage of Opal and Black Douglas. Here it is proposed that Black Douglas and Lustre (B) are the result of hybridisation between Williams Favourite and Beauchamps Black while Opal resulted from hybridisation between Williams Favourite and Waterloo. (Note that Waterloo (B) was a dark cherry and incorrectly labelled).

Clements Pride and Up-to-Date are closely related having 1 isozyme difference between them. It is proposed that Up-to-Date, is in fact, a parent of Clements Pride for two additional reasons, (a) it is an older cultivar which was in commercial use at the time Clements Pride was developed (W.N. Bishop pers. comm.) and (b) both cultivars are closely related to Williams Favourite (M). Early Lyons is most likely the other parent of Clements Pride. Up-to-Date has the parentage of Williams Favourite (M) and Waterloo. Burgsdorf is the only other closely related local cultivar tested but it is eliminated as a parent by the fact that its flowering period would not overlap those of Waterloo or Williams Favourite, the latter two cultivars having similar flowering times and historically planted in close proximity for cross pollination.

The probability of sports or mutations being the derivation of local cultivars was considered to be low because the compact mutants of Lambert and Stella were both separated by 5 isozyme differences from parent material and this represents greater genetic difference than that observed between local cultivars and likely parents. The existence of genetically different types of Williams Favourite, Beauchamps Black and other local varieties made tracing parentage more difficult. Ashari et al (1988) showed differences between like named mandarin cultivars and suggested possible causes as mutation, multiple origin of cultivar material, mistaken labelling or propagation via monoembryonic seed. Parental identification was further complicated by marketing different genotypes from different regions under the same name, no records of parental types and an inability to use morphological characters as discriminating features. All these problems, excepting monoembryonic seed, apply equally to cherries. Propagation via seed does explain some variation. Subsequent to isozyme analysis it was discovered that Williams Favourite (M) mother was in fact derived from a seedling which arose from discarded Williams Favourite fruit (R. Mieglich pers. comm.) Further variation has been caused by mislabelling because the Williams Favourite (M) held in the Lenswood Collection was supposedly sourced from the Williams Favourite (M) mother tree and yet they had different isozyme genotypes. Similarly, Williams Favourite (L) and Williams Favourite (B) were separated by 3 differences and Williams Favourite (L) was recorded as being sourced from Williams Favourite (B). Waterloo (B) was observed to

have black skin and flesh and red juice. It was separated by one isozyme difference from Beauchamps Black (B) and Williams Favourite (L) and could be either of these types or a hybrid.

Verification of parentage by comparing the isozyme genotypes of cultivars with that of their documented parents confirmed those reports, and showed that isozymes were controlled by heritable genes which added further support for the assignment of genotypes to the observed banding patterns.

Examination of allelic frequencies showed only one statistically significant divergence away from expected, and that was for the FDP locus. Both the Swiss and Australian samples showed a higher proportion of the a allele. Both these groups seemed to have diverged more rapidly compared to other groups. This could have been caused by selection for a characteristic closely linked to the FDP a-allele. Alternatively, the FDP-b allele may be linked to or itself be the basis of a selective disadvantage in both of these countries. The other non-significant but noticeable divergence associated with the Swiss group was that of the SKDH b-allele with a frequency of 0.5, while other groups had frequencies ranging from 0.81 - 0.94 for this allele. Again this may have been caused by selection pressures within the breeding program against this allele. Another association to be noted was the high frequency of the GOT b-allele with the Canadian cultivars, most of which originated from self-fertile parents, and while not statistically significant, was significant in light of the work conducted in apple by Manganaris and Alston (1987) which suggested that the GOT alleles were linked to the self incompatibility locus. Furthermore, Leach (1988) showed that disturbed segregation ratios between isozyme loci have been caused by linkage to self-incompatibility genes according to Leach (1988). This is probably the reason behind the deficiency of bb homozygotes for GPI. In this regard, linkage to the self-incompatibility gene generates results that would also be expected if isozymes were linked to a lethal character such as albinism. In this situation fixation of an isozyme allele could occur as was the case in the results for the a-allele of IDH. The deficiency of bb homozygotes for PGM may be a result of the bb phenotype

being indistinguishable from the heterozygous ab genotype due to comigration of another PGM locus, as reported in other species (Kephart, 1990).

Using Neis' dissimilarity index based on allele frequencies pairwise comparisons showed similar genetic distances indicating that divergence from the last common ancestor has been at a similar rate but in different directions. This could in part be attributed to the selection pressure exerted by climatic differences between the different localities. Many of the U.S.A. cultivars include Bing in their background; and this may have resulted in this group showing greater divergence from other groups. Genetically closest were the Australian and Canadian groups. Because the two countries share common links in terms of human settlement this may have followed through whereby the same cultivars were imported from England to start cherry culture in both countries. The low genetic distance between Australia and Switzerland reflects the significant result for the allele frequency of FDP and the similar divergence that Australian and Swiss cultivars have followed for this isozyme.

CHAPTER 4: INHERITANCE AND LINKAGE OF ISOZYMES IN *PRUNUS AVIUM*

4.1 Introduction

The interpretation of isozyme banding patterns in the majority of laboratories relies on experience and comparison with other related species which have previously undergone isozyme analysis (Kephart 1990). The interpretation and assignment of genotypes should be tested by hybridisation experiments, but this is not always possible. (Richardson et al 1986). Tree crops are poorly suited to genetic studies because they are large, have a long generation time and extended juvenile period. Consequently, there is limited knowledge of even simply inherited morphological traits as compared to cereal crops. Apples and peaches have been the subject of most genetic studies in tree crops, peach having more than 30 identified simply inherited characters (Byrne 1989). While, in comparison, Knight (1969) reports eleven plant, leaf, flower and fruit characteristics as simply inherited and likely to be under the control of a single gene in sweet cherry. There is no data for the majority of tree fruit crops regarding the position of genes on chromosomes or linkage with other traits.

The quantification of isozyme inheritance through the examination of segregation ratios in the progeny of controlled crosses is necessary for verification of genotype assignment to isozyme loci. Such work will underpin more rapid genetic gain in this species in the future, especially if isozymes are found to be markers for commercially desirable characteristics.

Disturbed segregation ratios were observed in the results reported in the previous chapter and were probably caused by linkage to the self-incompatibility locus. Self-incompatibility results in the failure of the plants own pollen to grow through the stylar tissue of the mother plant. Crane and Brown (1937) assigned the S-alleles to the self-incompatibility gene in sweet cherry. Pollen grains are haploid and carry only one S-allele. If this allele is the same as one of those in the diploid tissue of the style, pollen tube growth is arrested.

Leach (1988) developed a mathematical calculation to estimate linkage between isozymes and a gametophytic incompatibility locus. With this approach, only those crosses that were 50% compatible could be used. This is because the effect of an isozyme allele being linked to the incompatibility locus is similar to being linked to a recessive lethal gene in that if a cross is fully compatible there is no evidence or expression of self-incompatibility in the isozyme segregation ratios of the resultant progeny. If a cross is 50% incompatible and isozymes are linked to the incompatibility locus a deficiency of homozygous isozyme genotypes is observed.

4.2 Materials and Methods

Isozyme analysis, as described earlier, was conducted on the leaves and embryos of first generation progeny derived from a breeding program at Lenswood Horticultural Centre. Single locus chi-squared analysis for goodness-of-fit to expected segregation ratios was conducted for each cross. Contingency chi-squared analyses were then used to test for independent assortment between jointly segregating loci. If significant deviation was detected, recombination fractions and their standard errors were calculated. Analyses were carried out using the computer program LINKAGE-1 (Suiter et al 1983). The procedure was for comparison amongst the isozyme loci when parents were both fully and 50% compatible because random assortment of genes at meiosis would not lead to the situation in which the S alleles over-rode all other linkages. Note, however, that for the analysis to detect linkage, at least one parent must be a double heterozygote for the two loci being examined, or each parent could have been heterozygous for one of the two loci.

For estimation of the recombination frequency and its variance between the isozyme genes and a incompatibility gene the methods of Leach (1988) were used. Van x Bing (S_1S_3 x S_3S_4) and Stella x Stella (S_4S_4' x S_4S_4') were known to be 50% compatible and could be used in the analyses. The skewed segregation ratios of isozyme loci and deficiency of homozygotes in the progeny of Black Douglas x Stella and Williams Favourite x Stella were also indicative of a 50% compatible cross and linkage to a gametophytic incompatibility locus (Leach 1988). While the S-genotypes of Black

Douglas and Williams Favourite were unknown, on the above evidence, it was assumed that a 50% cross had been made and the data was included in the analyses.

4.3 Results

In the event that parents of a particular cross were homozygous for any of the isozyme loci, that locus was omitted from the analyses and does not appear in the results. Table 13 shows the segregation ratios found in progeny for each isozyme locus and the outcome of chi-squared analysis for goodness-of-fit to expected Mendelian ratios and its accompanying probability at the 0.05 level. Examination of Table 13 reveals that most of the chi-squared F-tests were highly significant, leading to a rejection of the hypothesis that observed and expected ratios were the same. For instance, in the first cross listed in table 13, Black Douglas x Stella, both parents have the heterozygous bc genotype for the GOT isozyme and would be expected to produce progeny with the bb, bc, and cc genotypes in the ratio of 1:2:1. Results show that no cc progeny were observed and only 2 bb homozygotes were recorded while 94 bc genotypes were recorded. Further examination of Table 13 shows the same result for all of the crosses listed. That is the combination of parents heterozygous for the GOT c allele and the absence of the cc genotype in progeny. In contrast, some isozyme loci did display expected segregation ratios. For instance FDP in the progeny of Black Douglas x Stella and Williams Favourite x Stella crosses. Other families showed disturbed segregation ratios for FDP. Similar outcomes were observed for GPI, G6PD, IDH and SKDH. While the Van x Stella cross was completely compatible most of the isozymes displayed disturbed segregation ratios.

Table 13. Chi-squared test for Goodness-of-fit to expected Mendelian segregation ratios of individual loci.

Locus	Parents	Parental genotypes	Progeny genotypes	Expected ratio	X^2	P
G6PD	Black Douglas x Stella	ab x ab	42aa:49ab:12bb	1:2:1	17.72	0.000
GOT		bc x bc	2bb:94bc:0cc	1:2:1	88.25	0.000
6PGD		aa x ab	62aa:38ab	1:1	5.76	0.016
FDP		aa x ab	42aa:46ab	1:1	0.18	0.669
GPI		aa x ab	49aa:55ab	1:1	0.35	0.556
IDH		aa x ab	81aa:11ab	1:1	53.26	0.000
PGM		aa x ab	66aa:38ab	1:1	7.54	0.006
MDH		aa x ab	60aa:42ab	1:1	3.18	0.075
G6PD	Williams Favourite x Stella	ab x ab	46aa:80ab:40bb	1:2:1	0.65	0.722
GOT		bc x bc	0bb:83bc:0cc	1:2:1	83.00	0.000
6PGD		ab x ab	56aa:81ab:29bb	1:2:1	8.88	0.012
FDP		aa x ab	70aa:66ab	1:1	0.12	0.731
GPI		aa x ab	103aa:64ab	1:1	9.11	0.003
IDH		aa x ab	119aa:40ab	1:1	39.25	0.000
PGM		ab x ab	86aa:83ab:0bb	1:2:1	87.58	0.000
SKDH		ab x aa	106aa:59ab	1:1	13.39	0.000
MDH		ab x ab	107aa:61ab:1bb	1:2:1	146.00	0.000
G6PD	Stella x Stella	ab x ab	51aa:139ab:0bb	1:2:1	68.14	0.000
GOT		bc x bc	2bb:185bc:0cc	1:2:1	179.13	0.000
6PGD		ab x ab	63aa:97ab:30bb	1:2:1	11.55	0.003
FDP		ab x ab	105aa:64ab:7bb	1:2:1	122.23	0.000
GPI		ab x ab	66aa:116ab:7bb	1:2:1	46.62	0.000

Locus	Parents	Parental genotypes	Progeny genotypes	Expected ratio	X^2	P
IDH		ab x ab	174aa:8ab:7bb	1:2:1	453.48	0.000
PGM		ab x ab	144aa:42ab:0bb	1:2:1	278.90	0.000
MDH		ab x ab	110aa:71ab:9bb	1:2:1	119.51	0.000
G6PD	Van x Bing	ab x ab	135aa:65ab:0bb	1:2:1	206.75	0.000
GOT		ac x bc	42ab:81ac:57bc:0cc	1:1:1:1	39.48	0.000
GPI		ab x ab	113aa:86ab:1bb	1:2:1	129.36	0.000
PGM		aa x ab	60aa:140ab	1:1	32.00	0.000
SKDH		ab x aa	56aa:144ab	1:1	38.72	0.000
MDH		ab x ab	18aa:182ab:0bb	1:2:1	137.72	0.000
6PGD	Van x Stella	aa x ab	149aa:11ab	1:1	119.02	0.000
G6PD		ab x ab	114aa:48ab:0bb	1:2:1	187.33	0.000
GOT		ac x bc	57ab:79ac:25bc:0cc	1:1:1:1	90.30	0.000
FDP		aa x ab	107aa:39ab	1:1	31.67	0.000
GPI		aa x ab	62aa:101ab	1:1	9.33	0.002
IDH		aa x ab	79aa:75ab	1:1	0.10	0.747
PGM		ab x ab	48aa:99ab:0bb	1:2:1	49.04	0.000
SKDH		ab x aa	50aa:63ab	1:1	1.50	0.221
MDH		ab x ab	50aa:99ab:0bb	1:2:1	49.67	0.000

Table 14. Probability and recombination values between loci observed to be linked in progeny from various controlled hybridisations within sweet cherry.

Loci	Parents	Parental Genotypes	Progeny	Genotypes							P	r^{+}SE
G6PD/MDH	[+]B.D. x Stella	ab/aa x ab/ab	34aa/aa	8aa/ab	24ab/ab	1bb/aa	10bb/ab				0.000	0.1698±0.0528
6PGD/FDP	B.D. x Stella	aa/aa x ab/ab	20aa/aa	31aa/ab	22ab/aa	11ab/ab					0.014	0.3691±0.0527
FDP/PGM		aa/aa x ab/ab	31aa/aa	11aa/ab	24ab/aa	22ab/ab					0.036	0.3977±0.0522
GPI/PGM		aa/aa x ab/ab	26aa/aa	23aa/ab	40ab/aa	15ab/ab					0.038	0.3944±0.0479
IDH/MDH		aa/aa x ab/ab	52aa/aa	28aa/ab	3ab/aa	7ab/ab					0.032	0.3440±0.0501
G6PD/FDP	W.F. x Stella	ab/aa x ab/ab	21aa/aa	14aa/ab	36ab/aa	28ab/ab	10bb/aa	24bb/ab			0.017	0.3477±0.0584
G6PD/GPI		ab/aa x ab/ab	32aa/aa	14aa/ab	42ab/aa	38ab/ab	29bb/aa	11bb/ab			0.048	0.5000±0.0549
G6PD/IDH		ab/aa x ab/ab	38aa/aa	4aa/ab	56ab/aa	20ab/ab	23bb/aa	15bb/ab			0.008	0.3375±0.0535
6PGD/SKDH		ab/ab x ab/aa	36aa/aa	18aa/ab	58ab/aa	22ab/ab	12bb/aa	16bb/ab			0.017	0.3658±0.0535
6PGD/MDH		ab/ab x ab/aa	55aa/aa	14aa/ab	1aa/bb	35ab/aa	31ab/ab	0ab/bb			0.003	0.3955±0.0593
PGM/SKDH		ab/ab x ab/ab	64aa/aa	21aa/ab	42ab/aa	38ab/ab	0bb/aa	0bb/ab			0.009	0.2470±0.0475
PGM/MDH		ab/ab x ab/ab	66aa/aa	19aa/ab	1aa/bb	41ab/aa	42ab/ab	0ab/bb	0bb/aa	0bb/ab	0bb/bb	0.004	0.1978±0.0247
G6PD/FDP	Stella x Stella	ab/ab x ab/ab	34aa/aa	7aa/ab	0aa/bb	71ab/aa	57ab/ab	7ab/bb	0bb/aa	0bb/aa	0bb/bb	0.014	0.2865±0.0298
G6PD/MDH		ab/ab x ab/ab	44aa/aa	7aa/ab	0aa/bb	66ab/aa	64ab/ab	9ab/bb	0bb/aa	0bb/ab	0bb/bb	0.000	0.2493±0.0265
6PGD/IDH		ab/ab x ab/ab	53aa/aa	7aa/ab	3aa/bb	93ab/aa	1ab/ab	2ab/bb	28bb/aa	0bb/ab	2bb/bb	0.012	0.4360±0.0355
FDP/PGM		ab/ab x ab/ab	88aa/aa	0aa/bb	41ab/aa	22ab/ab	0ab/bb	3bb/aa	4bb/ab	0bb/bb		0.009	0.1982±0.0245
FDP/MDH		ab/ab x ab/ab	79aa/aa	25aa/ab	1aa/bb	19ab/aa	37ab/ab	8ab/bb	2bb/aa	5bb/ab	0bb/bb	0.000	0.1901±0.0237
IDH/PGM		ab/ab x ab/ab	141aa/aa	29aa/ab	0aa/bb	0ab/aa	8ab/ab	0ab/bb	2bb/aa	5bb/ab	0bb/bb	0.000	0.1034±0.0168
IDH/MDH		ab/ab x ab/ab	104aa/aa	63aa/ab	7aa/bb	1ab/aa	5ab/ab	2ab/bb	5bb/aa	2bb/ab	0bb/bb	0.016	0.2461±0.0264
PGM/MDH		ab/ab x ab/ab	98aa/aa	41aa/ab	5aa/bb	12ab/aa	26ab/ab	4ab/bb	0bb/aa	0bb/ab	0bb/bb	0.000	0.1872±0.0229
G6PD/GOT	Van x Bing	ab/ac x ab/bc	42aa/ab 46aa/ac	31aa/ac 16aa/ac	0ab/ab 35ab/ac	26ab/bc	4ab/cc	0bb/ab	0bb/ac	0bb/bc	0bb/cc	0.000	-*
GOT/PGM		ac/aa x bc/ab	6ab/aa	36ab/ab	25ac/aa	56ac/ab	15bc/aa	42bc/ab	14cc/aa	6cc/ab		0.000	-
GOT/SKDH		ac/ab x bc/aa	17ab/aa	25ab/ab	18ac/aa	63ac/ab	10bc/aa	47bc/ab	11cc/aa	9cc/ab		0.002	-
GPI/MDH		ab/ab x ab/ab	12aa/aa	101aa/ab	0aa/bb	5ab/aa	81ab/ab	0ab/bb	1bb/aa	0bb/ab	0bb/bb	0.021	0.3820±0.0327

