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MOLECULAR GENETIC ANALYSIS OF VALUABLE NATIVE TIMBER SPECIES

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Executive Summary

Very little is known about the geographic distribution of genetic variability of tropical timber trees. Such information will be key for the development of tropical forestry efforts based on native species. This project was a collaborative effort among a Costa Rican molecular biologist, a Costa Rica-based U.S. tropical forest ecologist, and a premier genetic analysis laboratory in the U. S., to establish the foundations for a long-term program to assess the levels and distribution of genetic variability within Costa Rica's populations of economically important native timber trees. Our goals were to establish a plant genetics laboratory facility at the University of Costa Rica, to develop and test molecular techniques for assessing the genetic structure of Costa Rican native timber tree species, and to carry out pilot studies with these techniques on three populations of native timber species, *Minquartia guianensis, Hyeronima alchorneoides* and *Simarouba amara.* Procedures were developed for the analysis of genetic variation using two techniques: isozyme expression variation and DNA fingerprinting. Extensive results are presented on the isozyme analysis of *Minquartia guianensis* populations and preliminary results are shown on the other two species. DNA extraction procedures, and the standardization of the RAPD fingerprinting are reported. Key issues were the development of a simple and reliable technique for the extraction of DNA from tree samples suitable for PCR amplification, and the study of the optimum conditions for PCR amplification, where repeatability of results is paramount for our analysis. The project demonstrated the importance of collaborative efforts involving forest ecologists, molecular biologists and population geneticists. A Costa Rican lab is now fully equipped and operational for the analysis of genetic variability on plant populations, and local personnel were trained in leading laboratories in the USA on genetic analysis techniques and on the optimization of DNA fingerprinting techniques using PCR.

I. Research Objectives

The problem of erosion of genetic diversity is a major one for all developing countries, as pressure on natural resources increases and promising native species for development suffer major habitat loss. The goal of this project was to lay the foundations for a long-term continuing research program in Costa Rica to assess the levels and geographic distribution of genetic variation in the region's promising native timber species. Our objectives were threefold. First, we sought to establish a laboratory facility for genetic analysis of trees within the University of Costa Rica's Centro de Investigación en Biología Celular y Molecular (CIBCM), a key center in Latin America for graduate training in molecular biology. Secondly, through collaboration with a premier genetic analysis laboratory in the United States, and through extensive on-site testing, we sought to develop protocols based on the most recently available molecular techniques (using both DNA and isozymes), that would enable genetic evaluation of Costa Rican native timber tree species. Finally, we planned to carry out a first genetic analysis of three economically-valuable native tree species - *Minquartia guianensis* (Olacaceae; "Manú"), *Hyeronima alchorneoides* (Euphorbiaceae; "Pilón"), and *Simarouba amara* (Simaroubaceae;"aceituno"), taking advantage of intensively-studied natural populations that have been under long-term study in N.E. Costa Rica since 1983.

Several aspects of this project gave it direct applicability to important needs in developing countries:

1) Analysis of the geographic distribution of genetic variability in timber trees (and other economic plants and animals) is a fundamental need world-wide in order to identify and protect the genetic potential of each species. Such studies are urgently required wherever major habitat types are being greatly eroded by deforestation or other factors. As part of this project, we sought to develop model protocols for addressing this problem with native timber tree species wherever developing countries are undergoing drastic habitat alteration.

2) The focal tree species chosen for our case study analyses all have wide ranges within the neotropics (from southern Mexico in the north to Amazonia, lowland Ecuador, and as far south as southern Brazil), and they are of current or potential economic interest across their range.

3) For extremely few tropical tree species have recent DNA molecular techniques been used for genetic analysis. We know of no cases of tropical tree genetic studies where these newly-evolved DNA methods have been compared to the

prevailing isozyme-based techniques in terms of their cost effectiveness and efficacy for addressing population genetic issues.

