1. **HEADING**

Isozyme Analysis of Three Tropical Trees at the University of Perpetual Help System- DALTA Las Piñas Campus

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2. **ABSTRACT**

A study of the diversity and genetic structure of tropical rain forest trees is a major pre requisite to their conservation and management. Analysis of the genetic variation through isozyme electrophoresis is a powerful tool to achieve that purpose. The study determined the genetic relationship existing on three tropical trees found at UPHSD-LP namely, *Bauhinia purpurea* (Butterfly tree), *Delonix regia* (Fire tree) and *Cassia fistula* (Golden Shower tree) using electrophoretic isozyme analysis based on two substrates, Acid Phosphatase and Esterase. Frequency of common bands, similarity index, Rf values and genetic distance were determined among the three species. The results revealed that distinct bands were observed for Esterase while no distinct bands were noted for Acid Phosphatase. Results of similarity index and genetic distance are inversely proportional between any two of the three species. The study concludes that *Delonix regia* and *Cassia fistula* are closely related. This is followed by *Bauhinia purpurea* and *Delonix regia*. *Bauhinia purpurea* and *Cassia fistula* showed distant genetic relationship.

3. **KEYWORDS**

Genetic variation, genetic relationship, genetic distance, similarity index, isozyme electrophoresis

4. **INTRODUCTION**

Reports are indeed alarming that many of the world’s tropical rainforest areas succumb annually to deforestation. Originally, tropical rainforest areas covered 15-18 million square kilometres of land surface. According to FAO’s most comprehensive forest review to date, *The Global Forest Resources Assessment 2015*, some 129 million
hectares of forest - an area almost equivalent in size to South Africa - have been lost since 1990. Further, while in 1990 forests made up 31.6 percent of the world's land areas, or some 4 128 million hectares, this has changed to 30.6 percent in 2015, or some 3 999 million hectares. Africa and South America accounts for the highest net annual loss of forests in 2010-2015, with 2.8 and 2 million hectares, respectively. In the Philippines, dipterocarp forests were reduced from 16 million hectares in 1960 to less than one million hectares twenty years later (Repetto, 1990). If this scenario will continue, all rainforests will disappear in less than 50 years – a condition which is intolerable such that every effort should be done to set aside whatever area is left of the denuded forests for proper conservation and management.

A study of the diversity and genetic structure of tropical rain forest trees is a major prerequisite to their conservation and management. Analysis of the genetic variation through isozyme analysis is a powerful tool to achieve the purpose. A sufficient knowledge of its genetic structure will give ecologists and foresters enough basis in making crucial decisions whether or not to introduce new species of trees. Further, it will also provide the basis for establishing appropriate tree-planting models with respect to pollinators which in one way or another, are responsible for establishing a particular genetic structure. Evolutionary histories of tree species are also given plausible explanations through isozyme analysis which may be further used in phylogenetic and systematic studies.

Isozymes or isoenzymes refer to the presence of multiple molecular forms of enzymes with similar or identical substrate specifically occurring within the same
organism. It could be any of several forms of an enzyme that all catalyze the same reaction but may differ in reaction rate, inhibition by various substances, electrophoretic mobility or immunologic properties. Several enzymes, particularly alkaline phosphatase, lactate dehydrogenase and creatine kinase, have clinically important isoenzymes.

In a study conducted by Hamrick and Loveless on isozyme variation in tropical trees, genetic variation in 29 woody plant taxa common to Barro Colorado Island, Republic of Panama, was surveyed by starch gel electrophoresis. Results of the study indicate that the techniques described allow the application of genetic analyses to several questions of interest to tropical biologists.

In a similar study conducted by Maranan and Mendioro on sixteen accessions of Philippine cashew (*Anacardium occidentale* L.), results revealed that the clustering together of accessions from Zambales, Palawan and Los Baños indicate genotypic similarity among cashew accessions considered.

Genetic characterization of pili (*Canarium ovatum* Engl.) from Albay, Camarines Norte and Camarines Sur through isozyme analysis was conducted by Mendioro, et. al. Results of their study revealed that based on esterase (EST), acid phosphatase (ACP) and alkaline phosphatase (ALP), genetic variability was noted in 19 accessions of pili from Albay, Camarines Norte and Camarines Sur and in the 11 accessions of unknown origin. Further, results of the study indicate that accessions were genetically different. This could be due to the fact that pili being dioecious is an obligate cross-pollinating crop and
that genetic variability observed can be explained through recombination occurring during sexual reproduction.

5. FRAMEWORK

Tropical rainforest areas are of great interest to biologists due to their high species diversity and their complicated patterns of community organization (Loveless, 1992). Data on isozyme variation in tropical trees provide the basis for establishing appropriate tree-planting models with respect to pollinators which in one way or another, are responsible for establishing a particular genetic structure. A study on the genetic structure of tropical rain forest trees is a major prerequisite to their conservation and management. Further, knowledge of its genetic structure will give enough basis to ecologists as well as foresters in making crucial decisions whether or not to introduce new species of trees.