Loci	Parents	Parental Genotypes	Progeny	Genotypes								P	r^{+}SE
PGM/SKDH		aa/ab x ab/aa	32aa/aa	28aa/ab	24ab/aa	116ab/ab						0.000	0.2600±0.031
PGM/MDH		aa/ab x ab/ab	11aa/aa	49aa/ab	0aa/ab	7ab/aa	133ab/ab	0ab/bb				0.010	0.3888±0.0488
6PGD/G6PD	Van x Stella	aa/ab x ab/ab	113aa/aa	36aa/ab	0aa/bb	1ab/aa	10ab/ab	0ab/bb				0.000	0.0088±0.0104
6PGD/GOT		aa/ac x ab/bc	48aa/ab	76aa/ac	25aa/bc	0aa/cc	8ab/ab	3ab/ac	0ab/bc	0ab/cc		0.05	-
6PGD/IDH		aa/aa x ab/ab	77aa/aa	66aa/ab	2ab/aa	9ab/ab						0.023	0.4415±0.0400
G6PD/GOT		ab/ac x ab/bc	23aa/ab 72aa/ac	19aa/bc 0aa/cc	34ab/ab 7ab/ac	6ab/bc	0ab/cc	0bb/ab	0bb/ac	0bb/bc	0bb/cc	0.000	-
G6PD/FDP		ab/aa x ab/ab	61aa/aa	37aa/ab	45ab/aa	2ab/ab	0bb/aa	0bb/aa	0bb/ab			0.000	0.3775±0.0569
G6PD/IDH		ab/aa x ab/ab	68aa/aa	40aa/ab	11ab/aa	35ab/ab	0bb/aa	0bb/ab				0.000	0.3703±0.0550
G6PD/MDH		ab/ab x ab/ab	46aa/aa	55aa/ab	0aa/ab	4ab/aa	44ab/ab	0ab/bb	0bb/aa	0bb/ab	0bb/bb	0.000	0.2198±0.0279
GOT/FDP		ac/aa x bc/ab	48ab/aa	7ab/ab	39ac/aa	29ac/ab	18bc/aa	3bc/ab	0cc/aa	0cc/ab		0.001	-
GOT/GPI		ac/aa x bc/ab	30ab/aa	27ab/ab	28ac/aa	51ac/ab	4bc/aa	21bc/ab	0cc/aa	0cc/ab		0.015	-
GOT/IDH		ac/aa x bc/ab	15ab/aa	39ab/ab	44ac/aa	33ac/ab	20bc/aa	3bc/ab	0cc/aa	0cc/ab		0.000	-
GOT/MDH		ac/ab x bc/ab	8ab/aa 44ab/ab	0ab/bb 25ac/aa	48ac/ab 0ac/bb	17bc/aa	6bc/ab	0bc/bb	0cc/aa	0cc/ab	0cc/bb	0.000	-
FDP/PGM		aa/ab x ab/ab	43aa/aa	48aa/ab	0aa/bb	3ab/aa	36ab/ab	0ab/bb				0.000	0.652±0.0306
FDP/SKDH		aa/ab x ab/aa	8aa/aa	49aa/ab	33ab/aa	6ab/ab						0.000	0.1459±0.0360
FDP/MDH		aa/ab x ab/ab	45aa/aa	54aa/ab	0aa/ab	1ab/aa	33ab/ab	0ab/bb				0.000	0.0217±0.0179
IDH/PGM		aa/ab x ab/ab	40aa/aa	39aa/ab	0aa/bb	7ab/aa	55ab/ab	0ab/bb				0.000	0.1489±0.0424
IDH/SKDH		aa/ab x ab/aa	22aa/aa	53aa/ab	27ab/aa	5ab/ab						0.000	0.2524±0.042
IDH/MDH		aa/ab x ab/ab	50aa/aa	22aa/ab	0aa/bb	0ab/aa	75ab/ab	0ab/bb				0.000	0.000±n.d.
PGM/SKDH		ab/ab x ab/aa	5aa/aa	36aa/ab	45ab/aa	27ab/aa	0bb/aa	0bb/ab				0.000	0.1220±0.0435
PGM/MDH		ab/ab x ab/ab	34aa/aa	7aa/ab	0aa/bb	16ab/aa	77ab/ab	0ab/bb	0bb/aa	0bb/ab	0bb/bb	0.000	0.0917±0.0917
SKDH/MDH		ab/ab x aa/ab	0aa/aa	45aa/ab	0aa/bb	50ab/aa	5ab/ab	0ab/bb				0.000	0.0000±n.d.

+ Abbreviations for cultivar names B.D. = Black Douglas W.F. = Williams Favourite.
* Possible multiple parent linkage and unknown phase of alleles precludes estimation of r.

Results of the chi-squared test for independent assortment between jointly segregating loci have been tabulated in Table 14. While each pair of loci have been tested in contingency tables, only those showing significant departures have been included in the table along with estimates of the recombination fraction and standard error.

Estimates of the recombination frequency between isozymes and the S-gene of the self-incompatibility locus are shown in Table 15.

Table 15. Crosses as defined by Leach (1988) between cultivars of known S and isozyme genotypes, observed ratios of isozymes in progeny and the estimated recombination fraction and its variance.

Cross*	Parents (♀ x ♂)	Isozyme	Progeny			Estimation of recombination frequency r	Variance (r)
			aa	ab	bb		
Type 1	Van x Bing	PGM	60	140	-	0.3	0.001
Type 2+	Van x Bing	SKDH	56	144	-	-	-
Type 3	Van x Bing	G6PD	135	65	0	0.0	0.000
		GPI	113	86	1	0.009	0.000
		MDH	18	182	0	0.0	0.000
	Stella x Stella	G6PD	51	139	0	0.0	0.000
		GOT	2	185	0	0.0	0.000
		6PGD	63	97	30	0.32	0.002
		FDP	105	64	7	0.0625	0.0007
		GPI	66	116	7	0.0959	0.0009
		IDH	174	8	7	0.0387	0.0004
		PGM	144	42	0	0.0	0.000
		MDH	110	71	9	0.0756	0.0007

* Refer to Table 1 Leach (1988) for a description of types of cross.

+ Note for Cross type 2 it is not possible to calculate estimates of the recombination frequency.

4.4 Discussion

Tests for independent assortment between the isozyme loci showed that 6PGD, G6PD, MDH, PGM, SKDH, FDP, GOT and IDH occur within one linkage group. GPI is linked to G6PD with a recombination frequency of 0.5, suggesting that it resides on another chromosome. Different

families showed different recombination frequencies which was expected because different individuals used as parents are expected to have different distances between the same genes and alleles along the DNA. In the case of PGM, which displays close linkage to most of the other isozymes (Table 14), caution needs to be exercised when interpreting these results because of the absence of the homozygous bb genotype. According to Kephart (1990) PGM phenotypes in many species are a result of comigration of 2 loci. So for cherries the bb genotype may be present but indistinguishable because of banding from another PGM locus. In this situation the recombination frequency would be under-estimated.

The isozymes examined are all involved in essential metabolic cycles such as the citric acid cycle and so it seems reasonable that they are all inherited as one linkage group. In some instances there is overlap between cycles where reaction products in one cycle may react in another. In this case linkage between isozymes in overlapping cycles would also be advantageous to the survival of the organism.

Most of the isozymes segregated into non-Mendelian ratios for the crosses examined. Only FDP, GPI and MDH in the progeny of Black Douglas x Stella crosses and IDH and SKDH in the progeny of Van x Stella crosses (Table 13) showed agreement between expected and observed segregation ratios. The cross between Van and Stella was fully compatible (S_1S_3 x S_4S_4') and it was expected in this case that, assuming inhibition of pollen tube growth did not occur, that all of the isozyme loci studied would exhibit expected Mendelian ratio's. This was not the case and may have been caused in part by the close linkages shown between the isozyme loci.

In the case of crosses with 50% compatibility such as Stella x Stella (S_4S_4' x S_4S_4') and Van x Bing (S_1S_3 x S_3S_4), disturbed segregation ratio's were both expected and observed, and as previously mentioned the basis of this is linkage to the self-compatibility locus. Linkage analysis as described by Leach (1988) showed that GOT, 6PGD, IDH, G6PD, GPI, FDP, PGM and MDH are all linked to the self-incompatibility gene (Table 15). In some cases the recombination frequency was

estimated at zero suggesting that the isozymes were a functional part of the S-gene, particularly since both characteristics show codominant inheritance. If this were so the radiation treatment of pollen to generate the S_4' mutant should have produced a point mutation resulting in at least a single base change in the DNA sequence. This would have lead to a change in charge of the encoded isozyme and subsequently changed its distance of migration in an electric field. Because there were no changes in any of the isozyme phenotypes in samples from self-fertile cultivars, it tended not to support the role of isozymes as a functional part of the S-gene. Although it is possible in the case of a point mutation that a change in molecular charge did not occur. What the evidence does suggest is that the S-gene occurs in at least two parts, because it is linked by an estimated recombination fraction of 0 and 0.096 to the G6PD and GPI loci, respectively while those two loci were linked by a recombination fraction of 0.5. de Nettancourt (1977) describes a theory which was developed by Lewis in the 1960's and suggests that the incompatibility gene as found in monofactorial gametophytic systems such as in cherries, consists of three linked segments. The three segments are thought to involve a specificity part of recognition for pollen and style s-genes. The other two segments have been attributed to regulatory genes for pollen and stylar activity. The linkage relationships between isozymes and the S-gene found here in sweet cherry support the multi-segment locus theory for the gametophytic self-incompatibility gene.

In apple, GOT and IDH have been identified as markers for the S-gene and combined S and isozyme genotypes have been proposed (Managanaris and Alston 1987). Data presented here has established close linkage between the GOT locus and the S locus in sweet cherry. Closer examination of segregation ratios in Table 13 for GOT shows an almost complete absence of homozygotes. This is expected with close linkage to the incompatibility locus because like S-alleles cannot combine to form homozygotes. In the case of Stella, which carries the self-fertile allele S_4', it was expected that selfing would produce homozygous self-fertile (S_4'S_4')progeny and that this would be reflected by a greater number of progeny with homozygous GOT genotypes. Results in Table 13 show this was not so; that is, progeny from Stella x Stella crosses had an absence of homozygotes in the GOT isozyme data. Thus, just as S_4S_4 homozygotes were not

formed under the gametophytic incompatibility system of cherry nor were S_4'S_4' homozygotes. That is, the co-dominant inheritance of the S-alleles has not been altered by the self-fertile mutation. Given this, the linkage genotypes of the S and GOT alleles are proposed as S_4b/S_4'c. There were a small number of GOT, bb homozygotes recorded (Table 13) and these are probably a result of crossing over between chromatids of homologous chromosomes resulting in the rearrangement of the linked alleles ie S_4b/S_4'b.

Following this proposal of linked genotypes it should be possible to use the GOT genotypes to determine S genotypes of cherry cultivars. This can be tested by comparing the GOT genotypes of cultivars with known S-alleles. For instance in Table 13 Van is listed as having the ac GOT genotype and Bing the bc genotype. According to the initial proposal both Van and Bing should be carrying the S_4' allele. In fact the S allele they have in common is S_3. This suggests that the S_4' is a mutation of the S_3 allele. To prove this beyond doubt the S genotypes of the parents and progeny from controlled hybridisations would have to be determined by pollinating flowers with pollen of known S genotypes. Analyses to test for independent assortment between jointly segregating S and isozyme loci could then be conducted to determine linkage relationships.

CHAPTER 5 SELF-INCOMPATIBILITY IN SWEET CHERRY

5.1 Introduction.

Pollination in sweet cherry is subject to a gametophytic homomorphic system controlled by a gene complex known as the S-locus. It is a mechanism of self rejection, whereby any pollen grain may germinate and accomplish fertilisation provided the S-allele it carries is not present in the diploid tissue of the female organs. Usually incompatible crosses result in inhibition of pollen tube growth through the style (de Nettancourt, 1977).

Results from the previous chapter showed there was not only linkage amongst the isozyme loci studied, but their was also linkage between the isozyme loci and the self-incompatibility or S-locus. Linkage between isozyme loci and the S-locus has been described previously in apple (Manganaris and Alston 1987) and the GOT locus served as a marker for the S-alleles in apple. A similar situation was found in cherry whereby the linkage of the isozymes to the S-gene was so close it suggested that the isozymes may have been a functional part of the self-incompatibility reaction in cherry. Previous models developed to explain the self-incompatibility reaction were based on the interaction of enzymes. de Nettancourt(1977) reviewed the literature for these models, and for instance, the Kroers theory proposed a presence and absence of enzymes in different reproductive tissue. Lewis proposed the dimer repressor theory, whereby enzymes produced by the same S-allele in pollen and style tissue combined to produce a molecule which inhibited pollen tube growth. Pandey proposed a more direct mechanism in which S-alleles coded for peroxidase isozymes and they were suggested to be the basis of the incompatibility reaction in *Nicotiana*. In each theory the S-allele encodes the synthesis of a polypeptide (or absence in the Kroers theory). And in each theory the polypeptides produced by the S-gene are thought to be enzymes. As discussed in the previous chapter, the absence of an observable banding difference at any of the isozyme loci for the self-fertile mutant Stella ruled out isozymes as a functional part of the S-locus. Instead the S-gene in cherry was thought to consist of two segments of DNA interspersed by the isozyme loci. Recent work by McClure et al (1990) provided a visualisation of

the S-locus for *Nicotiana alata* which has similarities with that put forward here for sweet cherry. Using cDNA encoding the S_2 allele of *N. alata* they found that only 56% of amino acid identity between 3 alleles was identical. "The amino acid substitutions, and deletions or insertions are scattered throughout the sequence, but are concentrated in the amino half of the sequence in a series of hypervariable domains. These variable regions are interspersed with conserved regions that are analogous to the framework domains characteristic of a number of hypervariable gene systems".

Glycoproteins have been found in the pistils of *P. avium* (Mau, Raff and Clarke 1982) *Petunia hybrida* (Kamboj and Jackson, 1986) and *N. alata* (McClure et al, 1989). In addition, the glycoproteins were shown to be under the control of the S-alleles. The latter workers showed that the S-allele associated glycoproteins in *N. alata* were ribonucleases. Stylar ribonucleases are also associated with self-incompatibility genes in Japanese pear *Pyrus serotina* (Sassa et al, 1993). In *N. alata* the ribonucleases are secreted into the mucilage of the transmitting tissue of mature styles, along the path followed by pollen tubes growing toward the ovary (McClure et al, 1990). In the Japanese pear study, ribonucleases were detected in the style and ovary but not in leaf, pollen or germinated pollen. McClure et al(1990) observed the degradation of pollen RNA in incompatible matings of *N. alata*, but not in compatible matings. These workers then proposed that style S-RNases produced by *N. alata* are the primary cause of growth inhibition of self pollen tubes through an S-allele specific cytotoxic reaction. Following this Gray et al (1991) conducted experiments with *in vitro* grown pollen tubes of *N. alata* and found that S-RNases were taken up into the cytoplasmic compartment of pollen tubes where protein biosynthesis was inhibited, through the inhibition of RNA translation. There was no evidence of a specific mRNA substrate for the S-RNases. It was thought that allelic specificity was dependant on selective uptake of S-RNases into pollen tubes. Other workers have investigated the role of glycan side chains on the ribonucleases of *Petunia hybrida* (Broothaerts et al, 1991) and sugar side chains on ribonucleases in the recognition process of Japanese pear (Sassa et al, 1993). In both cases the ribonuclease

activity appears to be unaffected by these portions of the molecule, again suggesting that selective uptake by the pollen tube cell wall is the basis of recognition for the S-genes.

Thus, the currently accepted theory (McClure et al, 1991) is slightly different as compared to previous models. The latter were based on the S-alleles coding for different enzymes which determined specificity of the incompatibility reaction. Whereas, the current theory proposes that the S-alleles code for pollen tube cell wall structural differences which results in the selective uptake of SRNases. Under this model the activity segment of the S-gene codes for different molecular forms of RNase while the specificity segment of the S-gene codes for pollen tube cell wall structure. Therefore, experiments were conducted to examine RNase isozymes in the reproductive tissue of sweet cherry. Analysis of other isozymes in the reproductive tissue of sweet cherry was also included to detect any effect self-incompatibility maybe having on them because they are involved in cycles essential for growth and maintenance of life in the plant. For example GOT, MDH and IDH are in the Citric Acid Cycle which amongst other things produces the amino acids glutamate, aspartate, alanine and glycine. The cycle also produces intermediates for many other reactions. Thus it can be seen that directly or indirectly, say in the event that RNase did halt protein synthesis, isozyme expression could be effected in these tissues, realising of course that at the same time they have known functions other than that of self-incompatibility.

A second related issue was revealed by the segregation of the GOT isozyme genotypes in the progeny of Stella x Stella hybridisations as presented in the previous chapter. Stella had the unconfirmed S-genotype S_4S_4'. The expected outcome of selfing would be $S_4S_4':S_4'S_4'$ in a ratio of 2:1. The S_4S_4 homozygote cannot be formed because the same S-alleles cannot combine. In addition, Stella is heterozygous for GOT (bc) and the expected outcome of selfing is bb:bc:cc in the ratio 1:2:1 in the progeny. Contrary to the expected only the bc genotype was observed. Given the linkage ($r=0.0$) between the S-locus and GOT it can be concluded that the $S_4'S_4'$ homozygote was not formed. Otherwise one or other of the homozygous classes for GOT would also have been observed. Note that the ovary of sweet cherry carries two ovules, each one is haploid and carries one of the S-alleles of the parent. For Stella one ovule would be S_4 and the other S_4'. In the

fertilisation process only one ovule develops into an embryo while the other degenerates. Viable embryos were produced after selfing Stella and the S-genotype for embryos was probably S_4S_4'. Therefore, the S_4' allele must have been recognised at another site in the pistil. It remained to physically observe the inhibition of S_4' pollen tubes with microscope studies. Microscopic examination of crosses could also be used to elucidate the S-genotype of Stella which based on its reported parentage being Lambert (S_3S_4) by JI2420 (S_3S_4') (Lapins 1971) it could be either S_4S_4'or S_3S_4'. By crossing Stella with Early Rivers and Van, both having the S_1S_3 genotype any incompatibility reactions of pollen tubes can be interpreted as meaning that Stella possesses an S_3 allele, no incompatibility reactions would indicate that Stella has the S_4S_4' genotype.

5.2 Materials and Methods.

5.2.1 Isozymes

Pistils were extracted and analysis conducted for 6PGD, G6PD, GOT, PGM, GPI, MDH, PER and IDH isozymes using the procedures described in Chapter 1. Peroxidase was of interest because of the work by Pandey (1967) in which PER was implicated in having a direct role in protein biosynthesis. Assays were also included for ribonuclease to determine whether or not it plays a primary role in the self-incompatibility reaction in sweet cherry. Standard extraction procedures were used and buffer and staining procedures were as follows;

5.2.2 Gel buffers

Studies in humans have shown that ribonucleases have specific pH ranges and to optimise resolution on cellulose acetate gels the correct pH must be maintained during a gel run (Karn et al, 1979). A range of buffers were used to generate different environments and included: 0.02M Phosphate pH 7.0, 0.0015M Tris-maleate pH 7.2, 0.1M Tris-EDTA-Maleate-$MgC1_2$ pH 7.4, 0.1M Tris-Citrate pH 7.6, 0.1M Tris-Citrate pH 7.68, 0.05M Tris-Maleate pH 7.8. (Richardson et al, 1986).