4) The laboratory facility where these techniques would be developed, the University of Costa Rica's CICBM, is a research center of regional significance in Latin America. It has been selected as one of six Latin American centers for graduate training by the Latin American Plant Sciences Network (Red Latinoamericana de Botánica), a Latin American organization working to build regional academic excellence in plant sciences by providing graduate fellowships for young Latin Americans.

On several fronts this project was innovative. The creation of a new laboratory facility and the development of repeatable analysis protocols brought the use of these techniques for the genetic analysis of tropical trees into an already very productive "magnet" research facility in Latin America. The collaboration between an experienced molecular biology (G. Macaya) and a tropical forest ecologist (D. Clark) created a cross-disciplinary project, joining expertise in two very disparate fields. The project took advantage of a unique pre-existing data resource - long-term study populations of three focal timber tree species for which we had detailed growth histories and extensive site data (Clark and Clark 1992; Clark and Clark 1994). The research also incorporated the increasingly-important tool for spatial analysis, a Geographic Information System (Arc-INFO, running on Sun workstations) to analyze the tree populations' spatial relationships and site characteristics. The existence of a detailed soils map for the study site, an exceptional situation for a tropical wet forest, made it possible for us to ask a new question for natural populations of tropical rain forest trees - is there evidence of genetic sub-structuring of the trees' natural populations in relation to the great within-landscape edaphic (topographic and soil) heterogeneity now known to exist within neotropical rain forests? (see Clark et al. 1995, and included references). Our long-term reproductive data also included sex-typing for a large sample of *Hyeronima alchorneoides*, a dioecious species (one where trees are either male or female). This dataset offered the opportunity to search for genetic markers for sex identity in this economically important species; such markers could be of great interest for the management of plantations and seed orchards for this species. The molecular approaches to be used in this study were also innovative. Through consultation and cross-training in U.S. laboratories, we planned to develop in our laboratory some of the most current DNA genetic analysis techniques for comparison with the more standard isozyme techniques - by using both approaches with the same set of study populations, we could compare the relative difficulty and scientific efficacy of these two very different molecular approaches for studying the spatial structure of genetic variation within natural populations of tropical timber trees.

The project was significantly enhanced by contributions from multiple collaborators and institutions. Over the course of the project several interested specialists joined in the research. Dr. Peter Gresshoff of the University of Tennessee-Knoxville visited the project at the CIBCM with funding from the International Atomic Energy Agency (IAEA). He provided valuable consultations regarding material preparation for DNA-based genetic analysis, as well as donating reagents he had developed in his laboratory. His visit to our laboratory allowed us to improve on the use of RAPDs and silver staining of polyacrylamide gels. This collaboration led to the visit of Heidy Villalobos, our project technician, to Gershoff's laboratory in Tennessee, to learn first-hand his new techniques for RAPD-type genetic analysis (in particular, a new silver staining technique for greatly improved band resolution, as well as different concentration protocols designed to improve repeatability, a major hurdle for RAPD studies). Dr. Barbara Schaal, the director of an outstanding plant genetics laboratory at Washington University (St. Louis, MO), was also an important collaborator in our project. In addition to providing initial input into the design of the research, Dr. Schaal also hosted Heidy Villalobos in her lab for intensive hands-on training in the most current DNA techniques for genetic analysis of plant populations. Another valuable collaboration was provided when David Tobin, winner of Princeton University's all-campus outstanding senior award in 1993, elected to use his prize scholarship to spend nine months in 1994 working within our project. David carried out the preliminary testing for DNA sex markers in *Hyeronima*. The isozyme analyses of *Minquartia*, our principal study species, formed the Master's thesis of Mabel Paz, a Colombian graduate student, who spent 3 years working on our project with additional financing provided by a graduate fellowship from the Red Latinoamericana de Botánica. Even now, although the AID PSTC funding has ended for our project, the completion of the DNA analyses of our three target species is being funded through a collaboration with other colleagues at the University of Costa Rica. The logistics of field sampling and mapping the tree study populations were greatly aided by the existence of a precise landscape-scale grid system installed by the Organization for Tropical Studies (OTS) at the study site. Similarly, the computer analysis of inter-tree distances and tree site conditions was made possible by the Geographical Information System that has been developed at the site by OTS, with major funding contributed by the U.S. National Science Foundation, the Andrew W. Mellon Foundation, the Environmental Systems Research Institute, and Sun Microsystems. OTS also provided invaluable support for the project by carrying out a large proportion of the purchasing of reagents and equipment in the United States and then arranging their transport to Costa Rica.