6. OBJECTIVE OF THE STUDY

The main objective of the work reported here was to determine/estimate levels of genetic variation in the three tropical trees namely, *Bauhinia purpurea* (Butterfly tree), *Delonix regia* (Fire tree) and *Cassia fistula* (Golden Shower tree) by applying the technique of electrophoresis of plant isozymes.

7. METHODOLOGY

MATERIALS AND METHODS

The experimental method of research was used to determine/estimate levels of genetic variation in the three tropical trees namely, *Bauhinia purpurea* (Butterfly tree),
Delonix regia (Fire tree) and Cassia fistula (Golden Shower tree) by applying the technique of electrophoresis of plant isozymes.

A. Collecting the Samples

Leaf blades of the three tropical trees were collected. The leaf samples were placed in a plastic bag and into a cooler filled with ice. This prevented the breakdown of proteins in the leaf section and the activity of enzymes in the samples.

B. Sample Preparation

The extraction buffer was prepared a day before the actual run and stored in the refrigerator.

A crude extract was made from 1 to 1.5 grams of the cut leaf tissue by grinding in a mortar with a pestle placed in an ice bath tray with 1.0 ml of extraction buffer. The crude extract from leaf samples were placed in small vials and into a freezer to preserve enzymatic activity until ready for use.

C. Gel Preparation and Sample Loading

Polyacrylamide, a synthetic polymer of acrylamide monomer, was used as a medium for gel electrophoresis.

The preparation of the set-up involved assembling the glass plates of the vertical electrophoresis chamber. The separating and stacking gels were prepared as prescribed in the proceeding table.
The above ammonium persulfate solution was freshly prepared and added just before casting the gel. The percentage used for the stacking and separating gel acrylamide were 3% and 10%, respectively. The separating gel was poured between the glass plates and isobutanol was overlayed afterwards. The gel was allowed to polymerize at room temperature for 30-45 minutes at 37°C. After the separating gel has polymerized, the isobutanol was removed and the surface of the gel was washed with distilled water and allowed to dry using filter paper. The stacking gel was then poured into the glass plates and a comb was inserted. The gel was allowed to polymerize at room temperature for 60 minutes. After the stacking gel has polymerized, the inserted comb was removed and the wells formed were washed with an electrode buffer. After washing was done, the crude extract samples mixed with the sample buffer in ratio of 1:2 were loaded into the wells.

<table>
<thead>
<tr>
<th></th>
<th>Separating gel (10%)</th>
<th>Stacking gel (3%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(1)</strong> Monomer</td>
<td>10.0 ml.</td>
<td>1.0 ml.</td>
</tr>
<tr>
<td><strong>(2)</strong> Separating gel buffer</td>
<td>3.8 ml.</td>
<td>1.25 ml.</td>
</tr>
<tr>
<td><strong>(3)</strong> Double distilled water</td>
<td>15.9 ml.</td>
<td>7.6 ml.</td>
</tr>
<tr>
<td><strong>(4)</strong> TEMED</td>
<td>15.0 ul.</td>
<td>5.0 ul.</td>
</tr>
<tr>
<td><strong>(5)</strong> 40% Ammonium persulfate</td>
<td>20.0 ul.</td>
<td>25.0 ul.</td>
</tr>
</tbody>
</table>

The above-prepared gel solutions were degassed for five minutes before adding other components of the gel solutions.
After loading was done, voltage was set to constant 15 volts until the tracking dye had reached the separating gel and voltage was adjusted to constant 30 volts until the tracking dye had gone done completely along the gel. After the run, power supply was turned off and the recovered gel was placed in a tray.

D. Stain Preparation and Staining

Two isoenzyme substrates were prepared namely, Esterase and Acid phosphatase.

After placing the recovered gel in a tray, the freshly prepared isoenzyme substrates were poured over the gel and then placed inside an incubator with a temperature of 37°C for 30 minutes or until bands have appeared. (Note: There were separate electrophoresis run for the two isoenzyme substrates).

G. Recording the Gel

After incubation was done, the gel was washed with distilled water and then fixed with a fixative consisting of 30% ethanol and 10% glacial acetic acid. The gel was scored immediately using a light box, dried and photographed for documentation purposes.
8. RESULTS AND DISCUSSIONS

Electrophoresis as a tool for isozyme analysis

The technique of electrophoresis in polyacrylamide gel uses a buffer system designed to dissociate all proteins into their individual polypeptide subunits.

During electrophoresis, heat is generated by the passage of electric current through the gel at a rate dependent to the net charge and on the size and shape of protein molecules. The end result of electrophoresis is fractioning and separating a complex mixture of proteins into a series of discrete protein bands arranged in order of molecular weight. The proteins/enzymes are detected by methodology in which protein staining and fixation occur simultaneously.

Occurrence of Isozymes in plants

The occurrence of isozymes among plants even in animals is widespread. Such that isozymes are the rule rather than the expection. The number of enzymes known to occur in isozymic forms and the number of plant species examined has more than tripled.