5.2.3 Staining

Two staining procedures were used specifically for ribonucleases. One was adapted from starch gel staining methodology as described by Shaw and Prasad (1970), stain components being Yeast RNA (5mg), Black K salt (2mg), Acid phosphatase (200mg) and 0.05M acetate buffer, pH 5.0 (2ml).

The other method was adapted from a positive RNase zymogram method developed for cellulose acetate gels using granulocyte extracts from human spleen samples (Karn et al, 1979). Stain components were applied in a 0.01% agarose gel made up using Sorensen's phosphate buffer, pH 7.5 or applied directly to the gel in the usual manner. Stain components were UpA + ApA (10mg/ml in distilled water) 50ul, PMS (5mg/ml) 200 ul, MTT (5mg/ml) 100 ul, Nucleoside phosphorylase 10 ul, Xanthine oxidase 10 ul, Adenosine deaminase 20 ul and phosphate buffer pH 7.5 and an additional gel/electrode buffer of Na-barbital pH 8.6was used with this stain recipe. This was run at 250V for 3-4 hours.

Staining was also carried out for glycoproteins given their association with incompatibility S-genes (Kamboj and Jackson, 1986). Their function revealed to be that of ribonucleases (McClure, 1989). The procedure used was the Periodic acid solution staining as described by Segrest and Jackson (1972) for polyacrylamide gels and adapted for use with cellulose acetate.

5.2.4 Treatments

In 1990 the sweet cherry cultivar Sam was used as a source of pistils. Three treatments were used.

(1) No pollination (control)

(2) Selfed

(3) Compatible cross with the self-fertile mutant Stella.

In 1991 varieties of known S-genotype were used and the crosses expanded, as listed:

(1) Van (unpollinated) S_1S_3

(2) Van x Stella, compatible, S_1S_3 x S_4S_4'

(3) Van x Bing, semi-compatible, S_1S_3 x S_3S_4

(4) Van x Venus, incompatible, S_1S_3 x S_1S_3

(5) Van x Van, incompatible, S_1S_3 x S_1S_3

(6) Van x Merton Glory, compatible, S_1S_3 x universal pollinator.

In 1992 the crosses made in 1991 were repeated except that Vista was substituted for Merton Glory as a universal pollinator.

All pollinations were carried out at field sites on Lenswood Horticulture Centre, Lenswood South Australia. Insect proof cages were placed on limbs before flowering. Flowers were emasculated and pollinated at late balloon stage just prior to flower opening and anther dehiscence. Flowers not involved in the experiment were pinched off and removed to prevent contamination from self pollen. Treated flowers were labelled with small weather-proof tags. At least one hundred flowers were used for each treatment to ensure enough material was available for isozyme analysis.

Pollen and pistil collection was carried out as previously described. Note that pistil here refers to stigma and style and does not include the ovary.

5.2.5 Microscopy

Pollen tube growth in pistils of sweet cherry were observed using fluorescence microscopy techniques developed by Martin (1959). Pistils (including ovaries) were removed from flowers and fixed in Carnoy's fluid : alcohol (absolute) : chloroform : acetic acid 6:3:1, for a minimum of 2 hours. Pistils could be stored for extended periods in Carnoy's fluid but best results were achieved if the following steps were completed within one day: Hydration- samples were placed in 70% alcohol for 10 minutes, followed by 30% alcohol for 10 minutes and 2 exposures to distilled water each for 10 minutes; Softening- pistils were placed in a solution of 0.8N sodium hydroxide for 1

hour at 60°C; Staining- this was achieved using 0.1% W.S.aniline blue in 0.1N K_3PO_4 (allowed to mix overnight and then filtered) for 10 minutes. Pistils were then removed and mounted onto a glass slide. A drop of 80% glycerol was placed on pistils. A glass coverslip was pushed down against the slide to squash pistils.

Observation of samples was made using a Zeiss Axiophot with reflected light from an HBO 200 high pressure mercury vapour lamp. The filter combination was specifically for the 410nm aniline blue excitation peak.

Photographs were taken using Ilford Delta professional, ASA 400 black and white film or Kodak Ektachrome 160T, colour reversal film, ASA 400.

5.2.6 Treatments

In the spring of 1993, branches of selected cherry cultivars were cut when flowers were at the pre-popcorn stage and placed in beakers containing a 5% sucrose solution. Branches were recut and solutions changed on a daily basis.

Two treatments were carried out at room temperature. Fully compatible crosses Stella (S_4S_4') x Early Rivers (S_1S_3) and the semi-compatible cross Stella (S_4S_4') x Stella (S_4S_4'). Branches of Stella were used and all flowers emasculated. Unused flowers were removed. Pistils were sampled 6 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days and 7 days after pollen was applied to stigmas.

Cuttings from the cultivars Van and Stella were treated in the same way except that they were placed in a controlled temperature room, with 12 hours light and 12 hours dark at 18°C and 25°C. Pollinations were fully compatible Van (S_1S_3) x Stella (S_4S_4'), semi compatible Stella (S_4S_4') x Stella (S_4S_4') and incompatiable Van (S_1S_3) x Van (S_1S_3). These crosses were also repeated in the field on trees of the cultivars Van and Stella. Clusters of flowers were chosen and flowers at

balloon stage emasculated, all other flowers were removed. Pollinations were made and emasculated flowers were covered with waxed paper bags to prevent contamination of flowers with unknown pollen and to prevent desiccation of exposed flower parts. Sampling was taken over the same time intervals, as the laboratory experiments.

If the S_3 gene was carried by Stella 50% of pollen would be expected to be incompatible in the crosses with Van and Early Rivers. Secondly, the semi-compatible cross resulting from selfing Stella serves two purposes, to observe expected incompatible pollen tube reactions and to identify a possible second site of S_4' pollen tube inhibition. Thirdly, for completeness, incompatible selfing of Van was carried out to observe the expected inhibition of pollen tubes about one third the way down the style, which typically occurs in gametophytic incompatibility reactions (de Nettancourt, 1977).

5.3 Results

5.3.1 Isozymes

Pistil extracts from the Sam sweet cherry cultivar in 1990 showed no changes or differences for 6PGD, GOT, GPI, SKDH, PGM, G6PD or MDH for pistils pollinated with either Stella or Sam (self) pollen or left as unpollinated controls. Peroxidase was not analysed in 1990 but was included in the subsequent two seasons 1991 and 1992. In both these seasons, neither peroxidase nor any of the other isozymes showed any differences or changes for any of the treatments.

Ribonuclease was present in both pistils and ungerminated pollen. Bands were usually resolved close to the origin. Two zones of banding were noted both being monomorphic. Best results for resolution of ribonuclease bands were achieved using the phosphate pH 7.0 electrode buffer described by Richardson et al (1986) and staining with the Shaw and Prasad (1970) method, for pollen. For pistils the same staining procedure combined with Tris-maleate pH 7.8 electrode buffer (Richardson et al, 1986) gave comparable results. Ribonuclease did not show any changes or differences between the different treatments in 1991 or 1992.

5.3.2 Microscopy

Experiments conducted in controlled temperature rooms produced little result. Pollen germination was zero on flowers from cuttings held at 25°C and on agar plates held at room temperature. Negligible pollen germination occurred on flowers from cuttings held at 18°C. Similarly, field experiments at Lenswood showed very little activity. During flowering below average temperatures and rainfall were experienced. This led to a widespread and generally low fruit set in the Mount Lofty Ranges cherry production region. In crosses between Stella and Early Rivers and Stella and Van, no inhibition of pollen tubes was observed, that is they were fully compatible (Table 16). Selfing of Stella resulted in approximately half of the germinated pollen tubes being inhibited in the first ⅓ of the pistil (Plate 6 and Table 16), as expected in a semi-incompatible cross. The remaining compatible tubes did not display any further incompatible reactions in the tissue between the first one-third of the pistil and ovary (plate 7). While some branching of pollen tubes on approach to the ovary was observed (plate 8), this was interpreted as compatible and has been recorded as such in other species, for instance avocado *(Persea americana)* (Sedgley, 1979). Some spiralling of pollen tubes approaching the ovary was also observed and it was only after observing micropyle penetration by spiralled pollen tubes that it was considered as compatible pollen tube growth (plate 9). Van x Van crosses showed inhibition of all pollen tubes, as expected for a fully incompatible cross (Table 16)

Table 16. Results from hand pollination experiments showing the average number and standard deviation of pollen grains observed on the stigma and subsequent germination or inhibition as observed microscopically.

Cross	Pollen grains on stigma	Germinated	Inhibited	Pollen tube growth to base of style
Van x Van	21 ± 4.24	17 ± 1.41	17 ± 1.41	0.0
Stella x Stella	20 ± 10.55	11.75 ± 8.88	5 ± 4.24	6.75 ± 5.25
Stella x Early Rivers	29.5 ± 29	5.5 ± 0.71	0.0	5.50 ± 0.71
Stella x Van	82.25 ± 41.92	10.75 ± 13.67	0.0	10.75 ± 13.67

Plate 6 (x54) Upper third of a Stella style hand pollinated with Stella pollen five days after pollination in a laboratory experiment conducted at Lenswood Horticultural Centre. Pollen tubes are fluorescent and incompatible tubes show an inhibition of growth.

PT - pollen tube ; I - incompatible pollen tube.

Plate 7 (x58) Stella style hand pollinated with Stella pollen three days after being applied in a laboratory experiment at Lenswood Horticultural Centre. Compatible pollen tube growth extends the entire length of the style.

PT - pollen tube

Plate 6

Plate 7

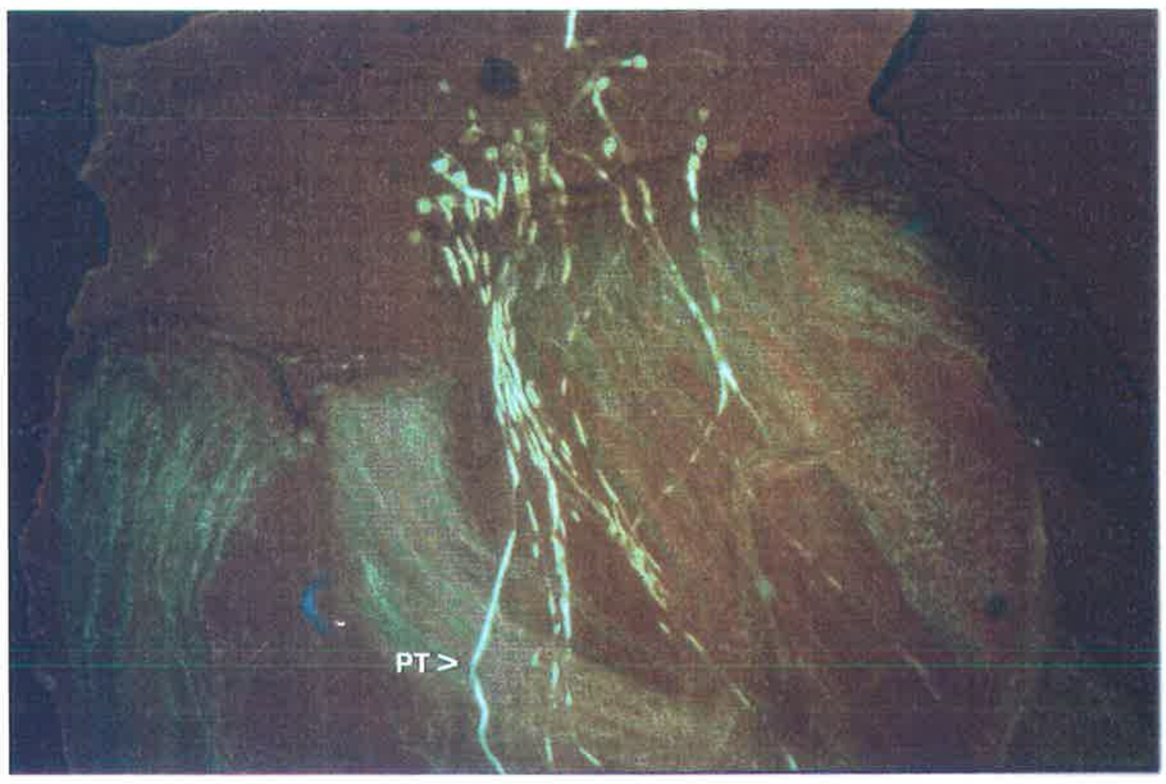


Plate 8 (x534) Branched pollen tube growth observed 7 days after applying pollen from Stella to the styles of Van in the field at Lenswood Horticultural Centre.

PT - pollen tube ; B - branching

Plate 8

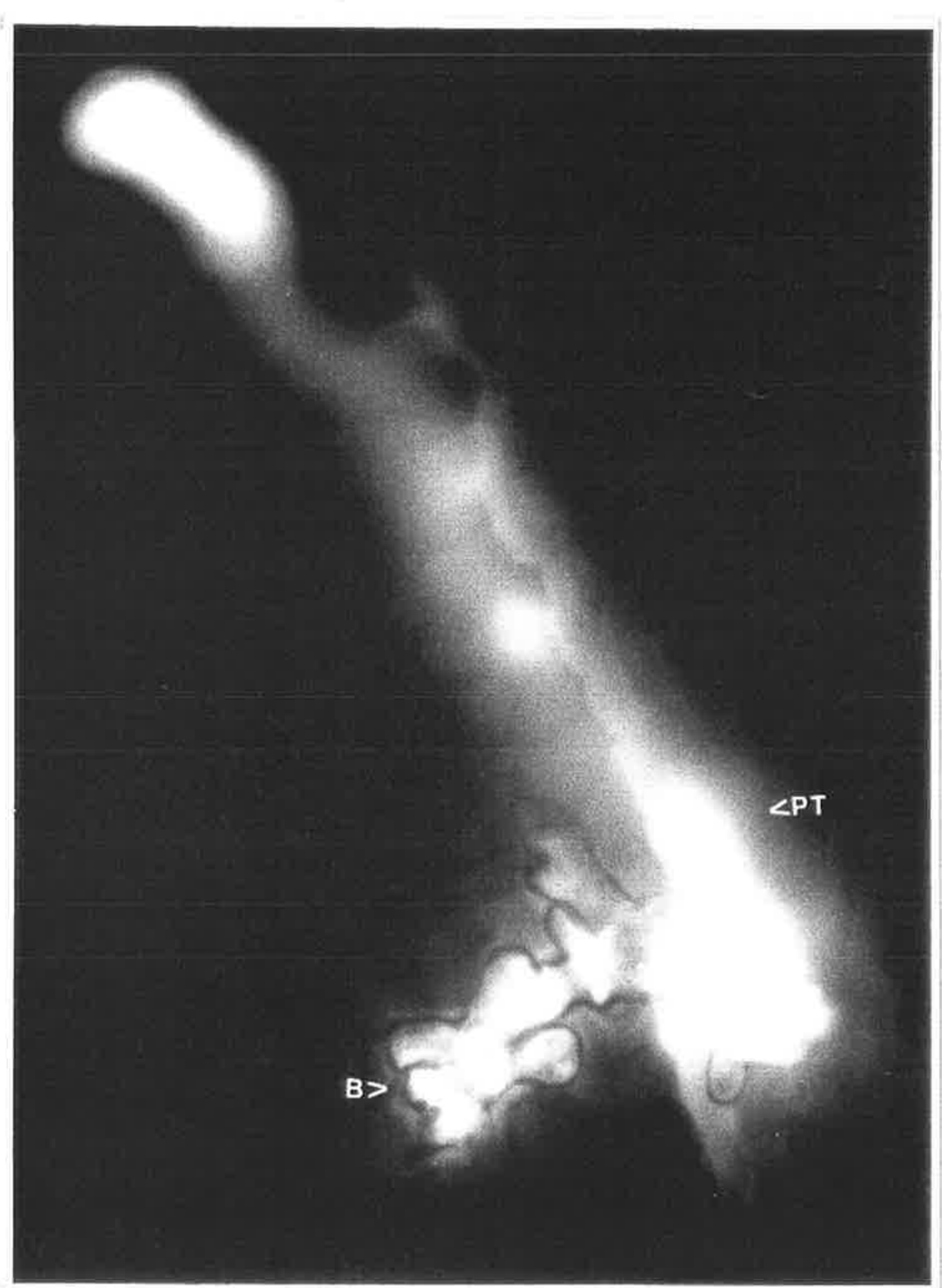


Plate 9

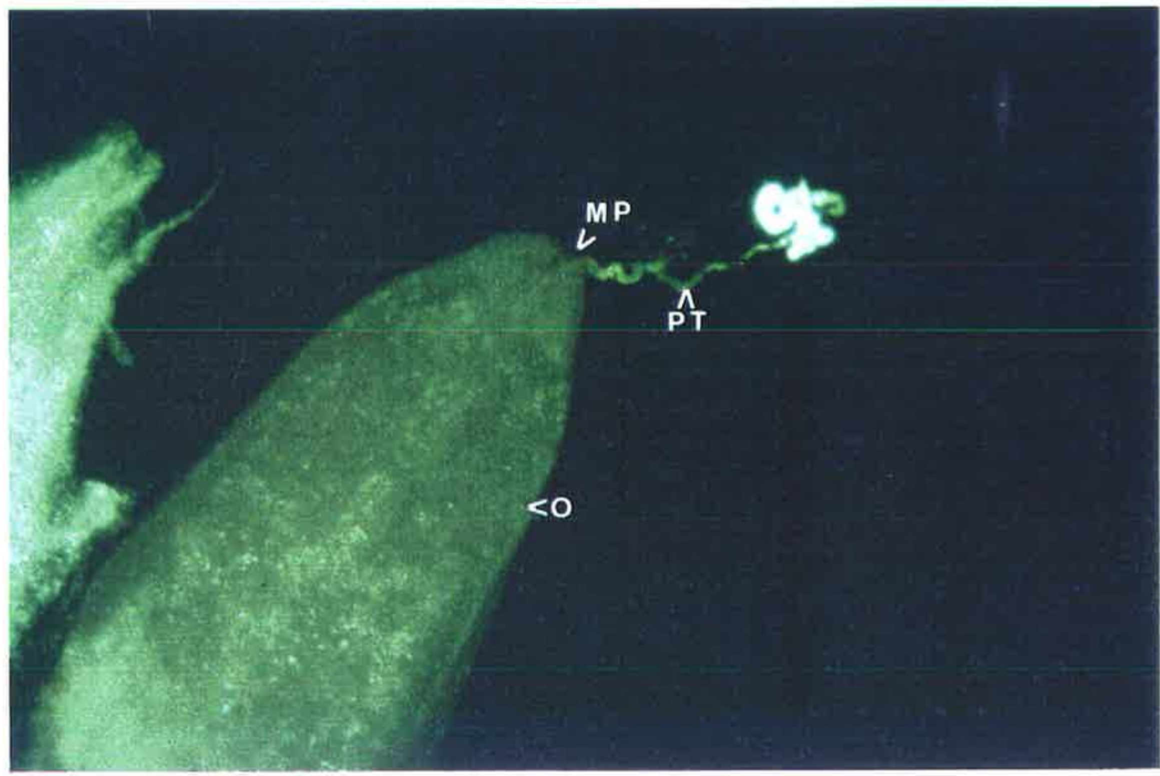


Plate 9 (x115) Stella pollen tube entering the micropyle of a Stella ovule.

