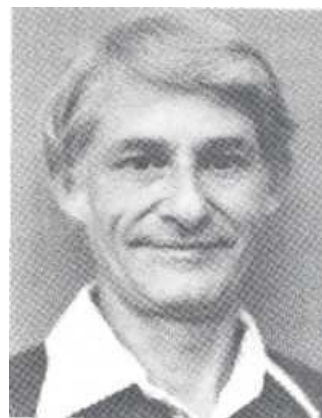


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## **ISOZYMES AS INDICATORS OF OUTCROSSING AMONG 'PINKERTON' SEEDLINGS**

### **Introduction**

The 'Pinkerton' cultivated variety of *Persea americana* is of growing importance in the California avocado industry because of its extensive breeding program to improve this remarkable cultivar further.

Mr. Wesley Pinkerton (*The following account was kindly provided by Messrs. Wesley Pinkerton and Clarence Holloway. Mr. Allan Pinkerton furnished several significant details of the ranch layout in 1973 and 1976.*) of Santa Paula, California, noted in the late 1940's that Topa Topa and other traditionally used rootstocks were not entirely compatible with scions he wished to propagate, and he began a search for other rootstock sources. In 1956 he grew some 'Rincon' seedlings of unknown pollen parents in open-ended tarpaper pots, to be used as rootstocks for tip grafting on the avocado ranch that he owned cooperatively with his brother, the late Mr. John Pinkerton. These were to be grafted by the longtime ranch foreman, Mr. Clarence Holloway. Surplus seedlings were dumped into a wash to control erosion. That year or the following year Mr. Holloway noted that one discard had emerged with considerable vigor, and when it first fruited, probably in 1959, he observed that it had a good medium-thick skin and very small seed-to-pulp ratio. He drew it to the attention of Mr. John Pinkerton and both began occasionally to check it. It was found to be very heavy bearing, free from russetting, and of fine flavor. Because of its morphology, and for other reasons, it was thought to be a 'Rincon' x 'Hass' hybrid (4). Observation continued for about 10 years and budwood was taken on a limited scale. The new cultivar was called 'Pinkerton' and became more and more widely appreciated. It was patented in 1975, and in 1977 Brokaw Nursery, Inc. of Saticoy obtained exclusive propagation rights. To date, approximately 30,000 'Pinkertons' have been grafted. In normal plantings these would cover some 300 acres, and since there are presently about 45,000 acres of avocados in California, acreage in 'Pinkerton' is less than 1 %. Nevertheless, it is of growing importance as shown by the fact that 'Pinkerton' represents around 25% of total propagation by the Brokaw Nursery, the largest in California.

To assure consistency of fruit quality, 'Pinkerton' like all avocado cultivars (varieties), is propagated by grafting. Propagation from seed results in highly variable and uncertain production and fruit quality, because of genetic recombination. Therefore, it is usually practiced only to produce sexual rootstocks, or in the search for superior new cultivars. It is a logical strategy to attempt further improvement of the better available cultivars by self-pollination. But because of the high cost of hand pollination and the low probability of success, desirable cultivars are usually self-pollinated by

different techniques including isolation plots and enclosing a tree in a screenhouse with a bee hive (1). In both cases, self-pollination is anticipated because the normal barrier to selfing enforced by the avocado's unique breeding system (synchronous dichogamy) can be overcome by certain environmental conditions (1, 3). A third method, and the one used in the present study, is to select only those fruits which are probable selfs because of their position on the tree. The 'selfed' seedlings which result upon germination develop to fruiting and are then evaluated for various tree and fruit qualities. Despite great precautions, cross pollination can occur even with screening—through tears in the screening, by feral bees pollinating through the screen, by flowers of nearby trees brushing against the enclosure so that bees within can get pollen from them. In our study, outcrossing would be much more likely. Because of such uncertainties, it is of considerable importance to the breeder to determine which seedlings are products of self pollination and which are outcrosses. Some pollen parents may be desirable but others, because of past performance, are to be avoided. Ideally, then, it would be helpful to know exactly which cultivar was the pollen parent. Such determinations have traditionally been based on the morphological features of the progeny's fruits and leaves. There is a heavy reliance on the experience and judgment of the breeder, but there is also the inescapable attendant difficulty that most such characters result from the interaction of many genes, thus depriving the breeder of clear-cut, single gene markers.