II. Methods and Results

a. Sampling Design and Field Collection

1. *Minquartia guianensis*

Our research effort during the project was principally focused on this highly valued timber tree species. We first sought to develop DNA and isozyme protocols to enable genetic analysis of this species. Our ultimate goal was then to evaluate the degree of genetic substructuring within the study population under intensive demographic study since 1983 in lowland old-growth tropical rain forest of N.E. Costa Rica (at the La Selva Biological Station; see Clark and Clark 1992). Our questions were: 1) to what degree does within-population genetic variation related to geographic distance within the 150 ha study area?; 2) is there evidence of inter-cohort differentiatiation in genetic characteristics? (i.e., between reproductive-sized adults and successively smaller juveniles?); 3). is there evidence of within-population genetic differentiation in relation to the contrasting soil types found within the study area?

To investigate these questions, we selected from the long-term demographic sample 110 trees stratified across three major soil types (infertile ultisols and two inceptisols of differing, higher fertility: respectively, "Ultisol", "Arboleda," and "Alluvium"; see Sollins et al. 1994), distributed across size classes (1-10 cm diameter, 10-20 cm diameter, and >20 cm diameter, the size of first reproduction), and distributed geographically across the 150 ha study area (see Fig. 1).

Individual trees are indicated by circles and squares. Alluvial soils: dark gray-shaded areas. Ultisols: Lightly shaded areas. Arboleda soils: white zone

All trees were located with reference to the 50 x 100 m grid surveyed over the study area, and were mapped into the Geographic Information System. The study individuals, their diameter and soil type are given in Appendix 1.

Fresh vegetative tissue (meristems, young unexpanded leaves, intermediate-aged leaves, old leaves, and fallen leaves) was collected from an initial subsample of trees; based on the results of isozyme screening and on availability/accessibility of material in the field, all subsequent collections were of intermediate-aged leaves. These were collected with clipper poles or by shotgun, depending on the tree's height. In the field, the leaf material was gently cleaned (to remove surface epiphylls and debris), labeled by individual tree number and immediately frozen in liquid nitrogen. The frozen material was delivered to the CIBCM the same day of collection and was stored in an ultracold freezer until processing for analysis.