PT - pollen tube ; MP - micropyle ; O - ovule.

5.4 Discussion

None of the pollination combinations used in these studies resulted in a change in isozyme genotypes. This included ribonuclease which was detected in both ungerminated pollen and styles, and showed no polymorphism. This suggests that ribonucleases detected here are not the primary factor in the incompatibility reaction in sweet cherry, because, under existing theories, the S-alleles code for different SRNases with different molecular weights and charges which should have manifested themselves as varying banding patterns on gels. Furthermore, the presence of RNase was not expected in ungerminated pollen, as other studies have shown it to be absent in ungerminated pollen. This fits the current thinking whereby selective uptake of different forms of SRNase into the pollen through the cell wall results in the inhibition of pollen tube growth. The exception to this is if the RNase were compartmentalised in the pollen and involved in reactions other than incompatibility. The action of grinding tissue during the extraction procedure could destroy this.

Results from microscope studies of pollen tube growth showed that Stella pollen was not inhibited when placed on pistils of Van or Early Rivers and this proved that Stella has the S_4S_4' genotype, it inherited the S_4 allele from the Lambert female parent and the S_4' allele from the JI 2420 male parent.

After selfing Stella, both compatible and incompatible pollen tube growth was observed in approximately equal proportions. Typically incompatible tubes showed a cessation of growth in the first third of the style. Compared to compatible tube growth, incompatible pollen tubes had enlarged tips and greater callose deposition as reflected by increased fluorescence. Inhibition of pollen tube growth at other sites prior to the ovary was not detected. This is evidence that pollen carrying the S_4' haplotype is not recognised in the conducting tissue of the style. This evidence is nothing new until coupled with the isozyme data from Chapter four which showed that GOT was a marker for the S-alleles, and that after selfing Stella, only embryos heterozygous for GOT

resulted. This means that those same embryos were heterozygous for the S-alleles. That is S4' pollen never successfully fertilised an S4' ovule. Thus, recognition must have occurred at fertilisation when the cytoplasmic contents of the gametes came into contact. And supports the idea that recognition is associated with the pollen tube cell wall.

It is also important to note that the self-fertile mutation was not expressed in any of the isozyme loci examined. That is, there were no unique or missing bands associated with the S_4' allele. Thus, in sweet cherry isozymes, including ribonucleases, the evidence suggests they do not have a primary function in the self-incompatibility process.

CHAPTER 6: GENE FLOW IN SOUTH AUSTRALIAN CHERRY ORCHARDS

6.1 Introduction

The majority of sweet cherry cultivars are self-incompatible. For the commercial production of cherries, orchardists must plant compatible pollinator cultivars nearby. In South Australia the main area for cherry growing is in the Mount Lofty Ranges, east of Adelaide. In traditional plantings solid rows of a cultivar are planted and 1-2 rows of another cultivar planted adjacent and parallel to this. In some circumstances the terrain may require a special planting pattern resulting in a tree of a certain cultivar being surrounded by 3-4 different cultivars. Little attention was paid to the need for cross pollination in these older orchards and significant reductions in yield have been traced to a lack of suitable pollinisers(Sweedman, 1987). Older cultivars such as Williams Favourite, Napoleon and St Margaret dominate these plantings and cross compatibilities are generally unknown. The importance of good cross pollination has been established in other tree crops such as almonds (Jackson and Clarke, 1991). These workers showed with the use of isozyme markers in almond embryo's, that cross-pollination was highest between neighbouring compatible trees on facing sides. Nut set was lower on the opposite side and on trees in rows more distant from the polliniser being studied.

This effect was thought to be directly related to the behaviour of the honeybee. McGregor (1976) states that "sweet cherry pollen must come from another and compatible cultivar, therefore a high degree of bee activity on the tree and between trees is required to adequately pollinate the crop". McGregor (1976) also cites many publications reporting decreased production with increased distance from the polleniser row. Various polleniser to recipient ratio's from 1:9 to 1:1 have been recommended for cherry plantings. In commercial almond orchards in South Australia, it is a general practice to plant a range of polliniser cultivars in rows adjacent to the main cultivar, Nonpareil, to ensure adequate nut set. Work by Jackson and Clarke(1991) indicated that Nonpareil should be planted in a one-to-one ratio with pollinisers.

More recently in cherries the need for cross pollination has been overcome to some extent by the introduction of self-fertile cultivars such as Stella (Lapins 1971), Lapins and Sunburst (Lane and Schmidt 1984). Initially, there were fears that fruit set levels in self-fertile cultivars would be too high and result in small fruit size. This has not been the case with Stella. In theory it is now possible to plant solid blocks of a single cultivar without the need for polliniser trees. In contrast, investigations in sour cherries *Prunus cerasus*, a predominantly self-fertile species have shown that fruit set can be increased by the presence of pollen from other cultivars in the orchard compared to self pollen (McGregor 1976). Sour cherries do require an outside vector to transfer self pollen from the anthers to the stigma, for a significant amount of self-pollination to occur. Thus, planting solid blocks of self-fertile sweet cherries would probably require the presence of bees in the orchard to provide a vector for pollen transfer. The inclusion of different and compatible cultivars as an alternative pollen source in the orchard may also increase fruit set.

Two broad questions exist which have important implications for the cherry industry.

1. What are the relative proportions of fruit derived from selfing and outcrossing on self-fertile cultivars?
2. What is the pollen gene flow in traditional orchards, and specifically, which cultivars are contributing most to final fruit set?

6.2 Materials and Methods

The cherry orchard at Lenswood Horticultural Centre (LHC) consists of a large number of trees, some are planted in randomly mixed plantings of different cultivars while others have been planted as a row of a single cultivar. This raised opportunities for the investigation of gene flow using isozyme analysis. In 1991 investigations began into two cultivars with pollination problems. Sam is a late season, large, firm fruited cultivar, with an attractive appearance that is well accepted on local and overseas markets. At Lenswood Horticultural Centre it has proven to be highly crack resistant with not more than 8 per cent of the crop being damaged by rainfall in any one year for the period 1986 to 1989 (Granger and Frensham 1991). In many instances Sam cannot be grown

profitably by commercial growers because of low fruit set. At LHC a row of Sam trees set satisfactory crops in most years. It was surrounded by a range of other cultivars as depicted in Figure 1. To realise the full potential of the Sam cultivar isozyme analysis of embryo's harvested from Sam trees was undertaken to determine what cultivar(s) was contributing most to seed set. Similarly, an established and locally selected cultivar, Clements Pride, while highly valued by the cherry industry primarily for its large size and appearance, often produced poor crops. An orchard at Basket Range, managed by a Mr G Crammond was identified as having a Clements Pride tree which carried satisfactory crop loads. This tree and surrounding trees where mapped (Figure 2) and isozyme analysis carried out on leaves to establish a genotype for each cultivar and then analyses were carried out on embryo's to determine gene flow between trees. On another cherry orchard at Basket Range, owned by a Mr W. Bishop the cultivar Beauchamps Black was surrounded by Williams Favourite trees and was representative of the site where the chance seedling of Black Douglas was discovered (Figure 3). The gene flow to Beauchamps Black could be compared to that of Black Douglas to gain further insight into the probability of Beauchamps Black and Williams Favourite being parents of Black Douglas.

With the predicted increased use of self-fertile cultivars such as Stella it was also pertinent to investigate the breeding system of Stella and determine the numbers of embryos produced by selfing and those by outcrossing. Again, the LHC orchard contained Stella and in the period 1985-1989 it produced well above average yields. For instance, in 1987, Stella trees produced the equivalent of 25 tonnes per hectare (Granger and Frensham 1991), compared to the highest yield of 10 tonnes per hectare in commercial orchards (Sweedman 1987). This indicates satisfactory crop levels were occurring with the breeding behaviour, it remained to determine what role inbreeding (selfing) and outbreeding played, which in turn would form the basis of recommendations made to the cherry industry with regard to the planting of self-fertile cultivars and whether or not there was a need for the inclusion of pollenising cultivars in new orchard plantings.

Figure 1. Layout of Cherry orchard at Lenswood Horticultural Centre.

South →

	57[+] Venus / Stockton	58 Venus / Stockton	59 Venus / Stockton	60 Venus / Stockton	61 Venus / Mazz[*]	62 Venus / Mazz	63 Venus / Mazz	64 Venus/ Mazz	65 Venus/ MAH	66 Venus/ MAH	67 Venus/ MAH	68 Venus/ MAH
	69 Stella / MAH	70 Stella / MAH	71 Stella / MAH	72 Stella / MAH	73 Stella / Stockton	74 Stella / Stockton	75 Stella / Stockton	76 Stella/ Stockton	77 Stella/ Mazz	78 Stella/ Mazz	79 Stella /Mazz	80 Stella/ Mazz
	81 Sam / Stockton	82 Sam / Stockton	83 Sam / Stockton	84 Sam / Stockton	85 Sam / Mazz	86 Sam / Mazz	87 Sam / Mazz	88 Sam/ Mazz	89 Sam/ MAH	90 Sam/ MAH	91 Sam /MAH	92 Sam/ MAH
Van	Van	Noir de Guben	Noir de Guben	Magda	Spur Lambert	Lambert	Lambert	Lambert	Bedford	Lustre	Hender-son	V-690620
Baslerl-ongsti-eler	Baslerlong-stieler	Oregon	Oregon	Larian	Larian	Schauen-burger	Schauen-burger	Early Burlat	Early Burlat	V-69062	V-69061	V-690620
Merton Crane	Merton Crane	Merton Glory	Merton Glory	-	Summit	V-690618	Compact Stella	V-690618	Early Purple Guigne	Vittoria MAH	-	-
Vista	Vista	Hedelfingen	Hedelfingen	Salmo	Salmo	Vic	Vic	Rainier	Rainier	Bing OB260	Bing OB260	-
Krasa-rica	V69068	Sunburst	Sunburst	Burgsdorf	Burgsdorf	Black-boy	Black-boy	Delta	Delta	-	Seneca	-

[+] Numbers refer to tree number
[*] Mazz abbreviation for Mazzard rootstock
Stockton abbreviation for Stockton Morello rootstock
MAH abbreviation for Mahaleb rootstock

Embryos were collected by harvesting mature fruit, immediately removing the flesh and cleaning seeds of any adhering flesh under running water. Seeds were then dusted with dry fungicide power (Captan®) and sealed in plastic bags and stored in the refrigerator. Analysis began as soon as possible after harvest. Seeds were cracked, the seed coat removed and extractions made using 0.1g embryo tissue, 500ul Normal buffer and 0.0375g PVPP.

The Lenswood Horticultural Centre trees were trained as close planted palmettes and formed a continuous hedge along each row. Thus, random samples of fruit were taken from each half of selected trees, of Sam and Stella. The main planting was totally enclosed by bird proof netting. While rows of cultivars outside of the net were directly in line with tree rows inside the net, they have not been included in Figure 1.

The cultivars Clements Pride and Beauchamps Black were planted in traditional cherry orchards having the typical vase shape made up of several main leaders. These trees were subdivided into North, South, East and Western quadrants and fifty fruit from each quadrant sampled at random, for subsequent isozyme analysis.

In 1991 samples were taken from Sam at LHC, tree numbers 81, 82, 86 and 92. Stella, tree numbers 72, 74 and 80 were also sampled at LHC. Only trees from the Crammond orchard were sampled in 1991 and included Clements Pride, Lanes Seedling and Napoleon (Figure 2). In 1992 sampling on LHC was confined to Stella, numbers 71 and 72. In 1992 the crop from Crammond's property was removed before samples could be taken. Samples were taken on Bishops' property from an eighty year old Beauchamps Black tree in a remanent part of a 2 hectare plot. Waterloo at this site, synonymous with Florence (Granger et al, 1993) in South Australia turned out to be incorrectly identified. Instead of producing white fleshed fruit it produced dark fruit. Inspection of the fruit suggested it was a Williams Favourite type.

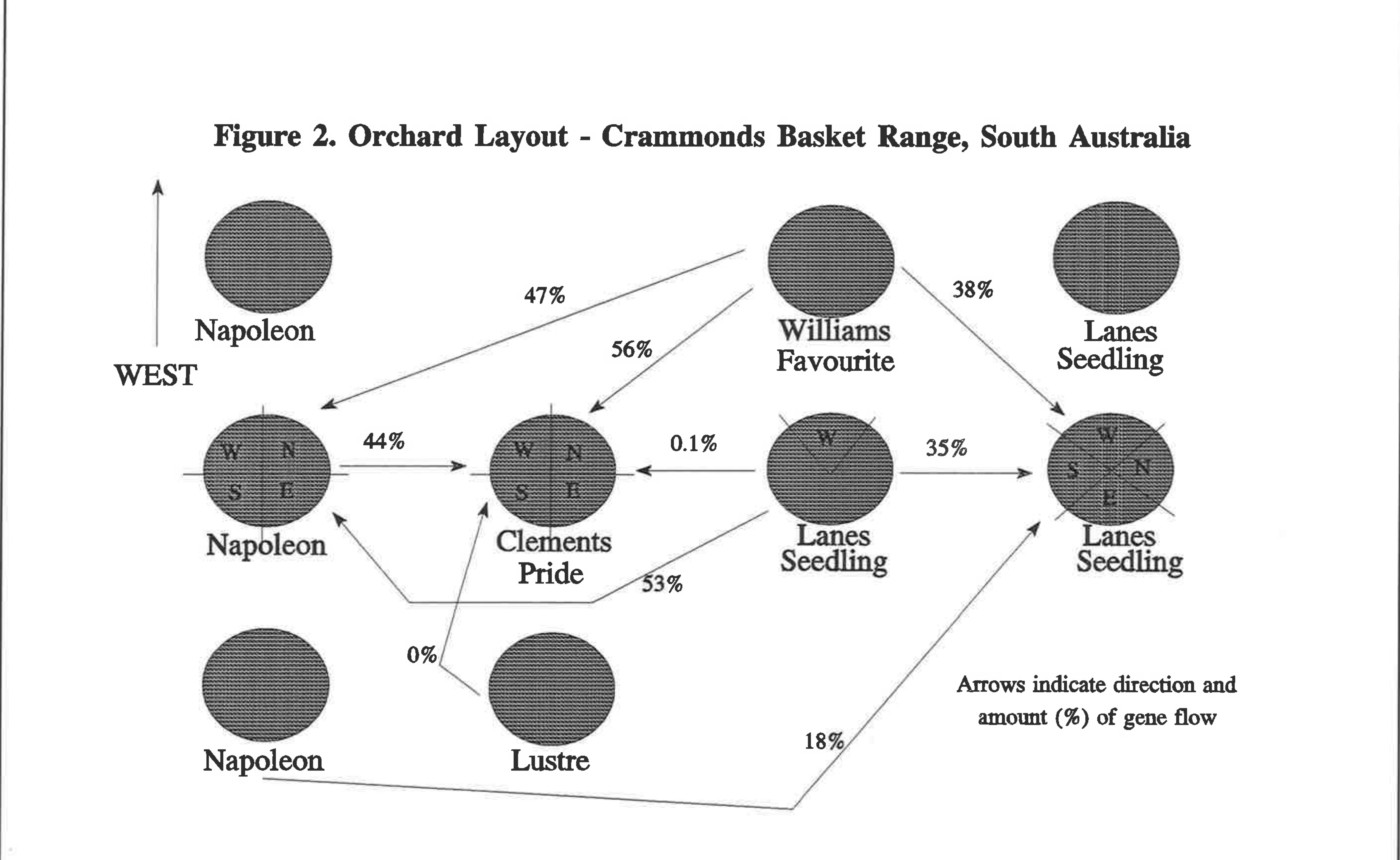


Figure 1

Figure 3. Orchard Layout - Bishop Basket Range, South Australia

Waterloo

NORTH

10m

(5m between canopies)

Williams Favourite

Beauchamps Black

Williams Favourite

Opal

Sampling at LHC in 1993 included Sam tree number 87 and Stella tree numbers 70 and 75. Both grower properties were sampled in 1993, Beauchamps Black at Bishop's and Clements Pride at Crammonds. The number of intact embryos from grower properties were low. Many had been infected by fungal and bacterial pathogens probably because of above average rainfall during the 1993 season.

In each season the same isozymes were examined namely 6PGD, G6PD, GOT, FDP, GPI, PGM, SKDH, MDH and IDH. Full bloom dates were also recorded for the cultivars in each study area. Leaf isozyme analysis was conducted to establish the genotype of each cultivar under study.

6.3 Results

6.3.1 Sam

The number of each genotype scored in the progeny, in the form of embryos, was compared to the genotype of Sam and the genotypes of other surrounding cultivars flowering at the same time (Table 17). The S-genotype of Sam and some of the other cultivars is unknown. It was also thought to be reasonable to further narrow down suspected pollen donors on the basis of tree age. The orchard was visualised as a pool of male gametes, contributing to the row of maternal Sam trees, older, larger trees contributing a greater number of gametes because they produce a greater number of flowers.

Cultivars planted outside of the bird netted area that flowered at the same time as Sam, thereby making them possible pollen donors, included Vega, Waterloo (L) and Williams Favourite (L). Vega was eliminated as a major gene donor because, like Sam, it is an aa for GPI, yet progeny showed almost a 1:1 ratio for the aa:ab genotypes indicating that a cultivar with the ab genotype was involved. Waterloo (L) had the same isozyme profile as Vega and, thus, was also ruled out. Williams Favourite (L) is bb for G6PD and the vast majority of progeny were ab for G6PD. Because Sam is ab for G6PD it would have been expected to produce a higher number of the bb genotype had Williams Favourite (L) been a major pollen donor. Further evidence to discount

Williams Favourite (L) as a pollen donor is that they share the same genotype for GPI; being aa yet more than half of the progeny carried the b allele indicating that a cultivar with an ab genotype for GPI was dominating as a gene donor. Stella was ruled out on the basis of GOT, as both it and Sam have the bc genotype. Combination of the two cultivars could not produce the ab genotype,and yet, the majority of progeny from Sam had this genotype. Thus, the pollen donor must have carried the a allele for GOT. Given this, it seemed as though no gene flow was coming from outside the net. Although the detection of a small number of null alleles for PGM and SKDH (Table 18), not previously recorded in sweet cherry indicated that foreign genes, probably from another Prunus species, were being inherited. The most likely donor being Stockton Morello a sour cherry *(Prunus cerasus)* cultivar, planted outside of the net, which reached full bloom on the same day as Sam in 1991. The isozyme profile for Stockton Morello is given in Table 18 and shows an ac genotype for PGM, indicating it donated this allele to the Sam embryo.

Attention was then turned to cultivars within the enclosure. The majority of trees were only 3 years old from grafting. The most established being Summit, Merton Glory, Merton Crane and Vista planted in 1984. Merton Crane was discounted as a pollen donor because it is aa for GPI as is Sam (Appendix VI), yet the majority of embryos were ab for GPI. Summit was ruled out as a major pollen donor to Sam for the same reason. This left Merton Glory and Vista as the most likely pollen donors to Sam. Both cultivars are universal pollinators, with an unknown S-genotype (Knight 1969).