Such markers are available in the form of variant molecular forms of a special class of proteins, the enzymes (2). They have been widely used for many years by geneticists, population biologists, anthropologists and those in the health fields to address a wide variety of problems calling for genetic markers. Briefly, the rationale for their use is that the enzyme molecules are specified, or coded, by the gene, a section of chromosomal DNA. The DNA is a master copy of a code for determining the sequence of a protein's amino acid building blocks which in turn dictate the characteristics of the protein. The gene itself is transmitted through sex cells, and with rare exceptions does not change or mutate to other forms. When a mutation does occur, as it has for many genes of avocados over evolutionary time, the changed form is faithfully copied and transmitted. The mutation may result in an enzyme with detectably different characteristics, especially net electrical charge. When this occurs, the variant forms of the enzyme (isozymes) can be separated from each other in a supporting medium by subjecting them to an electric current. The technique is called electrophoresis, and one supporting medium widely used is starch gel (2).

Thus, 'Pinkerton' or any other cultivar, can be characterized not only by all of its morphological features, but also by its array of isozymes. If, for instance, 'Pinkerton' has the A form of a certain gene from its seed parent, it may also have received an A from its pollen parent, but could have received a B (or C or D) form through the pollen side depending on what forms of the genes (alleles) the paternal source had. If only two forms of the gene (that is, two alleles existed) A and B, its genotype for this gene could be A/A, A/B or B/B. But, if 'Pinkerton' were of genotype A/A for the hypothetical gene, then all of its progeny from selfing would have to be A/A. What then could explain a supposed 'Pinkerton' selfed seedling with A/B? Where did the B come from? One possible explanation would be a mutation, but we know this is an exceedingly rare event. Far more likely, some pollen parent with B was involved in the production of the

seedling. Now if the suspected pollen parent has A and B, then an A-bearing pollen grain was just as likely to form as one with B. But only the B's can be detected because those with A will result in progeny that are A/A which cannot be distinguished from pure 'Pinkerton' A/A. This means that only half of the outcrossed progeny could be detected. To detect more, we would have to use other gene/enzyme systems.

Ideally, the analysis described should be made using leaf tissue of young trees rather than fruits since fruiting normally does not occur until the plants are three or more years old. It is obviously expensive to grow and care for possibly useless trees for such a long time.

An earlier study demonstrated that avocados are rich in isozyme variation, also that such markers can be used to document parentages of cultivars and to "finger-print" or biochemically characterize any given cultivar (4).

The purpose of this article is to demonstrate the use of isozymes to measure outcrossing rates among a large population of seedlings from fruits collected by B. O. Bergh and Robert Whitsell in 1973 and 1976 on the ranch of Mr. John Pinkerton. The known seed sources were, of course, 'Pinkerton' trees. Since cross pollination was neither protected against nor totally unexpected, a portion of both collections resulted from pollination by cultivars other than 'Pinkerton'. For this study, three leaf gene/isozyme systems detectable in leaves were used: leucine aminopeptidase (LAP), phosphoglucose mutase (PGM) and peroxidase (PX).

## **MATERIALS AND METHODS**

All of the 'Pinkerton' seedlings were grown at the South Coast Field Station of the University of California at Riverside. Fully expanded, mature leaves were harvested, wiped clean and stored at 2-3 C. until used, usually within a day or two. Such leaves are useable for about 20 days. For LAP and PGM, about three 8 x 8mm pieces of healthy tissue were placed in a plastic weighing boat, a drop of 0.1 M pH 7.0 phosphate buffer with 12 % polyvinylpyrrolidone (PVP; M.W. 40,000) was added and the sample was crushed with slip-joint pliers. No PVP was needed for leaf peroxidases. The liquid sample was absorbed onto 5 x 7mm filter paper wicks and inserted into 12 % starch gels made with 0.02 M pH 7.7 tris citrate buffer for LAP and PGM, and 0.017M pH 8.0 for PX. The electrode tray buffer was 0.4 M pH 8.7 sodium borate. General electrophoretic procedures and the staining mixture for LAP and PGM have been reported earlier (4). The staining mixture for PX was 40mg 3-amino, 9-ethylcarbazole, 2ml dimethylformamide, 4ml 1M sodium acetate buffer pH 5.0, 1.6ml 0.1M CaCl<sub>2</sub>, 93ml H<sub>2</sub>O and 0.4ml 3% H<sub>2</sub>O<sub>2</sub>. The use of PVP in the crushing buffer was essential for the resolution of leaf LAP and PGM, but interfered somewhat with the isozyme pattern (zymogram) of leaf PX.