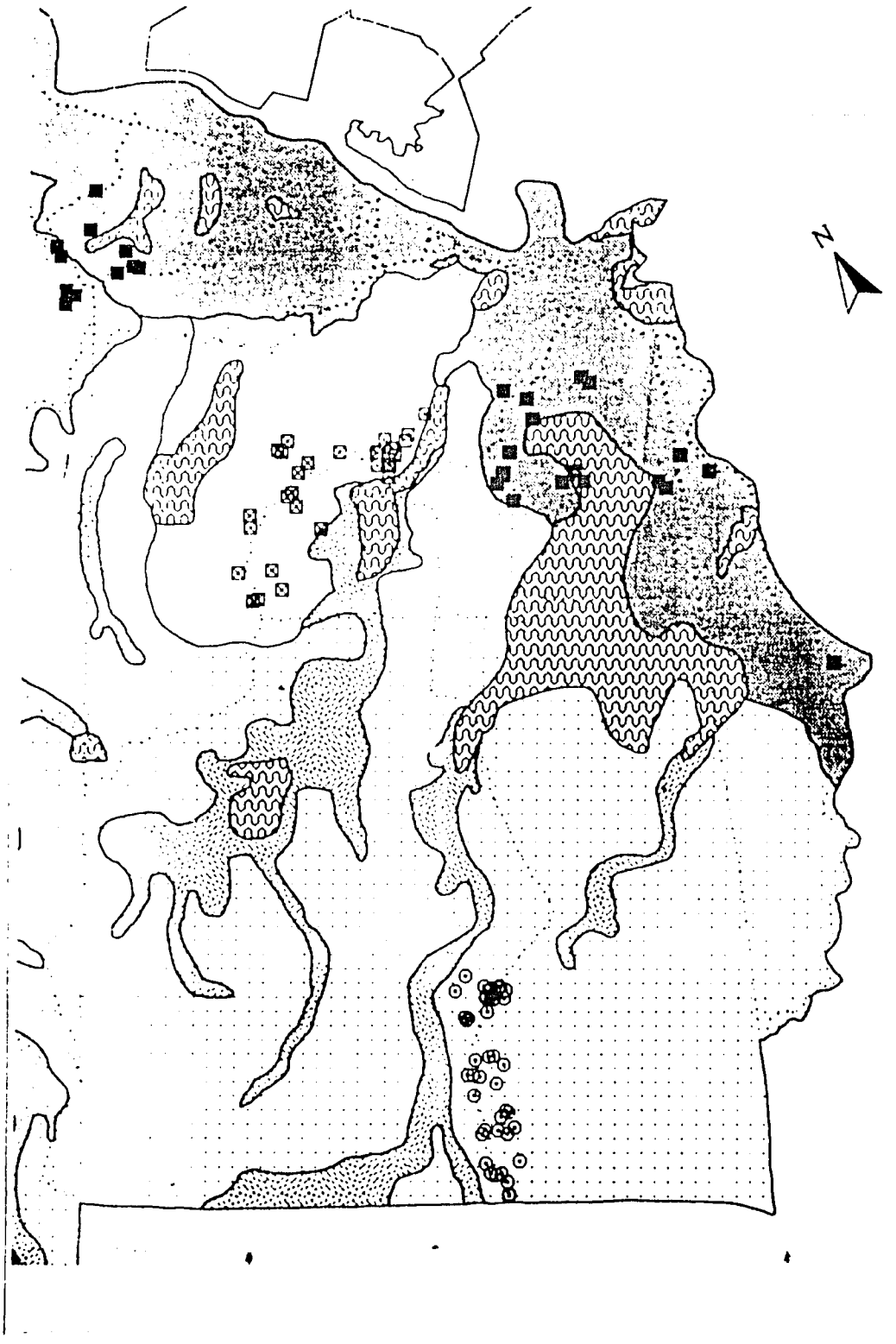

Figure 1: Demographic study population of *Minquartia guianensis* in La Selva Biological Station.

2. *Hyeronima alchorneoides.*

With this species, our two principal interests were to 1) search for genetic markers for sex identity; and 2) test for within-population genetic differentiation in relation to the contrasting soil types found within the study area. To this end, we collected from 89 adult *Hyeronima* individuals in the long-term study population; a large proportion were typed for sex based on our long-term observations, and all were precisely mapped with respect to soils and each other, as for *Minquartia*. Intermediate-aged leaves were collected from each individual and processed as described above.

3. *Simarouba amara.*

For a general screening of genetic characteristics of this species (both isozymes and DNA), we collected leaf material as described above, from trees in three subsamples distributed over the total study area (N=3D5 per subsample).

b. Genetic analyses

1. Isozyme studies

Minquartia guianensis

The initial step for this study was to obtain a clean extraction from *Minquartia* leaves for the isozyme analyses. As is commonly the case for tropical trees, the high concentrations of secondary compounds and latex in the leaves required considerable trial and error testing of methods before a workable extraction could be obtained. We tested 10 extraction buffers obtained from the literature and from colleagues. Of these, the best results with *Minquartia* were obtained with: buffer 9 of Hall et al. (1994), used for enzyme systems MEZ, MDH, HEX, β-GAL, APH, PER and PGI; and buffer 7 of Roose and Gottlieb (1978), used for enzyme systems EST and EST-F (see Table 1 for system names).