Sam is ab for FDP, Merton Glory bb and Vista aa (Table 18). Embryo analysis showed a ratio of 85:19 for the homozygotes bb:aa, so in this case, Merton Glory appears to be the major contributor of alleles. Most of the embryos were heterozygous for each isozyme, the two exceptions being IDH and SKDH. Sam, Vista and Merton Glory are all aa for IDH, so it is not surprising that all but a few of the progeny are aa for IDH. Sam is ab for SKDH and both Vista and Merton Glory have the aa, genotype yet the vast majority of progeny are aa with an

approximate ratio of 3:1 with the heterozygotes. With complete compatibility a 1:1 ratio would have been expected.

Some anomalies were observed in the data presented in Table 18. For instance, Sam is bb for 6PGD and yet 25aa progeny were recorded. Similarly, Sam is bc for GOT and 43 homozygous aa progeny were observed. Furthermore, Sam is homozygous aa for GPI and yet 12 bb progeny were observed. Also null alleles that had not been previously recorded were observed for PGM and SKDH in embryos.

Table 17. Full bloom dates for cherry cultivars at Lenswood Horticultural Centre.

	Full bloom date		
Cultivar	1991	1992	1993
Bing OB260	4 Oct	16 Oct	29 Sept
Bedford Prolific	4 Oct	18 Oct	30 Sept
Burgsdorf	30 Sept	5 Oct	30 Sept
Compact Stella	2 Oct	13 Oct	29 Sept
Delta	4 Oct	19 Oct	28 Sept
Early Burlat	7 Oct	14 Oct	30 Sept
Early Purple Guigne	9 Oct	6 Oct	14 Oct
Hedelfingen	11 Oct	10 Oct	19 Oct
Hendersons Bedford	2 Oct	5 Oct	25 Sept
Larian	7 Oct	14 Oct	28 Sept
Lambert	10 Oct	18 Oct	6 Oct
Lustre	3 Oct	14 Oct	28 Sept

	Full bloom date		
Cultivar	1991	1992	1993
Magda	7 Oct	11 Oct	29 Sept
Merton Crane	5 Oct	5 Oct	14 Oct
Merton Glory	7 Oct	16 Oct	30 Sept
Noir de Guben	2 Oct	5 Oct	29 Sept
Oregon	6 Oct	22 Oct	7 Oct
Rainier	3 Oct	19 Oct	30 Sept
Salmo	7 Oct	20 Oct	30 Sept
Sam	7 Oct	19 Oct	30 Sept
Schauenburger	-	22 Oct	30 Sept
Seneca	5 Oct	20 Oct	1 Oct
Spur Lambert	8 Oct	16 Oct	7 Oct
Stella	30 Sept	9 Oct	28 Sept
Summit	7 Oct	19 Oct	5 Oct
Sunburst	11 Oct	20 Oct	8 Oct
Van	6 Oct	16 Oct	30 Sept
Venus	30 Sept	8 Oct	24 Sept
Vic	5 Oct	16 Oct	30 Sept
Vittoria	2 Oct	7 Oct	29 Sept
Vista	6 Oct	19 Oct	30 Sept

Table 18. Isozyme genotypes and full bloom dates of Sam and probable pollen donors from an orchard at Lenswood Horticultural Centre. Isozyme genotypes for embryos harvested from Sam are at the bottom of the table.

Cultivar	Isozyme genotypes									Full bloom
	6PGD	GOT	G6PD	GPI	IDH	PGM	FDP	SKDH	MDH	date 1991
Sam	bb	bc	ab	aa	aa	ab	ab	ab	ab	7 October
Summit	ab	bc	ab	aa	aa	ab	bb	aa	ab	7 October
Merton Glory	aa	ac	ab	ab	aa	ab	bb	aa	ab	7 October
Vista	ab	ac	aa	ab	aa	aa	aa	aa	ab	6 October
Stockton Morello	ab	bc	ab	aa	ab	ac	bb	ab	aa	6 October
Progeny as										
embryo's from Sam:	25aa	43aa	18aa	127aa	155aa	38aa	19aa	223aa	49aa	
	210ab	278ab	278ab	166ab	17ab	251ab	159ab	74ab	220ab	
	68bb	9bb	9bb	12bb	1ac	1ac	85bb	6nn	41bb	
					1bb					
					19nn					

6.3.2 Stella

Figure 1 shows the position of Stella in the LHC orchard. Examination of full bloom dates in each of these years indicated that many of the other cultivars in the LHC planting, both inside and outside the bird enclosure, were reaching full bloom around 7 days after Stella. Venus coincided with the flowering of Stella, whereas Sam flowered later and coincided with other cultivars (Table 17).

This data alone implicates Venus as the most probable source of any outcrossing by Stella at LHC. Isozyme analysis was carried out on embryos sampled from Stella trees in the middle of the row ie those numbered from 70 to 75 in 1991, 1992 and 1993. Results are given in Table 19. The main points from the data in relation to the breeding behaviour of Stella require SKDH to be considered as a marker. This situation arises because Stella is heterozygous at all of the isozyme loci except one, SKDH, for which it carries the aa genotype (Appendix VI). To clarify further, if selfing is occurring, then the only SKDH genotype expected in embryo's sampled from Stella trees would be aa. This outcome could also be expected if outcrossing was occurring between Stella and another cultivar with the aa genotype. Outcrossing to another cultivar carrying the b allele for SKDH as either a heterozygote or homozygote would produce SKDH heterozygotes in embryo's sampled from Stella trees. As Table 19 shows, it is the latter situation which has occurred at LHC, with a total of 400 aa and 368 ab individuals for SKDH recorded in the 3 years of sampling. Venus is ab for SKDH (Appendix VI), is adjacent to Stella (Figure 1) and flowered at the same time (Table 17). Thus outcrossing is occurring with Stella in at least 50% of all successful fertilisations, because about half of the progeny carry the foreign b allele, and the most likely pollen donor at Lenswood is Venus. While SKDH is the best indicator of outcrossing, two other loci are showing the presence of foreign genes, namely, PGM and GOT. PGM shows two individuals with the fast aa genotype. Again this was probably sourced from *Prunus cerasus* outside of the netted area. And, as for GOT, it shows 86 embryos carrying the a allele as the heterozygotes ab and ac. Also, one embryo was found to have the homozygous aa genotype for GOT. Appendix VI indicates that Stella is bc for GOT. Thus the ab and ac genotypes have arisen from outcrossing. The donor in

this case cannot be Venus because it is also bc for GOT. These individuals may have arisen from outcrossing with ac cultivars in the same planting. These embryos account for about 11% of the total samples, and this provides an estimate on the amount of outcrossing Stella is undergoing with cultivars other than Venus.

If we accept that embryos from Stella have been produced by outcrossing in at least 50% of cases and that Venus is the primary pollen donor, more comparisons and estimates can be made. For instance, Venus is bb for 6PGD while Stella is ab (Appendix VI) yet Table 19 displays 230 of 794 embryos with the aa genotype. This is an estimator for the amount of selfing in Stella or it may indicate the amount of outcrossing with other cultivars possessing the a-allele for 6PGD. Alternatively, the individuals homozygous aa for 6PGD where derived from a combination of both. These individuals represent 29 per cent of the embryo's sampled for 6PGD.

In summary, the isozyme evidence suggests that 29% of embryo's harvested from Stella over 3 years were as a result of self-fertilisation. The remaining 71% were produced by outcrossing. The majority, 60%, shared Venus as the paternal parent while 11% were derived from other sources. Gene flow from outside the net was negligible 0.003% as indicated by the fast aa genotype observed for PGM (Table 19).

Table 19. Isozyme genotypes for embryos sampled at Lenswood Horticultural Centre from open pollinated Stella trees. The numbers of individuals with given a genotype for an isozyme are shown for each year and then all years combined as a grand total.

Isozyme								
6PGD	GOT	G6PD	GPI	IDH	PGM	FDP	SKDH	MDH
1991								
30aa	1aa	7aa	18aa	89aa	33aa	25aa	67aa	28aa
40ab	28ab	81ab	62ab	5ab	60ab	28ab	28ab	65ab
25bb	4ac	5bb	15bb	2ac	2 fast aa	22bb		1bb
	50bb							
	1cc							
1992								

92aa	1ab	187aa	84aa	207aa	48aa	45aa	39aa	154aa
137ab	53ac	113ab	197ab	72ab	250ab	93ab	255ab	136ab
68bb	227bc		19bb	18ac		99bb		
	10cc			3bc				
				1993				
108aa	5ac	106aa	114aa	358aa	91aa	37aa	294aa	294aa
198ab	377bc	275ab	251ab	33ab	290ab	229ab	85ab	125ab
96bb			27bb			103bb		
				Grand total				
230aa	1aa	300aa	216aa	654aa	172aa	107aa	400aa	476aa
375ab	29ab	469ab	510ab	110ab	600ab	350ab	368ab	326ab
189bb	57ac	5bb	61bb	20ac	2 fast aa	224bb		1bb
	604bc			3bc				
	50bb							
	11cc							

6.3.3 Clements Pride

Flowering in relation to Clements Pride was observed in 1992. Clements Pride (Figure 2) was in full bloom on 22 October, as did all of the Napoleon trees in the study area and the Williams Favourite tree (Figure 2). On the same day Lustre(Figure 2) had no remaining flowers. The Lanes Seedling adjacent to Clements Pride (Figure 2) had some viable flowers remaining. It was also discovered at this time that the western quadrant of this tree carried immature fruit the reason being that the Mazzard *(Prunus avium)* rootstock had sprouted from beneath the graft union and had become part of the canopy of the tree. The other two Lanes Seedling trees (Figure 2) were toward the end of flowering.

Leaf isozyme analysis was used to determine the genotype of trees in the study area. Table 20 displays the isozyme genotypes. Samples for embryo analysis were taken in 1991, 1992 and 1993. In 1992, embryos were stored as defleshed seeds at 4°C and all samples were degraded by bacterial and fungal pathogens. Similarly, in 1993 with above average rainfall during the growing

season, much of the fruit was damaged and rotting. Embryos removed from seeds were also infected by pathogens and only small numbers could be analysed. Table 21 gives the results of embryo isozyme analysis for Clements Pride for 1991 and 1993.

Table 20. Isozyme genotypes of cultivars in the study area located at Crammond's orchard, Basket Range, South Australia.

	Isozyme								
Cultivar	6PGD	GOT	G6PD	GPI	IDH	PGM	FDP	SKDH	MDH
Clements Pride	aa	ac	aa	aa	aa	ab	aa	aa	ab
Williams Favourite	ab	ac	ab	aa	-	ab	bb	aa	ab
Lustre	aa	ac	aa	ab	-	aa	aa	ab	ab
Napoleon (7)	aa	ac	aa	ab	-	ab	ab	aa	ab
Napoleon (8)	ab	ac	aa	ab	ab	ab	ab	aa	ab
Napoleon (9)	aa	ac	aa	ab	-	ab	ab	ab	ab
Lanes Seedling	aa	ac	aa	aa	-	aa	aa	aa	ab

Table 21. Number of embryos for each isozyme genotype harvested from Clements Pride in 1991 and 1993 at Basket Range, South Australia. Trees were divided into north, south, east and western quadrants and 50 fruit randomly sampled from each quadrant. Missing data have not been included so totals for each isozyme may vary.

Quadrant	Isozyme 6PGD	GOT	G6PD	GPI	IDH	PGM	FDP	SKDH	MDH
	1991								
North	15aa 35ab	3ab 43ac 4bc	7aa 42ab 1bb	40aa 10ab	50aa	1aa 49ab	10aa 37ab	40aa 10ab	37aa 13ab
South	40aa 10ab	3ab 47ac	4aa 43ab 1bb	26aa 24ab	37aa 13ab	1aa 49ab	17aa 28ab	46aa 4ab	25aa 23ab
East	50aa	3ab 47ac	4aa 46ab	16aa 33ab	25aa 24ac	4aa 46ab	3aa 47ab	41aa 9ab	27aa 23ab
West	47aa 1ab	43ac 7bc	1aa 44ab 5bb	29aa 20ab	50aa	3aa 46ab	6aa 44ab	36aa 11ab	26aa 20ab
	1993								
North	1aa 2ab	1ab 2bc	3ab	3aa	3aa	3aa	3ab	3aa	1aa 2ab
South	4ab	2ac 5bc 1cc		8ab	3ac 3bc	3ab	1aa 2bb	6ab	7ab
East	9aa 2ab	10ac 1bc	5aa 2ab	-	-	5aa 2ab	3aa 2ab	4aa 7ab	1aa 10ab
West	7aa 1ab	3ab	3aa	1aa 2ab	1aa 2ab	3aa	1aa 2ab	3aa	2aa 1ab
	Totals all quadrants 1991								
	152aa 46ab	9ab 180ac 11bc	16aa 175ab 7bb	111aa 87ab	162aa 13ab 24ac	8aa 190ab	36aa 156ab	163aa 34ab	115aa 79ab
	Totals all quadrants 1993								
	25aa 9ab	8ab 12ac 8bc 1cc	8aa 17ab	8aa 10ab	10aa 2ab 3ac 3bc	20aa 5ab	5aa 15ab 2bb	16aa 13ab	9aa 20ab

Results in Table 20 and Table 21 need to be compared at the same time to determine the source of pollen genes. Beginning with the 1991 totals and G6PD all of the cultivars in the study area except Williams Favourite are homozygous aa for G6PD, Williams Favourite is ab. Table 21 shows the majority of embryos were heterozygous for G6PD and the only source of the G6PD b-allele was Williams Favourite. Thus, on this evidence alone, Williams Favourite appears to be providing most of the male gametes to successful fertilisations. Numbers of genotypes at each of the other isozyme loci are in accordance with expectations if Williams Favourite was the main pollen donor. In particular, the IDH locus shows the vast majority of embryos to be homozygous aa which rules out Napoleon (ab) and Lanes Seedling (ac) heterozygotes as major pollen donors. But this does explain the source of genes for the heterozygotes recorded in both years. Williams Favourite is bb for FDP and Clements Pride aa. If this was the only cross pollination that occurred, all progeny would be heterozygous ab. This was almost the case in 1990 with only 36 of 192 embryos having a homozygous aa genotype.

The GPI locus showed that the majority of progeny had the aa genotype. This was expected with the aa genotype of Clements Pride and the paternal parent, Williams Favourite also being aa for GPI. However, 87 of 198 embryos were heterozygous ab, the only source of the b allele for GPI being the heterozygotes Lustre and Napoleon (Table 20). Observations at flowering time have shown that the flowering periods of Lustre and Clements Pride do not overlap. Thus, Napoleon was providing all of the GPI b-alleles, or approximately 44% of the gene flow to Clements Pride, while Williams Favourite was donating an estimated 56% of the gene flow to Clements Pride. The latter figure is only approximate because the results for GOT indicate the influx of the b allele, but none of the cultivars in the study area carry the GOT b allele (Table 20). Six embryos were retrieved from the Mazzard sucker on the Lanes Seedling tree (Figure 2) and analysed, results are presented in Table 22.

Table 22. Isozyme genotypes of embryos harvested from the Mazzard sucker, Crammond's Orchard,Basket Range, South Australia.

				Isozyme				
6PGD	GOT	G6PD	GPI	IDH	PGM	FDP	SKDH	MDH
2aa	6ab	5ab	1aa	1aa	1aa	1aa	5bb	4aa
3ab			5ab	3bc	5ab	3ab		2ab
				2cc		1bb		

The GOT locus showed only the ab genotype, indicating that this Mazzard rootstock carried the GOT b-allele. This allele occurred in 0.1% of the embryos sampled from Clements Pride. Seven embryos from each of the North and West quadrants were scored with the GOT-b allele, while 3 were scored in the South and East quadrants (Table 21). The North and West quadrants comprise that part of the tree facing toward the Mazzard sucker (Figure 2). Further, totals for 1991 show 24 embryos with the ac genotype for IDH. Not all of the IDH genotypes were determined for leaf samples. However Table 22 shows that Mazzard carries the c allele for IDH because two of its progeny were homozygous cc, and one allele must come from each of the parents. Again the number of embryo's recorded in 1991 carrying the IDH c-allele is about 0.1%.

In 1991, samples of embryos were also taken from Lanes Seedling, tree number 2 in Figure 2, and Napoleon, tree number 8 in Figure 2. Trees were sampled on a quadrant basis, but only total numbers for each isozyme are given in Table 23.

The totals for Lanes Seedling indicate that Mazzard, Napoleon and Williams Favourite all donated pollen genes. In particular, Mazzard is implicated by the presence of the GOT b-allele in 35% of the embryos sampled, Mazzard being the only source of the GOT b-allele in the study area. Lanes Seedling is homozygous aa for G6PD, yet 73% of the embryos sampled were heterozygous for G6PD (Table 23). This implicates Williams Favourite and Mazzard again as the main pollen donors. All other cultivars can provide only the a-allele for G6PD (Table 20). Some insight into the role Napoleon plays is given by the data for GPI in (Table 23) and examination of genotypes

in the study area. That is, 18% of embryos sampled from Lanes Seedling carried the GPI b-allele, both Lustre and Napoleon carry the GPI b-allele, but it could only have come from Napoleon, because Lustre did not flower with Lanes Seedling. Alternatively, the Mazzard may carry the GPI b allele as a heterozygote, and with the level of gene flow indicated by the GOT isozyme from Mazzard to Lanes Seedling, the embryos carrying the GPI b allele could have come from the same cross. It is difficult to separate the input of Napoleon from Williams Favourite when looking at the PGM locus because both have the same genotype. FDP provides some information in this regard. Lanes Seedling is homozygous aa for FDP (Table 20). The Mazzard sucker produced both homozygous aa and bb embryos, this means it must carry both the a and b allele of FDP (Table 22) and Napoleon is heterozygous. If either of these cultivars donated pollen to Lanes Seedling then both aa and ab progeny would be expected in a 1:1 ratio. The only other source of the FDP b allele is Williams Favourite, which is homozygous for this allele. However, this cross would result in only heterozygous progeny. The actual figures in Table 23 show an excess of heterozygotes of the order of 2:1. This indicates that the homozygous Williams Favourite is contributing genes and causing a skew in expected ratios at the same time the heterozygotes, Napoleon and Mazzard are also providing gene flow. Similarly, for SKDH, Lanes Seedling is aa homozygous and 67 heterozygous embryos were detected of the 159 analysed. The SKDH b-allele could have come from either the Mazzard or Napoleon, number 9.

Table 23. Isozyme genotypes of embryos sampled from Lanes Seedling and Napoleon trees at Crammond's Orchard,Basket Range, South Australia in 1991.