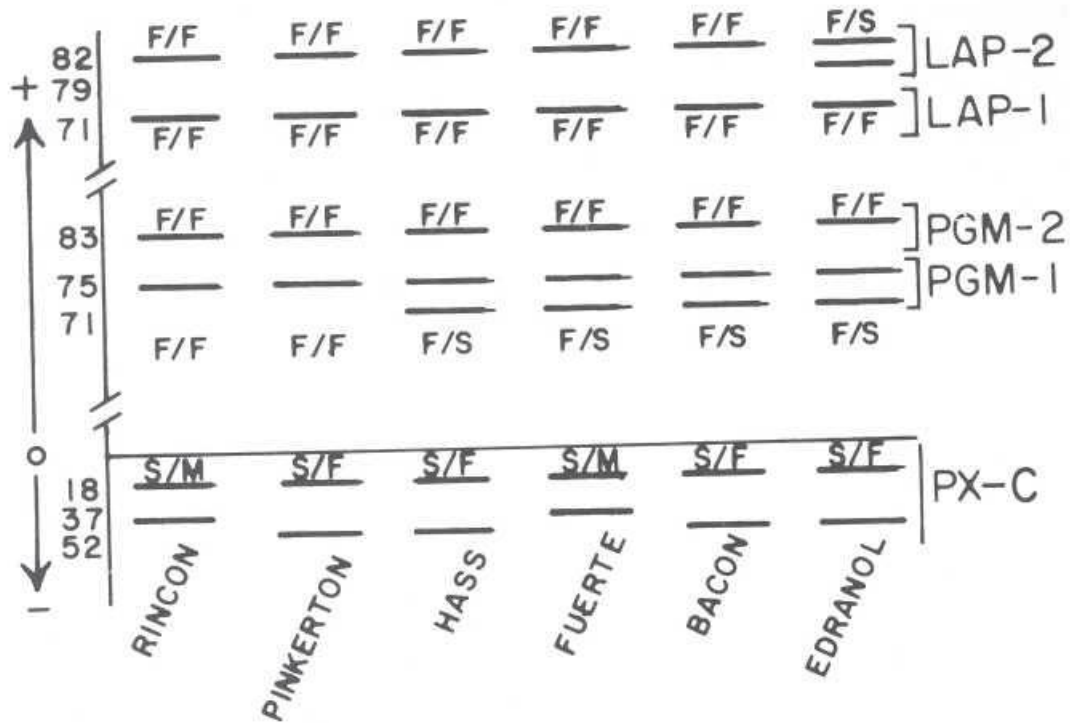


Figure 1. Schematic illustration of zymograms (patterns of isozymes) of pertinent cultivars. Origin of sample insertion in gel represented by 0; + = anode, - = cathode. Genes indicated on the right. Numbers at left are  $R_p \times 100$  values (ratio of migration of isozymes relative to the gel front).

### Results and Discussion

The isozyme profile of 'Pinkerton' is given in Table 1 along with those of the cultivars growing near the 'Pinkerton' trees used as seed sources, and which may therefore have been pollen parents. The corresponding zymograms are illustrated in Figure 1. By comparing the genotypes of 'Rincon,' 'Hass' and 'Pinkerton' it may be seen that no isozyme evidence contradicts the assumed parentage of 'Pinkerton'. Additional markers were reported earlier (4), and we have recently found evidence also from malate dehydrogenase that 'Pinkerton' isozymes are the expected sum of those of 'Rincon' and 'Hass' (Torres and Bergh, in preparation).