We used polyacrilamide gel electrophoresis to run leaf extract from each of the sampled trees on 20 enzymatic systems, the same set of systems used by Hamrick and Loveless (1986) in their studies of genetic variation in Panamanian tropical trees. The details of analysis protocols (e.g., pH, staining, etc.) are given in Paz (1996).

Table 2 shows the electrophoretic conditions for the isozyme analysis of young leaves of *Minquartia guianensis.*

Table 1: Enzyme systems tested for enzymatic expression by *Minquartia guianensis*.

Acid phosphatase	APH
Alcohol dehydrogenase	ADH
Esterase (colorimetric)	EST
Esterase (fluorescent)	EST-F
α–glycerophosphate dehydrogenase	α–GPDH
β-galactosidase	β-GAL
Glucose-6-phosphate dehydrogenase	G-6PDH
Glutamate dehydrogenase	GDH
Glutamate-oxaloacetate transaminase	GOT
Hexokinase	HEX
Isocitrate dehydrogenase	IDH
Leucine aminopeptidase	LAP
Malate dehydrogenase	MDH
Malic enzime	MEZ
Peroxidase	PER
Phosphoglucoisomerase	PGI
Phosphoglucomutase	PGM
6-Phosphogluconate isomerase	PGDH
Shikimate dehydrogenase	SHIK
Triosephosphate isomerase	TPI

Table 2: Electrophoretic conditions for the isozyme analysis of young leaves from *Minquartia guianensis*.

Tissue Intermediate leaf	Extraction buffer# (1:2)	Electrode and gel buffer	voltage % Bis	% acrylamide
Isozyme system				
Esterase (EST)	7	C	120	10 %
Fluorescent Esterase (EST–F)	7	C	120	10%
Phosphogluco isomerase (PGI)	9	C	200	9%
Peroxidase (PER)	9	C	220	10%
Acid phosphatase (APH)	9	C	150	10%
β–Galactosidase (β–Gal)	9	C	200	10%
Hexoquinase (Hex)	9	C	250	9%
Malate Dehidrogenase (MDH)	9	C	320	9%
Malic enzyme (MEZ)	9	C	320	9%

C: Gel buffer: 9:{ 0.05 M TRIS base, 0.007 M Citric acid pH 8.3 }: 1{Electrode buffer}. Tank buffer: 0.04M LiOH. 0.19 M Boric acid pH 8.3
7: TRIS base 0.1 M, KCl 10 mM, MgCl2 0.05M, EDTA 1mM, BMPE 14 mM, Ascorbic acid 0.1M, PVP 0.20gr/ml.
9: 0.011M EDTA, 0.001M KCl, 0.02M MgCl2, 4% PVP, 0.2M Phosphate buffer pH 7.5, 1.7 mM Sodium metabisulfite, 0.027 mM NADP, 0.003 mM NAD, 0.003 DTT, 10% Glycerol.

The sampled trees of *Minquartia* showed enzyme expression for nine of the 20 enzyme systems. Of these nine systems, two (EST and PGI) were polymorphic and highly resolvable (Figures 2,3). For system EST, our sample population produced 23 different bands in total, which were distributed among 40 distinct enzymatic patterns (Appendix 2). For PGI, there were two polymorphic regions with a total of 10 bands and 21 enzymatic patterns (Appendix 3). Two systems (β-GAL and APH), with 1 band and 3 bands, respectively, showed no variation among the entire sample population. The remaining systems did not show repeatable, interpretable banding patterns.

One of the EST enzymatic patterns, found in 13 individuals, occurred only in the subpopulation from the Arboleda soil. This extremely interesting finding indicates genetic substructuring of this natural population of *Minquartia* and suggests the possibility of very local adaptation to edaphic conditions. To establish such a soil-genetic relationship, however, will require further sampling of neighboring individuals from the same and contrasting soils, as well as experimental plantings.