Isozymes								
6PGD	GOT	G6PD	GPI	IDH	PGM	FDP	SKDH	MDH
Lanes Seedling								
135aa	9aa	39aa	131aa	127aa	60aa	42aa	92aa	35aa
28ab	30ab	108ab	30ab	16ab	93ab	94ab	67ab	103ab
1bb	98ac		8fastaa	18ac		3bb		9bb
	28bc							
	1cc							
Napoleon								
59aa	32ab	5aa	51aa	70aa	48aa	33aa	37aa	18aa
65ab	39ac	115ab	78ab	19ab	80ab	52ab	91ab	57ab
	14bc		1bb	3fastaa		23bb		16bb
	1cc							

To summarise, it appears that Mazzard and Williams Favourite are contributing most of the pollen genes to Lanes Seedling embryos, about 35 and 37 per cent respectively, and Napoleon provided another 18%.

The results for Napoleon in Table 23 are more straight forward. The data for G6PD show a predominance of heterozygotes, Napoleon number 8 is homozygous aa for G6PD (Table 20) as are Lustre, Lanes Seedling, Napoleon numbers 7 and 9 (Figure 2) and Clements Pride. On the basis of these results this rules these cultivars out as major gene donors to Napoleon. Looking at SKDH, Napoleon number 8 is homozygous aa, yet 76% of the embryos analysed are heterozygous. The b allele must have been donated by Mazzard, because Lustre and Napoleon number 9, the only other carriers of the SKDH b-allele, have been ruled out on the basis of the G6PD result. GOT is the other indicator of gene flow from the Mazzard sucker. All of the cultivars in the study area have the ac genotype except Mazzard, which carries the GOT-b allele. Table 23 shows that 46 of the 86 Napoleon embryos sampled or approximately 53% carry the GOT b-allele from Mazzard. It is difficult to separate out the effect of Mazzard and Williams Favourite at other loci where they share the same genotype ie. 6PGD, G6PD. GPI, PGM and FDP (Table 20 and Table 22). Thus, a first estimate of gene flow to Napoleon number 8 could be 53% from Mazzard seedling and the difference could be attributed to Williams Favourite.

6.3.4 Beauchamps Black

Isozyme genotypes of leaves analysed from cultivars in the Basket Range orchard, owned by Mr W.N. Bishop (Figure 3) are given in Table 24.

Table 24. Leaf isozyme genotypes of trees in Bishop's cherry orchard at Basket Range, South Australia from a study area shown in Figure 3. The genotypes of Black Douglas (Mother) and Waterloo (L) (Appendix VI) have been included for comparison.

	Isozyme								
Cultivar	6PGD	GOT	G6PD	GPI	IDH	PGM	FDP	SKDH	MDH
Beauchamps Black	ab	ac	ab	aa	aa	ab	aa	ab	aa
Waterloo*	ab	ac	ab	aa	aa	aa	aa	ab	aa
Williams Favourite (E)+	ab	ac	ab	aa	aa	ab	aa	aa	aa
Williams Favourite (W)	ab	ac	ab	aa	aa	ab	aa	ab	aa
Opal	ab	ac	ab	aa	aa	ab	aa	ab	aa
Black Douglas (Mother)	aa	bc	ab	aa	aa	aa	aa	aa	aa
Waterloo (L)	aa	bc	ab	aa	aa	ab	ab	aa	ab

* Waterloo in the study area was incorrectly labelled and was in fact a Williams Favourite type.

+ Letters in parentheses refer to the following E = East, W = West and L = LHC.

Table 25. Isozyme genotypes for embryos analysed from a Beauchamps Black Cherry tree as shown in Figure 3, in Bishop's orchard at Basket Range, South Australia. Totals are given for 1992 and 1993. Missing data have not been included so totals may vary.

Isozyme								
6PGD	GOT	G6PD	GPI	IDH	PGM	FDP	SKDH	MDH
				1992				
85aa	15ab							
96ab	117ac	101aa	56aa	125aa	83aa	32aa	63aa	146aa
14bb	1bb	91ab	121ab	45ab	113ab	135ab	128ab	
	11bc	4bb	11bb	25ac		17bb	3bb	
	52cc			1bc				
				1993				
35aa	5ab	48aa	110aa	104aa	19aa	24aa	110aa	51aa
71ab	43ac	62ab	10ab	14ab	101ab	84ab	11ab	23ab
10bb	74bc	8bb				6bb		

Table 25 provides the results of embryo isozyme analysis from the Beauchamps Black tree shown in Figure 3 for 1992 and 1993.

Seasonal variation is evident in the ratios between homozygous and heterozygous embryos. For instance, in 1992 there was an excess of heterozygotes for SKDH (Table 25), while in 1993 there were ten times the number of SKDH aa homozygous embryos compared to heterozygotes. Similarly, for GPI in 1992, analyses showed an excess of heterozygotes to aa homozygotes of approximately 2:1. Yet in 1993, homozygotes aa for GPI outnumbered the heterozygous embryos by ten times. It should be noted here that full bloom occurred at the same time for all of the cultivars in the study area in both years, and so on this basis, each had an opportunity to donate pollen to Beauchamps Black.

Comparison of the genotypes of the cultivars in the study area determined by isozyme analysis of leaves (Table 24), and those of the embryos harvested from the Beauchamps Black tree in 1992, reveal that at least somewhere between 70 and 83 per cent of embryos were derived from pollen sources other than the cultivars typed for isozymes. The evidence for this lies with the GPI and FDP loci. Within the study area all of the cultivars are homozygous aa for both loci (Table 24). Thus, the outcome of crosses within the study area for FDP and GPI would be expected to be homozygous aa embryos only. In contrast to this 70% of embryos tested carry the GPI b-allele and 83% carry the FDP b-allele, primarily as heterozygotes. Both loci show small numbers of the bb homozygotes in the embryos analysed. In 1993 the key indicators for gene flow from outside of the study area were GOT, with 65% of embryos carrying the GOT b-allele, and FDP, with 70% of embryos carrying the FDP b-allele and also MDH with 31% of embryos carrying the MDH b-allele. And as Table 24 shows, these alleles are absent from the cultivars analysed in the study area. In contrast, the outcome for GPI fitted the expected outcomes for cultivars in the study area being the major source of donated genes.

No estimate of the contribution of genes, if any, by the cultivars within the study area can be made. This is because of the high influx of foreign genes from outside of the study area.

6.4 Discussion

Embryo isozyme analysis has proved to be a good diagnostic tool, by using the process of elimination pollen donors and gene flow can be deduced for different orchards.

Segregation ratios of isozymes in embryos of open pollinated cherry cultivars provide a good guide as to the identity of other pollinating cultivars. In this situation, segregation analysis is not appropriate because the isozyme genotypes of both parents for a particular embryo are not certain and several cultivars are usually implicated in pollination of a single cultivar under consideration.

Jackson and Clarke (1991) showed that gene flow in almonds was concentrated between neighbouring parts of tree canopies, and that paternity analysis using isozymes gave an 'apparent, net' gene flow. In the case of sweet cherries, gene flow is occurring over much greater distances. For instance at LHC it was shown that the two universal pollinators Vista and Merton Glory were contributing about 50% each of the paternal genes of embryos harvested from Sam trees. Tree numbers 82, 86 and 92 were 9, 11 and 27 metres away from Merton Glory in a straight north west - south east line, respectively (Figure 1). And 11, 15 and 36 metres away from Vista in a straight north west - south east line, respectively. Thus, in this orchard 50% gene flow occurred over 50 metres compared to zero gene flow beyond 76 metres reported in almonds (Jackson and Clarke, 1991).

Bee behaviour has been reported as having the largest influence on gene flow in commercial almond orchards (Jackson and Clarke, 1991) and increasing sweet cherry production through increased pollination and fruit set (McGregor, 1971). In their movements to collect nectar and pollen from flowers worker bees transfer pollen from anthers to styles within and between flowers. On returning to the hive they utilise specialised leg parts to remove pollen grains adhering to the

body and place it into storage cells. At LHC gene flow between Sam and Stella trees enclosed by bird proof netting and trees outside of the netted area was insignificant. The presence of the netting probably modified the behaviour of honey bees. It may have been that of the hives provided some bee colonies were dedicated to flying through the netting and visiting only trees inside the exclosure, while worker bees from other hives were dedicated to visiting only flowers outside of the netted area. Because if worker bees from one hive were visiting cherry trees both inside and outside of the netted area a greater gene flow between trees inside and outside of the net would be expected. Pollen mixing does not occur in hives sited in almond orchards (Jackson pers comm) and it is thought that bees visit one cultivar return to the hive and clean adhering pollen from the body with specialised leg parts before visiting another cultivar. In the orchard at LHC this activity could be further exaggerated by the presence of bird netting. The small amount of gene flow observed between trees inside and outside of the exclosure could represent small amounts of pollen left on bees working trees inside and outside of the net or perhaps a worker bee dedicated to working outside of the net which had strayed into the netted area.

Embryos from Sam trees at LHC showed significant numbers of genotypes that could only arise through mutation or crossing over at an isozyme locus. For, example Sam is aa homozygous for GPI, yet 3.9% of the embryos surveyed displayed the bb genotype. Similarly Sam is bb for 6PGD and analysis produced 8.25% of embryos with the aa genotype. As discussed in Chapter 4, a high rate of mutation at the isozyme loci is not unexpected because of their linkage to the S-gene which also experiences high rates of mutation.

In examining the results of isozyme analysis for embryos sampled from Stella at LHC the importance of the effective flowering period was evident. McGregor (1971) states that when the cherry flower opens, the stigma is receptive, but the anthers are closed. And further that pollination on the first day after anthesis was much more effective than pollination on the second day. Prevailing weather conditions can further restrict the time available for pollination by effecting bee behaviour. As a consequence, Venus which flowers at the same time as Stella at

LHC, donated, on average, 60% of the pollen genes to Stella embryos. It was estimated that 29% of embryos were a result of selfing and another 11% from other sources. In all, 71% of embryos were a result of outcrossing, or 71% of gene flow was coming from 5 metres or more away and 29% from bee movement within the same cultivar. If Stella were not self-fertile this 29% would not occur and would represent a loss in production. Furthermore, this may or may not represent the amount of self fertilisation that would occur if Stella were planted as a solid block or enclosed by insect proof caging with a hive of honeybees. What these results do say is that pollen from other cultivars is more likely to effect fertilisation in Stella as compared to self pollen.

Gene flow studies in commercial cherry orchards displayed results which also appear to be dominated by bee behaviour. Both the Crammond and Bishop orchards relied on feral bee populations. Bee hives were not introduced into the orchard. In this situation satisfactory cropping levels have been achieved. At Crammonds orchard, Basket Range, South Australia, Clements Pride set good crops. The majority of cherry growers in South Australia are unable to do this. Comparison with the Clements Pride genotype in Appendix VI shows it is different to that on Crammonds orchard (Table 20) at three isozyme loci and is similar to the Williams Favourite situation in South Australia (Granger et al, 1993) where it is apparent that intracultivar variation exists. While all of the Lanes Seedling trees shared the same isozyme profile, one of the Napoleon trees, number 8, showed two isozyme differences to that of Napoleon 7 and 9 which shared identical profiles. The Williams Favourite tree in the Crammond orchard was also different to any of the Williams Favourite cultivars described in Appendix VI. Equally it did not share the isozyme profile of any of the other cultivars listed in Appendix VI. So the basis of the performance of Clements Pride in this orchard is probably related, at least in part, to intracultivar genetic differences and also to the positioning of Clements Pride in relation to a number of different cultivars, thereby effecting successful pollination. The directional influence found to be prevalent in almond orchards, where almost all pollination occurred between neighbouring trees, was not as obvious at Crammonds orchard. Although an indication of this same sort of directional influence was noted in the results in which a greater number of embryos were recorded from the

side of the Clements Pride tree facing the source of Mazzard pollen genes compared to the opposite side of the tree. Only small numbers were recorded with the gene from the Mazzard seedling and examination of data in Table 21 tends to indicate that the distribution of pollen genes is not restricted to the side of the tree facing the pollen gene donor as in almonds (Jackson and Clarke, 1991). Clements Pride at Crammonds orchard is directly exposed to a greater number of pollen donors compared to the almond situation described by Jackson and Clarke (1991); however, the results indicate that gene flow is occurring at higher rates and over longer distances in cherry orchards with various configurations of trees. For example, at Crammond's, Napoleon provided 18% of the gene flow over a distance of 21 metres to Lanes Seedling (Figure 1). Furthermore, the Mazzard seedling in the same orchard donated 53% of the pollen genes to Napoleon number 8 over a distance of 14 metres and the two trees were separated by Clements Pride (Figure 1), with Williams Favourite supplying the remaining 47% over a 16m distance. Similarly, the Beauchamps Black tree studied at Bishop's orchard also situated at Basket Range in South Australia indicated that practically all of the pollen genes were coming from outside of the study area at a distance of not less than 12 metres, and this included all sides of the tree.

The bulk of the gene flow at Crammond's orchard occurred in a south-westerly direction. This was the main trend but it did not exclude a significant amount of gene flow in other directions. In the commercial cherry orchards studied bee hives were not introduced. Feral bee colonies were relied upon for pollination purposes and were thought to reside in natural vegetation surrounding the orchards. In addition it must be remembered that bees are foraging for the food sources supplied by nectar and pollen, and do not deliberately pollinate tree crops. Given this, it seems that pollination in South Australian cherry orchards depends on bee colony placement in relation to each cultivar. For instance, the situation with Sam at LHC can be visualised as bees flying to the Vista and Merton Glory trees (Figure 1) unladen from the hive. Pollen and nectar collection would occur and bees would then fly back to the hive. Foraging could occur in the Sam trees either on the way to or from the Vista and Merton Glory trees or in both directions. Conceivably, bees weighted down by pollen and nectar may make a series of flights from one tree to the next on

route back to the hive, at each stop further foraging would occur thereby transferring pollen from previously visited flowers to the next.

With the configuration of trees at LHC in relation to the placement of hives, gene flow occurred in one direction, between Sam, Vista and Merton Glory. Gene flow to Stella at LHC was dominated by Venus which flowered at the same time, in an adjacent row (Figure 1). While at Crammond's gene flow occurred in many directions with a tendency toward a south west - north east axis. So, in contrast to an almond orchard, the evidence from cherry orchards suggests that bees not only fly along rows but also fly through rows. This change in behaviour is associated with changed environments. The main difference being a departure from solid rows of a single cultivar, as in almonds to a more random configuration in commercial cherry orchards. Isozyme analysis revealed genetic variation within cherry cultivars in comparison to planting material in almond orchards which is more uniform. This leads to a realisation that a random configuration of cultivars and bee colonies in relation to one another has resulted in higher gene flows over larger distances in South Australian cherry orchards compared to almond orchards (which have ordered planting schemes and uniform placement of bee hives is usually practiced).

In cherry orchards a greater number of cultivars over a wider area need to be analysed for successful paternity analysis. Because gene flow is occurring over much larger distances as compared to almond orchards and as in the case of Beauchamp's Black at Bishop's orchard is not necessarily confined to nearest neighbours with sufficient overlap in flowering time. The importance of direction of cherry cultivars in relation to one another tended to be expressed to a greater degree where bee hives were introduced, as at LHC where gene flow was aligned with cultivars and the bee hive. In contrast, gene flow at Crammond's orchard occurred in many directions although the majority tended to be in a south west - north east direction, which corresponds to the prevailing wind direction in the Mount Lofty Ranges.

6.5 Conclusions

Recommendations for the cherry industry from this work are as follows:

Where bird exclosures are used, both polliniser cultivars and bee hives should be placed inside the netted structure. Bees should be introduced to the orchard and hive position should not be restricted to the end of rows or a single apiary. A random distribution of hives would result in a greater amount of gene flow, but this approach would be regulated by apiary management requirements.

For pollination, a random assortment of many different cultivars in the orchard would be expected to produce best results. This is not acceptable to modern orchardists who prefer to plant solid rows of a single cultivar, and so a compromise should be reached. This would include planting half of a row with cross-compatible cultivars which flower at the same time and planting compatible cultivars in adjacent rows. The best compromise may be to plant pollenisers in the row in a 1:9 ratio offset by five trees, thereby forming a diamond pattern of pollenisers throughout the planting and ensuring that each tree is no more than 3 trees away from a polliniser. Similar planting designs for sweet cherry have been suggested by McGregor (1971) for cherry orchards in the United States.

Self-fertile cultivars such as Stella should not be planted as solid blocks of trees, because fruit set could be reduced. Although larger fruit size might be achieved with lower fruit set. Pollen from another source is more efficient in the production of fruit and specific cross pollinators should be included within plantings of self-fertile cultivars.

GENERAL DISCUSSION

World-wide sampling, protein extraction and electrophoretic procedures vary significantly. In particular, the use of different matrices for electrophoresis can produce different results. There is a need to standardise procedures so results from all laboratories can be used to further the knowledge of genetics for each group of species.

The question then arises, are isozymes the method that should be used, or should DNA based procedures be adopted? Procedures such as Restriction Fragment Length Polymorphism tend to produce too much information which can be uninterpretable. As a consequence many laboratories are now using methods based on the Polymerase Chain Reaction which basically involves the amplification of small segments of DNA. The strength of isozyme analysis lies with the fact that they are expressions of genes stable under a range of environmental conditions that can be analysed using methods based on Mendelian genetics and subsequently inheritance and linkage characteristics determined. This type of work would ideally be supported by karyotype analysis to determine the position of enzyme genes on chromosomes.

Isozymes showed a high degree of polymorphism which was a reflection of the outbreeding reproductive system of sweet cherry. This isozyme polymorphism was successfully used to uniquely identify cherry cultivars. Allele frequencies were also used to determine the genetic distance between cultivars grouped by country of origin which showed that all of the groups had undergone similar amounts of change. And, because the majority of the cultivars examined were products of breeding programs, it indicates that each country has effected a similar amount of genetic gain albeit associated with different characteristics.

The number of isozyme differences between any two cultivars was also used as a measure of genetic relatedness and corresponded well with allele frequency. Isozyme differences were used to determine the parentage of several local cultivars.

It has been established here for sweet cherry that some isozymes present in leaves are absent in embryo and pollen and vice versa. Further investigations should be conducted to determine if isozyme expression differs between tissues and developmental phases of the same tissue.

Linkage analysis of isozyme results showed that many of the isozymes were closely linked, and, in turn, some of the isozymes were tightly linked to the self-incompatibility locus. This had a cascade effect, the consequence of which was that all of the isozymes studied appeared to be linked to the self-incompatibility locus. One conclusion arising from this was that the isozymes themselves may be playing a role in the incompatibility reaction. This has been the basis of many models put forward by various workers to explain the self-incompatibility mechanism (de Nettancourt 1977). Close linkage between glutamate oxaloacetate transaminase (GOT) and the self-incompatibility locus was particularly evident in the progeny of the self-fertile cultivar, Stella, following selfing. A deficiency in progeny homozygous for any of the GOT alleles showed that recognition of the mutated S-allele (S_4') was occurring. Microscopy results showed normal pollen tube growth had occurred along the length of the style and entered the micropyle successfully. Thus, it was concluded that the S_4' allele was recognised at fertilisation and inhibition occurred at this point. This data is complimentary to the model proposed by McClure et al(1990) in which the pollen tube cell wall was implicated in the recognition process by acting as a selective barrier to S-allele specific RNase enzymes. In this model, pollen tube cell walls possessing an S-allele in common with that of the style were permeable to S-RNases produced by the style. These are taken up into the cytoplasm of the pollen tube where degradation of RNA occurs. This inhibits protein synthesis and halts pollen tube growth. Because S_4' pollen tubes of sweet cherry in this study were observed to grow the length of the style unnoticed, it suggests that the mutation has effected the pollen tube cell wall and other factors involved in the recognition process are being excluded. In sweet cherry,

isozyme analysis showed that ribonucleases were not the primary factor involved in the incompatibility reaction. While isozyme analysis has shown that ovarian inhibition is occurring in the case of selfing Stella, further microscope studies to capture the moment of ovarian inhibition of pollen tube growth would prove beyond doubt that this was occurring. Further, comparative studies between pollen tube cell walls of normal pollen and pollen carrying the S_4' allele should be conducted to determine their role in the incompatibility reaction.