Because it is well known that different plant tissues may have different isozymes, it was necessary to determine whether or not the LAP and PGM isozymes of fruits and leaves were electrophoretically identical. Several cultivars, including 'Pinkerton' and its seedlings, were analyzed for isozyme identity by comparing fruit and leaf samples from the same plants. Without exception, the zymograms of the two tissues matched in pattern and in electrophoretic position. It is clear that the variable *Lap-2* and *Pgm-1* genes could be analyzed as to genotype in leaves as well as fruits, thus obviating the need to wait for fruiting. To date, PX has been studied only in leaves.

For brevity, numbers will designate the genes, which are named for the enzyme they specify, and letters will denote the forms of the genes or alleles. F (for *Fast*) will stand for the allele that codes for the fast migrating isozyme, S the slow and M medium. The gene that specifies the slower migrating set of isozymes will be designated as 1; 2 will designate the gene that specifies the faster migrating isozyme set. For PX there is an isozyme set which migrates toward the cathode in the gel and its gene will be called

*Px-C*. To illustrate the naming system, *Lap-1* is the gene which codes for the slower anodally migrating isozyme set, *Lap-1<sup>S</sup>* is the allele of *Lap 1* which specifies the slowest migrating isozyme coded by this gene, and *Lap-1<sup>F</sup>*, the fastest. Similarly *Px-C<sup>S</sup>* is the allele which codes for the cathodally slowest migrating isozyme of the gene *Px-C*. There is a one-on-one correspondence between alleles and their products, the isozymes.

As the LAP and PGM isozymes and their genetic control had been reported earlier (4), here we need only discuss briefly the leaf PX zymogram (see Figure 1). Under the electrophoretic conditions described, PX isozymes migrate both cathodally and anodally. Cathodally, one or two isozymes are present. If one, it may be in any of three different positions, S, M or F. This pattern suggests that *Px-C* codes for an enzyme which functions as a single amino acid chain and that in the avocados examined to date there are three *Px-C* alleles, one coding for each isozyme. If a cultivar is homozygous (both copies of the gene the same) it may be so in three ways; *Px-C<sup>S</sup>/Px-C<sup>S</sup>*; *Px-C<sup>M</sup>/Px-C<sup>M</sup>* and *Px-C<sup>F</sup>/Px-C<sup>F</sup>*. Similarly, a cultivar could be heterozygous in three ways: (*C<sup>S</sup>/C<sup>M</sup>*; *C<sup>S</sup>/C<sup>F</sup>* and *C<sup>M</sup>/C<sup>F</sup>*). There are therefore, six different cathodal patterns or zymograms possible corresponding to the six possible genotypes. Curiously, the *Px-C* isozymes cannot be resolved until the leaves have aged under refrigeration for about two weeks. Apparently, they are either coded by a gene which turns on as the leaf senesces, or they become accessible as membranes break down. The formal genetic analysis of the *Px-C* gene/enzyme system will be presented elsewhere.

In the anodal region of the gel, two *Px* gene/enzyme systems can be analyzed. The zymograms specified by *Px-1* have either one or three isozymes. If one, it may occur in any of four different electrophoretic positions. The fact that there can be three *Px-1* isozymes in a sample from a single plant indicates that the functional enzyme is dimeric or consists of two subunits or polypeptide chains. For example, F and S can combine as F/F, F/S (= S/F) and S/S. Again, the formal genetics of *Px-1* will be presented elsewhere. In all of the cultivars with which we are presently concerned, only *Px-1<sup>S</sup>* is found; consequently, this gene is not useful for detecting outcrosses in this particular case. *Px-2* similarly occurs in multiple forms specified by five alleles, but all cultivars in this study are *Px-2<sup>B</sup>*. Evidence for alleles S, A, C, and F has been found in other cultivars.

In Table 1, it may be seen that 'Pinkerton' is F/F for *Pgm-1*. It cannot transmit the S allele through either pollen sperm or ovary egg since it does not have it. Thus, if S is detected in a 'Pinkerton' seedling it came from a different cultivar. This simple observation for *Pgm-1*, and the other genes as well, underlies the analyses that follow.