The isozymes substrates used in the study were Esterase and Acid phosphatase. Acid phosphatase isozyme system was used to test whether it is prevalent in the three species of tropical trees and to determine their genetic relationship. Unfortunately, no bands occurred in those three tropical trees with Acid phosphatase as the substrate. It
could be that such isozyme system is not present in those three species despite its occurrence in the other plant species.

In the isozyme system Esterase, there were bands produced in the three species of tropical trees. The occurrence of bands could be attributed to the fact that the three species of tropical trees are leguminous species and some of those plants mentioned in previous studies such as *Phaseolus* and pea are also leguminous and contained Esterase.

**Gel Interpretation**

Evidence in studies of electrophoresis in plant species is observed to be the appearance of bands of color in gel. These bands reveal the relative positions reached by different molecular forms of an enzyme (or a protein) which have migrated through the gel when voltage is applied. The enzyme variants are separated because they have different electrophoretic charges (function of the relative number of amino acids with positive and negative charges on the surface). Furthermore, their rate of migration through the gel also depends on their size or configuration. Following the separation step of the enzyme variants, the isozymes are identified by a staining reaction based on their catalytic activities. This particular combination step of electrophoresis and staining specificity makes it possible to distinguish a particular enzyme among hundreds that may be present in the crude extract sample.

In this present study the Rf values of the three species (*B. purpurea, C. fistula and D. regia*) were obtained. An Rf value refers to the distance a protein has migrated from origin divided by the distance from the origin to the reference point. *D. purpurea*
has the highest migration value of 0.72, followed by *C. fistula* which 0.61 and the lowest was observed in *D. regia*, 0.37. (Tables 1 and 2)

**Table 1. Distance travelled by protein from the origin to the point of reference in mm**

<table>
<thead>
<tr>
<th>Number of bands</th>
<th>Fire tree A</th>
<th>Butterfly B</th>
<th>Golden shower C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8 mm</td>
<td>7 mm</td>
<td>9 mm</td>
</tr>
<tr>
<td>2</td>
<td>10 mm</td>
<td>10 mm</td>
<td>13 mm</td>
</tr>
<tr>
<td>3</td>
<td>15 mm</td>
<td>26 mm</td>
<td>20 mm</td>
</tr>
<tr>
<td>4</td>
<td>28 mm</td>
<td>28 mm</td>
<td>25 mm</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>35 mm</td>
<td>34 mm</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>41 mm</td>
<td>40 mm</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>43 mm</td>
<td>46 mm</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>50 mm</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>52 mm</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>54 mm</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Rf values of A. *Delonix regia* (Fire tree), B. *Bauhinia purpurea* (Butterfly tree) and C. *Cassia fistula* (Golden shower tree)

<table>
<thead>
<tr>
<th>Number of bands</th>
<th>Fire tree A</th>
<th>Butterfly B</th>
<th>Golden shower C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1067</td>
<td>0.0933</td>
<td>0.12</td>
</tr>
<tr>
<td>2</td>
<td>0.1333</td>
<td>0.1333</td>
<td>0.1733</td>
</tr>
<tr>
<td>3</td>
<td>0.2000</td>
<td>0.3467</td>
<td>0.2667</td>
</tr>
<tr>
<td>4</td>
<td>0.3733</td>
<td>0.3733</td>
<td>0.3333</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.4667</td>
<td>0.4533</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.5467</td>
<td>0.5333</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.5733</td>
<td>0.6133</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0.6667</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>0.6633</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.7200</td>
<td></td>
</tr>
</tbody>
</table>

It is evident that the relationship of similarity index and genetic distance is inversely proportional. The greater the value for index of similarity, the closer is the genetic relationship. In this present study, it was proven that *D. regia* is closely related genetically to *C. fistula* through electrophoretic results while *B. purpurea* and *C. fistula* are distantly related.

An electrophoretic evidence is precise and directly quantifiable in terms of the number and kinds of enzymes studied, permitting the amount of genetic information utilized to be stated exactly. This is seldom possible with morphological or other characters. Another significant advantage is that comparisons are made with enzymes that are generally always present and less influenced by environmental factors.
9. CONCLUSIONS

Isozyme electrophoresis is a powerful tool and the most convenient available for detecting genetic differences close to the DNA level. The use of isozyme markers readily monitor the comparative diversity of various kinds of genetic resources. *D regia* and *C. fistula* are closely related. *D regia* and *B. purpurea* follow next in rank. On the other hand, *B. purpurea* and *C. fistula* are quite distantly related. Isozyme electrophoresis may be used also to study the genetic structure of population of plant species.

10. RECOMMENDATIONS

The following recommendations were formulated grounded on the findings of the study and the conclusions drawn:

1. that further studies be done to determine the genetic structure of the population of the plant species used in the study; and

2. the use of other enzyme substrates.

11. LITERATURE CITED