Gene flow studies using isozyme analysis of cherry embryos showed that the apparent net gene flow in cherry orchards is occurring over greater distances in comparison to almond orchards. It was thought that an interaction between bee behaviour and orchard design was having the greatest influence on pollination. In the same way, isozymes were used to examine the breeding behaviour of Stella. About 71% of Stella embryos were a result of outcrossing and this suggests that compatible pollen from other cultivars has a competitive advantage in the effective fertilisation of Stella ova. The practical implications of this is, rather than plant self-fertile cultivars in solid blocks a compatible polliniser should be included in the planting.

ABBREVIATIONS

AAAT	Aromatic amino acid transaminase	LAP	Leucine amino peptidase
AAT	Amino acid Transaminase	LDH	Lactate dehydrogenase
ACON	Aconitase	LHC	Lenswood Horticultural Centre
ACP	Acid Phosphatase	M	Molar
ADH	Alcohol Dehydrogenase	Ma	Milliamps
AK	Adenylate Kinase	MDH	Malate dehydrogenase
ALD	Aldolase	ME	Malic enzyme
ALPH	Alkaline Phosphatase	Mg	Milligram
α-AMY	alpha - Amylase	$MgCl_2$	Magnesium Chloride
AP	Alkaline Phosphatase	ml	Millilitre
ApA	Adenyl (3'5') Adenosine	MTT	Methyl-thiazolyl blue
AO	Aldehyde Oxidase	NAD	Nicotinamide adenine dinucleotide
ATP	Adenosine 5' - triphosphate	NADH	Nicotinamide adenine dinucleotide - reduced form
CA	Carbonate Dehydratase	NADP	Nicotinamide adenine dinucleotide phosphate
CAT	Catalase	NADPH	Nicotinamide adenine dinucleotide phosphate - reduced form
CK	Creatine Kinase	NaOH	Sodium Hydroxide
cm	Centimetre	NBT	Nitro blue tetrazolium
°C	Degrees celsius	PEG	Polyethylene glycol
DIA	Diaophorase	PER	Peroxidase
DNA	Deoxyribose nucleic acid	PGK	Phosphoglycerate Kinase
EDTA	Ethylenediamine-tetra-acetic acid	PGM	Phosphoglucomutase
EST	Esterase	PK	Pyruvate Kinase
FDP	Fructose 1,6 - diphosphatase	PMS	Phenazine methosulphate
g	Gram	PVP	Polyvinylpyrrolidone
GalDH	Galactose dehydrogenase	PVPP	Polyvinylpolypyrrolidone
GDH	Glutamate dehydrogenase	6PGD	6-phosphogluconate dehydrogenase
GPD	Glycerol-3-phosphate dehydrogenase	RNA	Ribose nucleic acid
GLDH	Glucose dehydrogenase	RNase	Ribose nuclease
GOT	Glutamate Oxaloacetate transaminase	SDS-PAGE	Sodium dodecyl -- polyacrylamide gel electrophoresis
GPI	Glucose phosphate isomerase	SKDH	Shikimate dehydrogenase
GPT	Glutamate-pyruvate transaminase	SOD	Superoxide dehydrogenase
G6PD	Glucose-6-phosphate dehydrogenase	SORDH	Sorbitol dehydrogenase
HCl	Hydrochloric Acid	TPI	Triose phosphate isomerase
HK	Hexokinase	TYR	Tyrosine
IDH	Isocitrate dehydrogenase	μl	Microlitre
IEF	Isoelectric focusing	XDH	Xanthine Dehydrogenase
K_3PO_4	Potassium phosphate	XO	Xanthine Oxidase
LAC	Laccase		

Appendix I. Gel and electrode buffers used for enzyme systems.

Gel buffer	Electrode buffer	Enzyme(s)	Reference
0.05M Tris 0.007M Citrate pH 8.3	0.038M Lithium hydroxide, 0.19M borate pH 8.3	APH, EST, GOT, PER, PGM, GPI, LAP	Arulsekar and Parfitt (1986)
1:9 electrode buffer:water	0.3M Tris citrate pH 7.0	ME, IDH, HK	Ashari et al (1989)
1:28 electrode buffer:water pH 7.2	0.223M Tris, 0.069M citrate pH 7.2	MDH, SKDH, 6PGD	Ashari et al (1989)
1:10 electrode buffer:water pH 7.4	0.1M Tris; 0.1M maleate, 0.01M Na_2EDTA 0.01M magnesium chloride pH 7.4	PGM, CAT, GDH, F1,6DP	Ashari et al (1989)
0.05M Tris; 0.09M boric acid	0.5M Tris; 0.6M boric acid, 0.016M Na_2EDTA pH 8.0	PER, SOD	Ashari et al (1989)
0.03 Tris Citrate pH 8.2	0.34M sodium borate pH 8.	GOT	Ashari et al (1989)

Appendix II. Staining solutions for enzymes electrophoresed on starch gels.

No.	Ingredients	Amount	Reference
1.	Acid Phosphatase Fast Garnet GBC salt Sodium acetate buffer (pH 5.0) 0.1M $MgCl_2$ 1% alpha-napthyl acid phosphate	 100mg 100ml 5ml 2ml	 Aruleskar and Parfitt (1986)
	0.05M Sodium acetate buffer (pH 5.0) 0.1M $MgCl_2$ alpha-napthyl acid phosphate, sodium salt Fast Garnet GBC salt	100ml 5ml 100mg 80mg	Soltis et al (1983) modified
2.	Catalase Solution A: water acetic acid potassium iodide	 100ml 2ml 2g	 Ashari et al (1989)
	Incubate at room temperature for 15 minutes, rinse several times with distilled water then add Solution B: water 30% Hydrogen peroxide	 100ml 1ml	
3.	Esterase Fast Blue RR salt (Dissolve in 40ml of deionised water) Phosphate (NaH_2PO_4)(27.8gl^{-1} solution) Phosphate (NaH_2PO_4)(53.6gl^{-1} solution) 0.1% alpha-napthyl acetate (0.05g per 50ml acetone)	 40mg 50ml 10ml 2ml	 Arulsekar and Parfitt (1986)
4.	Fructose 1,6 Diphosphate 1M Tris HCl pH 8.0 1M $MgCl_2$ Fructose - 1, 6 - diphosphate NADP MTT PMS Phosphoglucoseisomerase Glucose - 6 - dehydrogenase Water	 10ml 1ml 250mg 10mg 5mg 1mg 50units 50units 90ml	 Ashari et al (1989)
5.	Glutamate Dehydrogenase 1M Tris HCl pH 8.0 1M glutamic acid pH 8.0 NAD MTT PMS Water	 10ml 20ml 20mg 10mg 2mg 70ml	 Ashari et al (1989)
6.	Glutamate Oxaloacetate Transaminase Aspartic acid alpha-ketoglutaric acid (in 100ml of water pH adjusted to 8.0 with NaOH) Fast Blue BB salt	 250mg 150mg 150mg	 Arulsekar and Parfitt (1986)
	0.1M Tris pH 8.5 alpha-ketoglutarate Aspartic acid Pyridoxal - 5 - phosphate Fast Blue BB salt	100ml 100mg 200mg 10mg 150mg	 Vallejos (1983) modified
	Incubate at 36°C until bands appear.		

No.	Ingredients	Amount	Reference
7.	Glucose Phosphate Isomerase		
	NADP	10mg	Arulsekar and Parfitt (1986)
	PMS	5mg	
	MTT	15mg	
	1M Tris HCl (pH 8.0)	10ml	
	0.1M $MgCl_2$ ($6H_2O$)	10ml	
	Deionised water	80ml	
	0.18M fructose - 6 - phosphate	2ml	
	Glucose - 6 - phosphate dehydrogenase	40units	
8.	Hexokinase		
	0.05M Tris (pH 8.0)	4ml	
	Dextrose	130mg	Ashari et al (1989)
	ATP	16mg	
	NADP (0.013M 1% solution)	0.5ml	
	0.1M Magnesium chloride	0.66ml	
	0.01M NBT	0.33ml	
	0.01M PMS	0.03ml	
	Glucose - 6 - phosphate dehydrogenase	10units	
	Ingredients poured into a flask with 6ml 1% boiling agar. Mixture is then poured over the gel.		
9.	Isocitrate dehydrogenase		
	0.1M Tris HCl (pH 7.5)	50ml	Ashari et al (1989)
	1M $MgCl_2$	0.5ml	
	DL isocitric acid	50mg	
	NADP	5.5mg	
	MTT	10mg	
	PMS	2mg	
10.	Leucine aminopeptidase		
	Leucyl-napthyl amide HCI	20mg	Arulsekar and Parfitt (1986)
	Black K salt	20mg	
	Dissolve in 30ml of deionised water		
	0.2M MaOH	20ml	
	2M Tris-maleic	50ml	
11.	Maleate dehydrogenase		
	1.0M Tris-HCI (pH 8.0)	10ml	Soltis et al (1983)
	2.0M DL-malic acid (add NaOH to pH 8.0)	10ml	
	Water	80ml	
	NAD	10mg	
	MTT	10mg	
	PMS	2mg	
12.	Malic enzyme		
	DL-Malic acid	100mg	Ashari et al (1989)
	0.1M Tris-HCI pH 7 (readjusted with NaOH)	20ml	
	NADP (in 1ml H_2O)	5mg	
	MTT (in 1ml H_2O)	5mg	
	PMS (in 0.1ml H_2O)	0.5mg	
	25ml warm liquid agar poured over gel.		
13.	Peroxidase		
	3-amino-9-ethyl carbazole (dissolved in 5ml dimethyl formamide)	50mg	Arulsekar and Parfitt (1986)
	0.1M $CaCl_2$	2ml	
	30% sodium acetate (pH 5.0)	0.2ml	
	1.0M sodium acetate pH 4.7 (adjusted with 5ml glacial acetic acid)	95ml	
	95% ethanol	30ml	
	30% hydrogen peroxide	0.5ml	
	Water	65ml	

No.	Ingredients	Amount	Reference
14.	Phosphoglucomutase		
	NADP	10mg	Arulsekar and Parfitt (1986)
	PMS	5mg	
	MTT	15mg	
	alpha-D-glucose-1-phosphate	75mg	
	1M Tris-HCI (pH 8.0)	2ml	
	0.1M $MgCl_2$	5ml	
	Glucose-6-phosphate dehydrogenase	40units	
	Water	93ml	
15.	Shikimate dehydrogenase		Ashari et al (1989)
	Tris-HCI pH 8.5	10ml	
	Shikimic acid	100mg	
	NADP	10mg	
	MTT	20mg	
	PMS	2mg	
	Water	90ml	
16.	Superoxide dimutase		
	0.05M Tris-HCI (pH 8.0)	25ml	Arulsekar and Parfitt (1986)
	MTT	5mg	
	PMS	5mg	
	Added to 25ml boiled liquid agar (2%)		
17.	6-Phosphoglucose dehydrogenase		
	1.0M Tris-HCI pH 8.2	10ml	Ashari et al (1989)
	1.0M $MgCl_2$	2ml	
	6-Phosphogluconic acid	40mg	
	NADP	10mg	
	MTT	10mg	
	PMS	2mg	
	Water	90ml	

Appendix III. Summary of chemicals and procedures used in extraction buffers from 17 different publications. Ranked from most used to least (1-8)

Chemical/Procedure	Number of publications	Rank
Tris	3	6
Potassium Chloride	2	7
Magnesium Chloride	2	7
EDTA	4	5
β-Mercaptoethanol	2	7
Ascorbic acid	4	5
PVPP	5	4
PVP	10	1
2-Mercaptoethanol	7	2
Sucrose	1	8
Sodium Phosphate	1	8
Tris-Maleate	5	4
Glycerol	2	7
Triton-X-100	3	6
Tris-HCI	7	2
Gluthione	1	8
Citric acid	1	8
Polyethyleneglycol	1	8
Dithioreitol	1	8
Potassium Phosphate	2	7
Sodium Tetraborate	4	5
Sodium Metabisulphite	6	3
Diethyldithiol	2	7
Phenylmethylsulfonylfluoride	1	8
Bovine Serum Albumin	1	8
Sodium ascorbate	1	8
DIECA	3	6
DMSO	1	8
Geranium dioxide	1	8
Phosphate buffer	1	8
Maleic acid	1	8
Low pH 6.5 - 7.2	1	8
Etiolation	1	8
Grinding	2	7
Crude extract	2	7
Centrifuge	2	7
Liquid Nitrogen	3	7
Low temperature	1	6

Appendix IV. Results as measured by isozyme band activity and resolution on zymograms with banding variation as an indicator of usefulness for a combination of tissue, extraction buffer and electrode buffer treatments for given dates.

Date	Tissue	Extraction buffer	Gel & electrode buffer	Iso-zyme	Activity	Resolution	Variation
22-9-89	Leaves	Parfitt	0.05M Tris-Maleate pH 7.8	GOT	yes	poor	yes
				GPI	yes	poor	yes
				LAP	yes	poor	no
				IDH	yes	poor	no
			0.025M Tris-Glycine pH 8.5	PGM	yes	poor	no
22-9-89	Leaves	Normal	0.05M Tris-Maleate pH 7.8	GOT	yes	satisfactory	yes
				GPI	yes	satisfactory	yes
				LAP	yes	satisfactory	no
				IDH	yes	satisfactory	no
			0.025M Tris-Glycine pH 8.5	PGM	yes	satisfactory	no
22-9-89	Pistils	Parfitt	0.05M Tris-Maleate pH 7.8	GOT	yes	poor	yes
				GPI	yes	poor	yes
				LAP	yes	poor	no
				IDH	yes	poor	no
			0.025M Tris-Glycine pH 8.5	PGM	no	-	-
22-9-89	Pistils	Normal	0.05M Tris-Maleate pH 7.8	GOT	yes	satisfactory	yes
				GPI	yes	satisfactory	yes
				LAP	yes	satisfactory	no
				IDH	yes	satisfactory	no
			0.025M Tris-Glycine pH 8.5	PGM	no	-	-
28-9-89	Leaves	Normal	0.05M Tris-Maleate pH 7.8	GOT	yes	satisfactory	yes
				GPI	yes	satisfactory	yes
				LAP	yes	satisfactory	yes
				IDH	yes	satisfactory	yes
			0.025M Tris-Glycine pH 8.5	PGM	yes	satisfactory	yes

Date	Tissue	Extraction buffer	Gel & electrode buffer	Iso-zyme	Activity	Resolution	Variation
18-10-89	Leaves	Normal	0.05 Tris-Maleate pH 7.8	ADH	yes	good	no
				GPI	yes	very good	yes
				GOT	yes	good	yes
				IDH	yes	very good	?
				AK	yes	fair	yes
				EST$^+$	yes	good	no
				HK	yes	-	-
				MDH	yes	very good	yes
				LAP	yes	very good	no
				GDH	no	-	-
				GLDH	no	-	-
				CK$^+$	yes	poor	-
				PGM	yes	good	no
				SORDH	yes	poor	-
				CAT	no	-	-
			0.02 Phosphate pH 7.0	G-6PD	yes	fair	yes
				GPT$^+$	yes	poor	-
				LDH	yes	poor	-
				ME	yes	poor	no
				6-PGD	yes	good	yes
			0.025M Tris-Glycine pH 8.5	PGM	yes	very good	yes
12-12-89	Embryo stigmata and styles	Normal	0.05M Tris-Maleate pH 7.8	GPI	yes	good	yes
				IDH	yes	good	no
				LAP	yes	good	no
				6-PGD	yes	good	yes
			0.02m Phosphate pH 7.0	GOT	yes	poor	-
			0.025M Tris-Glycine pH 8.5	PGM	yes	good	no
13-12-89	Embryo, fruit stalk, fruit flesh	Normal	0.05M Tris-Maleate pH 7.8	ADH	yes	good	no
				GDH	yes	poor	-
				G-6PD	yes	good	yes
				GPI	yes	good	yes
				IDH	yes	good	no
				LAP	yes	good	no
				MDH	yes	good	no
				ME	yes	good	no
				6-PGD	yes	good	yes

Date	Tissue	Extraction buffer	Gel & electrode buffer	Iso-zyme	Activity	Resolution	Variation
24-1-90	Embryo	Normal	0.02M Phosphate pH 7.0	G-6PD	yes	good	yes
				LDH	no	-	-
				6-GPD	yes	good	yes
7-2-90	Embryo	Normal	0.02M Phosphate pH 7.0	GOT	yes	poor	yes
				6-PGD	yes	poor	yes
				G-6PD	yes	poor	yes
				GPI	no	-	-
15-2-90	Embryo	Normal	0.02M Phosphate pH 7.0	SOD	no	-	-
				6-PGD	yes	good	yes
				FDP	yes	poor	yes
				SKDH	yes	good	no
				GPT^{+}	no	-	-
			0.05M Tris-Maleate pH 7.8	GPI	yes	poor	no
				HK	yes	poor	yes
				EST	yes	poor	no
			0.015M Tris-EDTA-Borate-$MgCl_2$ pH 7.8	ACP	no	-	-
				DIA	yes	poor	no
				AP	no	-	-
20-2-90	Embryo	Normal	Tris-EDTA-borate-$MgCl_2$	PER	no	-	-
			pH 7.2	SOD	no	-	-
				ACP	yes	poor	yes
			0.223M Tris/0.069M Citrate pH 7.2	SKDH	yes	good	yes
				HK	no	-	-
				FDP	no	-	-
21-2-90	Embryo	Normal	0.223M Tris/0.069M Citrate pH 7.2	ACON	yes	poor	no
				LAC	no	-	-
				ALD	yes	good	no
				CAT	no	-	-
				PER	yes	poor	-

Date	Tissue	Extraction buffer	Gel & electrode buffer	Iso-zyme	Activity	Resolution	Variation
22-2-90	Embryo	Normal	0.015M Tris-EDTA-borate-$MgCl_2$-$6H_2o$ pH 7.8	LAC	no	-	-
				PER	no	-	-
				ACP	yes	fair	yes
				ALD	yes	poor	-
				ACON	no	-	-
				SKDH	yes	poor	-
				GPD	no	-	-
				CAT	no	-	-
27-2-90	Embryo	Normal	0.05M Tris-Maleate pH 7.8	FDP	yes	satisfactory	yes
				SKDH	yes	good	yes
				ACP	yes	poor	yes
				ACON	no	-	-
				ADH	yes	good	no
				CK^{+}	yes	-	-
				ALD^{+}	yes	fair	no
				AK	yes	poor	no
28-2-90	Embryo	Normal	0.05M Tris-Maleate pH 7.8	XDH	no	-	-
				SORDH	yes	fair	no
				GPT^{+}	yes	fair	no
				FDP	yes	poor	yes
				EST	yes	poor	no
				MDHb	yes	good	no
				ACP	yes	poor	-
				GLDH	yes	poor	no
28-2-90	Pollen	Normal	0.05M Tris-Maleate pH 7.8	XDH	yes	good	-
				SORDH	yes	fair	no
				GPT^{+}	yes	fair	no
				FDP	yes	poor	-
				EST	yes	good	-
				MDHb	yes	good	no
				ACP	yes	poor	-
				GLDH	yes	poor	-