The 1973 collection of fruits was from ranch rows where 'Hass' and 'Bacon' grew nearby and were possible pollen sources. Either of these could contribute *Pgm-1<sup>S</sup>* and we cannot distinguish the S of 'Hass' from the S of 'Bacon.' In general, if *Pgm-1<sup>S</sup>* is found in the 1973 collection, the pollen parents were likely 'Hass' or 'Bacon'. To obtain a minimal measure of outcrossing we need only analyze leaf samples for *Pgm*, but must remember that 'Hass' and 'Bacon' were just as likely to produce pollen bearing the F allele as bearing the S. Thus, we can detect only one-half of the likely number of outcrosses.

TABLE 1. Relevant genotypes of 'Pinkerton' and nearby cultivars on the John Pinkerton Ranch in 1973 and 1976.

|             | Genotype           |       |                    |                    |
|-------------|--------------------|-------|--------------------|--------------------|
|             | Lap-2 <sup>1</sup> | Pgm-1 | F.T-C <sup>1</sup> | Adh-2 <sup>2</sup> |
| 'Rincon'    | F/F                | F/F   | M/S                | F/F                |
| 'Pinkerton' | F/F                | F/F   | F/S                | F/F                |
| 'Hass'      | F/F                | F/S   | F/S                | F/F                |
| 'Bacon'     | F/F                | F/S   | F/S                | F/F                |
| 'Fuerte'    | F/F                | F/S   | M/S                | F/F                |
| 'Edranol'   | F/S                | F/S   | F/S                | F/F                |

<sup>1</sup>S of *Lap-2* and *Pgin-1* and M of *Px-C* are specific outcrossing markers because 'Pinkerton' does not have them.

<sup>2</sup>*Adh-2* illustrates a useless marker for this study because all cultivars are the same.

The 1976 collection was from the same trees as in 1973, but in addition, fruits from one tree near a machine shed were collected. There was a 'Fuerte' growing 75 feet, a 'Hass' 100 feet and an 'Edranol' 150 feet from the machine shed 'Pinkerton'. 'Edranol' has an *Lap-2* allele, S, not present in any other variety under consideration. If the *Lap-2<sup>s</sup>* allele's isozyme is detected, 'Edranol' is the likely pollen parent. Similarly, 'Fuerte' has the *Px-C<sup>M</sup>* allele not found in the other varieties. If it is found in 'Pinkerton' seedlings, 'Fuerte' was almost certainly the pollen parent. Again, since the *F* allele is shared by all varieties for LAP and PX, markers provide only a minimal measure of outcrossing and the real frequency is probably twice the minimal.

If *Pgm-1<sup>s</sup>* is found in the 1976 group, 'Bacon', 'Hass', 'Edranol' or 'Fuerte' could be pollen parents. If *Lap-2<sup>s</sup>* is found, the nearly certain pollen parent was 'Edranol', and *Px-C<sup>M</sup>* implicates 'Fuerte'.

Table 2 summarizes the results of the isozyme analysis of the 'Pinkerton' seedlings. The total 1973 group consists of 318 plants, most of which are now bearing. Of these, 108 (34%) were sampled and 32 were found to have the *Pgm-1<sup>F</sup>/Pgm-1<sup>s</sup>* genotype. Two were *Px-C<sup>s</sup>/Px-C<sup>M</sup>* which clearly suggests the pollen parent was 'Fuerte'. Yet, as far as we know, the nearest 'Fuerte' was about 1300 feet away. How a pollen grain was transported such a distance remains an enigma since bees, the chief avocado pollinator in California do not generally forage over such great distances once they have alighted and begun working flowers. In all, 34 of 108 seedlings, or 31%, were outcrosses. The actual outcrossing rate among the 1973 seedlings was probably twice that high, or 62%.