Date	Tissue	Extraction buffer	Gel & electrode buffer	Iso-zyme	Activity	Resolution	Variation
6-3-90	Embryo	Normal	0.05M Tris-Maleate pH 7.8	XDH	no	-	-
				EST	no	-	-
				G-6PD	yes	poor	-
				FDP	yes	poor	-
			0.015M Tris-EDTA-borate-$MgCl_2$ pH 7.8	XDH	no	-	-
				EST	no	-	-
				ACP	yes	good	yes
				FDP	yes	poor	no
7-3-90	Embryo	Normal	0.05M Tris-Maleate pH 7.8	SOD	no	-	-
				ALPH	yes	poor	-
				EST	no	-	-
				6-PGD	yes	very good	yes
			0.02M Phosphate pH 7.0	ALPH	yes	poor	-
				XDH	yes	warped/smeared	-
				DIA	yes	warped/smeared	-
				SOD	no	-	-
8-3-90	Embryo	Normal	0.02M Phosphate pH 7.0	AAAT	yes	good	yes
				DIA	yes	satisfactory	yes
				TYR	yes	poor	yes
				ALPH	yes	fair	no
			0.05M Tris-Maleate pH 7.8	GPI	yes	excellent	yes
				ALPH	no	-	-
				AO	no	-	-
				LAC	no	-	-
27-3-90	Embryo	Normal	0.02M Phosphate pH 7.0	ACP	yes	poor	-
				G-6PD	yes	good	yes
			0.05M Tris-Maleate pH 7.8	FDP	yes	feint	-
				SUCDD	yes	poor	-
				IA	no	-	-
				AAAT	yes	good	no
				?-GDP	no	-	-
				GPT+	no	-	-
				PK+	no	-	-
				ALPH	no	-	-
				EST	no	-	-

Date	Tissue	Extraction buffer	Gel & electrode buffer	Iso-zyme	Activity	Resolution	Variation
28-3-90	Embryo	Normal	0.05M Tris-Maleate pH 7.8	6-PGD	yes	poor	-
				G-6PD	no	-	-
				GPI	yes	poor (overloaded)	yes
						poor	
				ACP	yes	good	yes
				ADH	yes	good	no
				SKDH	yes	-	yes
				TYR	no	poor	-
				GPT$^-$	yes	-	-
				α-GDP	no	-	-
				GPT$^+$	no	-	-
				FDP	no	poor	-
				DIA	yes		-
3-4-90	Embryo	Normal	0.05M Tris-Maleate pH 7.8	XO	yes	poor/smeared	-
				TPI	yes	good	no
				EST	yes	poor	-
			0.02M Phosphate pH 7.0	XO	no	-	-
				TPI	yes	satisfactory	no
				EST	yes	poor	-

Date	Tissue	Extraction buffer	Gel & electrode buffer	Iso-zyme	Activity	Resolution	Variation
4-4-90	Embryo	Normal	0.015M Tris-EDTA-Borate-$MgCl_2$ pH 7.8	XO	no	-	-
				TPI	yes	poor	-
				EST	no	-	-
				PGK	no	-	-
				G-6PD	yes	poor	-
				SKDH	yes	good	no
				FDP	yes	poor	-
				DIA	yes	fair	yes
			0.05M Tris-Maleate pH 7.8	XO	no	-	-
				TPI	yes	poor	-
				EST	no	-	-
				PGK	no	-	-
				LAC	no	-	-
				ADH	yes	good	no
				XDH	no	-	-
				PER	no	-	-
				α-AMY	no	-	-
				CAT	no	-	-
				CA	no	-	-
				GalDH	yes	fair	no
			0.02M Phosphate pH 7.0	XO	no	-	-
				TPI	yes	fair	no
				EST	no	-	-
				PGK	no	-	-
			0.025M Tris-Glycine pH 8.5	XO	no	-	-
				TPI	yes	poor	-
				EST	no	-	-
				PGK	no	-	-

Date	Tissue	Extraction buffer	Gel & electrode buffer	Iso-zyme	Activity	Resolution	Variation
4-4-90	Pollen (one sample only)	Normal	0.015M Tris-EDTA-Borate-$MgCl_2$ pH 7.8	XO	yes	poor	-
				TPI	yes	poor	-
				EST	yes	poor	-
				PGK	yes	good	-
				G-6PD	yes	poor	-
				SKDH	yes	good	-
				FDP	yes	poor	-
			0.05M Tris-Maleate pH 7.8	DIA	yes	fair	-
				XO	yes	poor	-
				TPI	yes	poor	-
				EST	yes	good	-
				LAC	no	-	-
				ADH	no	-	-
				XDH	yes	fair	-
				PER	no	-	-
				α-AMY	no	-	-
				CAT	yes	good	-
				CA	no	-	-
			0.02M Phosphate pH 7.0	GalDH	yes	fair	-
				XO	yes	good	-
				TPI	yes	fair	-
				EST	yes	good	-
			0.025M Tris-Glycine pH 8.5	PGK	yes	good	-
				XO	yes	good	-
				TPI	yes	poor	-
				EST	yes	fair	-
				PGK	no	-	-
19-4-90	Leaf	Parfitt	0.05M Tris-Maleate pH 7.8	6-PGD	yes	good	yes
				ACP	yes	poor	?
				SKDH	yes	fair	yes
				G-6PD	yes	feint	yes
				XDH	yes	poor/smeared	-
				FDP	yes	good	yes
				AAAT	no	-	-
				GPI	yes	poor/smeared	-

Date	Tissue	Extraction buffer	Gel & electrode buffer	Iso-zyme	Activity	Resolution	Variation
27-4-90	Leaves	Parfitt	0.02M Phosphate pH 7.0	ACP	yes	good	no
	"	"		ALPH	yes	good	yes
	Leaves/ Embryos	Parfitt/Normal		EST	yes	poor	?
	Leaves/ Embryos	Parfitt/Normal	0.015M Tris-EDTA-Borate-$MgCl_2$ pH 7.8	ACP	yes	good	yes
	Leaves/ Embryos	Parfitt/Normal		ALPH	yes	good	yes
	Leaves	Parfitt	0.025M Tris-Glycine pH 8.5	PER	yes	poor	-
	Leaves	Parfitt		ACP	yes	fair	yes
	Embryo	Normal		ACP	no	-	-
	Leaves	Parfitt		PER	yes	poor/warped	-
	Embryo	Normal	0.01M Citrate-Phosphate pH 6.4	PER	no	-	-
	Leaves	Parfitt		ALPH	yes	good	no
	Embryo	Normal		ALPH	no	-	-
	Leaves	Parfitt		PER	yes	good	yes
	Embryo	Normal		PER	no	-	-
2-5-90	Leaves	Parfitt	0.05M Tris-Maleate pH 7.8	AK	yes	poor	-
				ACP	yes	fair	no
				GOT	yes	good	yes
				TYR	yes	poor	yes
			0.02M Phosphate pH 7.0	ALPH	yes	poor	-
				DIA	yes	good	yes
				AAAT	no	-	-
				PER	yes	fair	yes
			0.015M Tris-EDTA-Borate-$MgCl_2$ pH 7.8	ACP	yes	poor	-
				ACP	yes	good	no
				GOT	no	-	-
				TYR	yes	good	yes

Date	Tissue	Extraction buffer	Gel & electrode buffer	Iso-zyme	Activity	Resolution	Variation
15-5-90	Embryo	Parfitt	0.1M Tris-Citrate pH 8.1	TYR	no	-	-
				ACP	yes	poor	-
				ALPH	yes	poor	-
				PER	no	-	-
	Leaves	Parfitt		TYR	yes	poor	-
				ACP	yes	poor	-
				ALPH	yes	poor	-
				PER	yes	reasonable	no
	Embryo	Parfitt	0.015M Tris-EDTA-Borate pH 8.2	TYR	no	-	-
				ACP	yes	poor	-
				ALPH	yes	poor	-
				PER	no	-	-
	Leaves	Parfitt		TYR	yes	poor/smeared	-
				ACP	yes	poor	-
				ALPH	yes	poor	-
				PER	yes	poor/smeared	-

+ indicates staining procedure involving the production or loss of fluorescence. Requires a U.V. light source for observation.
- indicates an isozyme usually visualised by fluorescence, detected with an alternative MTT/PMS based reaction.

Appendix V. Staining protocols for isozymes used with cellogel.

Isozyme	Substrate(s), reagents, buffer	Linking enzymes	Reference
Adenylate Kinase (AK)	ADP, glucose, NADP, Magnesium Chloride, MTT, PMS, Tris-HCl pH 8.0	HK, 96PD	1
Fructose- 1,6 - Diphosphatase (FDP)	Fructose-1,6- Diphosphate, NADP, Mg,Cl_2, MTT, PMS, Tris-HCl pH 8.0	GPI, G6PD	2
Glutamate Oxaloacetate Transaminase (GOT)	Alpha-Ketoglutaric acid, L-aspartate, pyridoxal-5-phosphate, fast garnet, Tris-HCl pH 8.0	-	1
Glucose Phosphate Isomerase (GPI)	Fructose-6-phosphate NADP, Magnesium Chloride, MTT, PMS, Tris-HCl pH 8.0	G6PD	1,2 & 3
Glucose-6- Phosphate Dehydrogenase (G6PD)	Glucose-6-phosphate, NADP, Magnesium Chloride, MTT, PMS Tris-HCl pH 8.0	-	1
Isocitrate Dehydrogenase (IDH)	DL-isocitric acid, NADP, Magnesium Chloride, NADP, MTT, PMS, Tris-HCl pH 8.0	-	1
Leucine Amino Peptidase (LAP)	Leucine-3-napthyl HCl, Magnesium Chloride, 0.1M Tris-Maleate pH 6.5 incubate for 15 mins then restain with Fast Black, 0.1M Tris-Maleate	-	3
Malate Dehydrogenase (MDH)	Malic acid, Fast Garnet, NAD, Tris-HCl pH 8.0	-	1
Peroxidase (PER)	p-phenylalanine, ethanol, Manganese Sulphate, Sodium acetate pH 4.6 blot gel then restain with 30% Hydrogen peroxide		4
Phosphoglucomutase (PGM)	Glucose-1-Phosphate, NADP, Magnesium chloride, MTT, PMS, Tris-HCl pH 8.0		1,2 & 3
6-Phosphogluconate dehydrogenase (6PGD)	6-Phosphogluconic acid, NADP, Magnesium Chloride, MTT, PMS Tris-HCl pH 8.0		1,2 & 3
Shikimic Dehydrogenase (SKDH)	Shikimic acid, NADP, MTT, PMS Tris-HCl pH 8.5		2
Xanthine Oxidase (X0)	Hypoxanthine (in acetone), MTT, PMS, Tris-HCl pH 8.0		1

1. Richardson et al (1986)
2. Soltis et al (1983)
3. Arulsekar and Parfitt (1986)
4. Ashari et al (1989)

Appendix VI. Isozyme genotypes of cherry leaf extracts.

CULTIVAR	6PGD	GOT	G6PD	GPI	IDH	PGM	FDP	SKDH	PER	MDH
Merton Bigarreau	aa	ab	ab	aa	aa	ab	ab	aa	ab	ab
NY 3308	aa	ab	ab	aa	aa	ab	ab	ab	aa	ab
Black Tartarian	aa	ac	aa	aa	aa	ab	aa	ab	ab	ab
Blackboy	aa	ac	aa	ab	aa	ab	aa	ab	aa	aa
Noir de Guben	aa	ac	ab	aa	aa	aa	ab	ab	ab	aa
Van	aa	ac	ab	aa	aa	ab	aa	ab	ab	ab
JI 10981	aa	ac	ab	aa	aa	ab	ab	aa	ab	ab
Schneiders Spate Knorpelkirsche	aa	ac	ab	aa	aa	ab	ab	aa	ab	ab
Up-To-Date	aa	ac	ab	aa	aa	ab	ab	aa	aa	ab
JI 11247	aa	ac	ab	aa	aa	ab	bb	aa	ab	ab
Vernon	aa	ac	ab	aa	aa	ab	bb	aa	ab	ab
Magda	aa	ac	ab	aa	ab	aa	bb	bb	ab	ab
Hendersons Bedford	aa	ac	ab	aa	ab	ab	aa	aa	ab	ab
Clements Pride	aa	ac	ab	aa	bc	ab	ab	aa	aa	ab
Lyons	aa	ac	ab	aa	bc	ab	bb	ab	aa	ab
Oregon	aa	ac	ab	ab	aa	aa	ab	aa	aa	aa
Merton Glory	aa	ac	ab	ab	aa	ab	bb	aa	ab	ab
Nordwunder	aa	ac	bb	aa	aa	ab	bb	bb	ab	ab
Black Douglas Mother	aa	bc	ab	aa	aa	aa	aa	aa	aa	aa
Beauchamps Black (G)	aa	bc	ab	aa	aa	aa	aa	aa	aa	ab
Lustre (G)	aa	bc	ab	aa	aa	aa	aa	aa	aa	ab
V 690616	aa	bc	ab	aa	aa	aa	ab	aa	ab	aa
Rainier	aa	bc	ab	aa	aa	ab	aa	aa	ab	ab
Bedford Prolific (P)	aa	bc	ab	aa	aa	ab	aa	ab	aa	ab
JI 14007	aa	bc	ab	aa	aa	ab	ab	aa	ab	ab
Waterloo (L)	aa	bc	ab	aa	aa	ab	ab	aa	ab	ab
Vega	aa	bc	ab	aa	aa	ab	ab	aa	ab	ab
Mora di Vignola	aa	bc	ab	aa	aa	ab	ab	aa	ac	ab

CULTIVAR	6PGD	GOT	G6PD	GPI	IDH	PGM	FDP	SKDH	PER	MDH
Bedford Prolific (B)	aa	bc	ab	aa	aa	ab	ab	bb	ab	ab
Opal	aa	bc	ab	aa	aa	ab	bb	aa	aa	aa
Lustre (P)	aa	bc	ab	aa	ab	ab	ab	aa	ab	ab
Krasarica	aa	bc	ab	ab	aa	ab	aa	aa	ab	ab
Bing OB260	aa	bc	ab	ab	aa	ab	ab	aa	aa	ab
Delta	aa	bc	ab	ab	aa	ab	ab	aa	aa	ab
JI 11253	ab	ab	ab	aa	aa	ab	ab	aa	ab	ab
Early Rivers (L)	ab	ab	ab	ab	aa	ab	ab	ab	aa	ab
JI 11376	ab	ab	ab	ab	aa	ab	bb	aa	ab	ab
JI 12526	ab	ac	aa	aa	aa	aa	aa	aa	ab	aa
St Margaret	ab	ac	aa	aa	bc	aa	bb	aa	ab	aa
Vista	ab	ac	aa	ab	aa	aa	aa	aa	ab	ab
JI 11610	ab	ac	aa	ab	aa	aa	ab	aa	aa	aa
Sunburst	ab	ac	aa	ab	aa	ab	ab	ab	aa	ab
Beauchamps Black (B)	ab	ac	ab	aa	aa	aa	aa	aa	aa	aa
Mazzard F12-1	ab	ac	ab	aa	aa	aa	aa	aa	aa	aa
Williams Favourite (G)	ab	ac	ab	aa	aa	aa	aa	aa	aa	aa
Waterloo (B)	ab	ac	ab	aa	aa	aa	aa	aa	ab	aa
Williams Favourite (M) Mother	ab	ac	ab	aa	aa	aa	aa	aa	ab	aa
Victor	ab	ac	ab	aa	aa	aa	ab	aa	ab	ab
Williams Favourite (M)	ab	ac	ab	aa	aa	ab	aa	aa	aa	ab
Vittoria	ab	ac	ab	aa	aa	ab	ab	aa	aa	ab
Burgsdorf	ab	ac	ab	aa	aa	ab	ab	aa	ab	ab
Merton Crane	ab	ac	ab	aa	aa	ab	ab	aa	ab	ab
Early Rivers (B)	ab	ac	ab	aa	ab	ab	ab	aa	ab	ab
Seneca	ab	ac	ab	aa	ab	ab	bb	aa	ab	ab
Colt	ab	ac	ab	aa	fast ab	ab	aa	ac	aa	ab
Star	ab	ac	ab	ab	aa	aa	ab	ab	ab	ab
Salmo	ab	ac	ab	ab	aa	ab	aa	aa	aa	ab
Lapins	ab	ac	ab	ab	aa	ab	ab	aa	ab	ab

CULTIVAR	6PGD	GOT	G6PD	GPI	IDH	PGM	FDP	SKDH	PER	MDH
13S-24-28	ab	ac	ab	ab	aa	ab	bb	aa	aa	ab
Williams Favourite (B)	ab	ac	bb	aa	aa	aa	ab	aa	ab	bb
Williams Favourite (L)	ab	ac	bb	aa	aa	ab	ab	aa	ab	ab
Early Purple Guinge	ab	bb	ab	ab	aa	ab	bb	ab	ab	ab
Hedelfingen	ab	bc	aa	ab	aa	aa	aa	aa	aa	aa
Compact Stella	ab	bc	aa	ab	aa	aa	aa	aa	ab	ab
Schauenburger	ab	bc	ab	aa	aa	aa	bb	aa	ab	aa
Vic	ab	bc	ab	aa	aa	ab	aa	aa	ac	ab
Summit	ab	bc	ab	aa	aa	ab	bb	aa	aa	ab
Basler Longstieler	ab	bc	ab	aa	aa	ab	bb	ab	aa	ab
Compact Lambert	ab	bc	ab	ab	aa	aa	ab	ab	ab	aa
Larian	ab	bc	ab	ab	aa	ab	aa	aa	ab	ab
Napoleon	ab	bc	ab	ab	aa	ab	ab	aa	ab	ab
13S-18-15	ab	bc	ab	ab	aa	ab	bb	ab	ab	ab
Stella	ab	bc	ab	ab	ab	ab	ab	aa	aa	ab
Sue	bb	bc	ab	aa	aa	aa	ab	aa	aa	ab
Lustre (L)	bb	bc	ab	aa	aa	aa	ab	ab	aa	ab
Ulster	bb	bc	ab	aa	aa	ab	ab	aa	ab	ab
Sam	bb	bc	ab	aa	aa	ab	ab	ab	aa	ab
Lambert	bb	bc	ab	ab	aa	ab	aa	ab	aa	ab
Venus	bb	bc	ab	ab	aa	ab	ab	ab	ab	aa

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