TABLE 2. Outcrossing rates (%) in the 1973 and 1976 collections of 'Pinkerton' seedlings. (N = sample size)<sup>1</sup>.

| Year | N   | Lap-2<br>F/S    | Pgm-1<br>F/S | Px-C<br>S/AP | Outcrossing<br>Rate <sup>4</sup> |
|------|-----|-----------------|--------------|--------------|----------------------------------|
| 1973 | 108 | 0               | 32           | 2            | 31 % <sup>5</sup>                |
| 1976 | 213 | 30 <sup>2</sup> | 85           | 6            | 49%                              |

<sup>1</sup> The 1973 crop resulted in 318 plants, and there are 1210 in the 1976 collection.

<sup>2</sup> 'Edranol' was most likely pollen parent.

<sup>3</sup> 'Fuerte' was most likely pollen parent.

<sup>4</sup> These figures exclude all duplications from plants which have two genes indicating outcrossing.

<sup>5</sup> Actual rates are probably twice the rates shown.

The 1976 collection consists of 1210 presently growing seedlings. Of these, 213 (18%) were sampled and 85 of the samples had the S allele of *Pgm-1* indicating that 'Hass', 'Bacon', 'Fuerte' or 'Edranol' could be pollen parents. There were 30 with *Lap-2*<sup>S</sup> which were most likely 'Pinkerton' x 'Edranol' hybrids. The six seedlings with *Px-C*<sup>M</sup> were probably 'Pinkerton' x 'Fuerte' offspring. The measured outcrossing rate was a surprising 49 % and the actual rate is probably 98% for the 1976 collection.

Over the four year period between 1973 and 1976, the branches of 'Pinkerton' and its neighbors had grown much closer toward each other thus shortening the distances between them. It is interesting to note that the minimal out-crossing rate was considerably higher in 1976 than in 1973 (49 vs 31 % respectively) .

Because 'Pinkerton' is F/S for *Px-C*, the ratio of F/F.-F/S.-S/S seedlings would be expected to be 1:2:1. This ratio should not be significantly disturbed by the few outcrosses with 'Fuerte' and should be undisturbed by outcrosses to 'Bacon', 'Hass' or 'Edranol' since they are identical in genotype to 'Pinkerton' for *Px-C*. The 1:2:1 ratio is expected since 50 % of 'Pinkerton's sex cells (egg or sperm) should have F and 50 % S alleles. F and S would then combine at random 25% F/F, 50% F/S and 25% S/S, or 1:2:1. The actual 1973 ratio was 34 S/S:48 F/S:26 F/F. The theoretical ratio would be 27:54:27, and a statistical comparison (chi-square test) of actual vs. expected shows the deviation is not significant ( $X^2 = 2.5$ ;  $p > 0.2$ ). The 1976 figures, excluding the 'Fuerte' outcrosses, were 47 F/F.-97 F/S: 56 S/S (13 plants out of the 213 sampled could not be scored for this gene). The expected corresponding numbers with random segregation would be 50:100:50. Thus, there is no significant difference between observed and expected results ( $X^2 = 0.99$ ;  $p > 0.7$ ).

The very high outcrossing rates were totally unexpected. Bergh from past experience, would have expected perhaps 10 % . Most of the known outcrosses cannot be identified as such from their morphology, because even selfs have tremendous phenotypic plasticity reflecting the great amount of genetic variability that has been observed for centuries from morphology and is now demonstrated by isozyme studies.

This study has shown that isozymes can provide a powerful tool for addressing certain problems of avocado parentage. Further, it has been shown that the avocado, which is an unlikely choice for genetic studies (because of its breeding system and perenniality), is amenable through its isozymes to formal genetic analyses. We are hopeful that further work will provide additional genetic markers to refine and expand the possible types of problems that can be attacked using isozymes for genetic analysis in the avocado.

### **Summary**

Using three isozyme systems as genetic markers (leucine aminopeptidase phosphoglucose mutase, and peroxidase), two groups of 'Pinkerton' seedlings were analyzed to determine the rate of outcrossing. Identified outcrosses probably only one-half of the total, were 31 % in 1973 and 49 % in 1976. The higher 1976 figure probably reflects the shorter distances between the branches after three years of growth. 'Edranol' accounts for 14% of the 1976 outcrossing, 'Fuerte', 3%. In 1973, 'Fuerte' accounts for about 2% of the pollen. 'Bacon' and 'Hass' account for the remainder in both years.

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