For each of the two polymorphic systems, EST and PGI, we analyzed the relationship between size, soil, and genetic similarity used cladistic analysis of the presence/absence patterns of all bands occurring in a given system (using the statistical analysis package NTSys-pc, SIMQUAL routine to obtain genetic distances from band data, using Jacquard coefficient, and the SAHN routine for cluster analysis; see Fig. 4). In both cases, there was no relation between either size class or soil type with the genetically similar clusters of individuals in the resulting cladogram (i.e., all major genetic clusters included individuals from all soil types and from all three size classes).

Based on correlation analysis of the two matrices of genetic distance and geographic distance (routine MXCOMP of NTSys-pc), we did not find evidence of a decline of genetic similarity among individuals with increasing distance. This analysis, then, suggests a high degree of genetic mixing within the 150 ha study area.

In a second, post PSTC, phase of our studies of *Minquartia*, we are now using DNA techniques to assess the genetic characteristics of the same individuals studies with isozyme techniques. With this second stage of research, we will be able to use *Minquartia* as a case study of the scientific and financial cost-benefits of the two approaches for studying tree genetic variability.

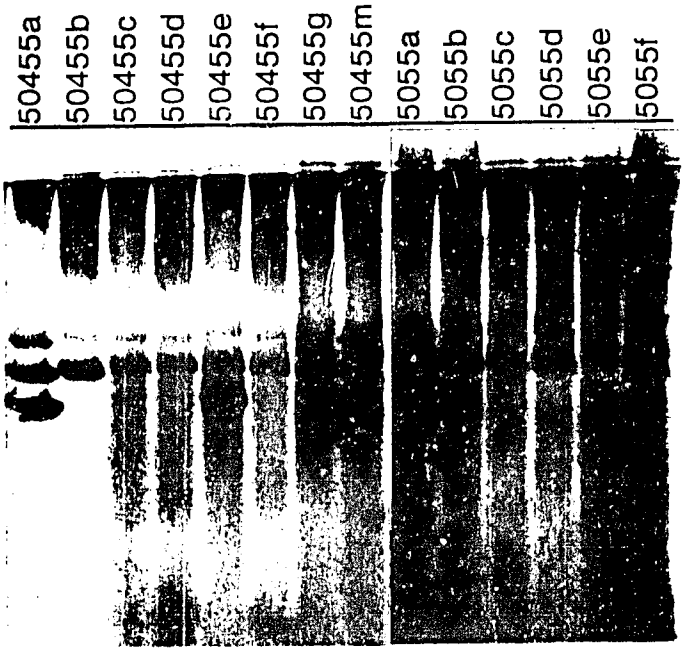

Figure 2: Photograph of a sample gel from electrophoresis of EST in intermediate leaves of *Minquartia guianensis.*

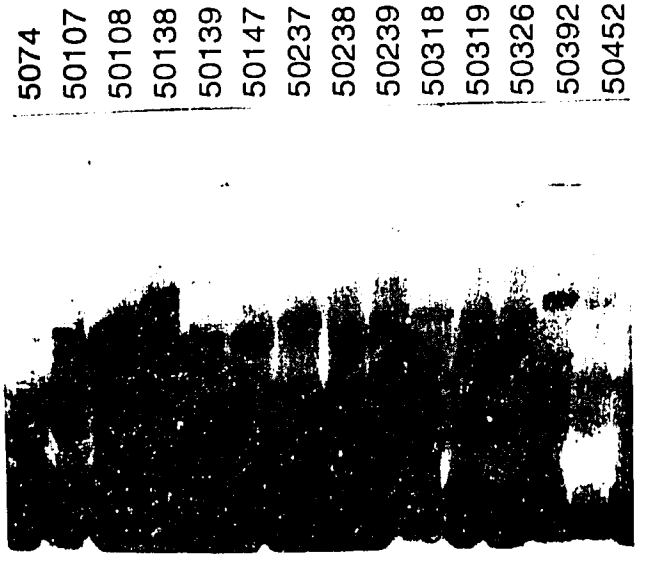

Figure 3: Photograph of a sample gel from electrophoresis of PGI in intermediate leaves of *Minquartia guianensis.*

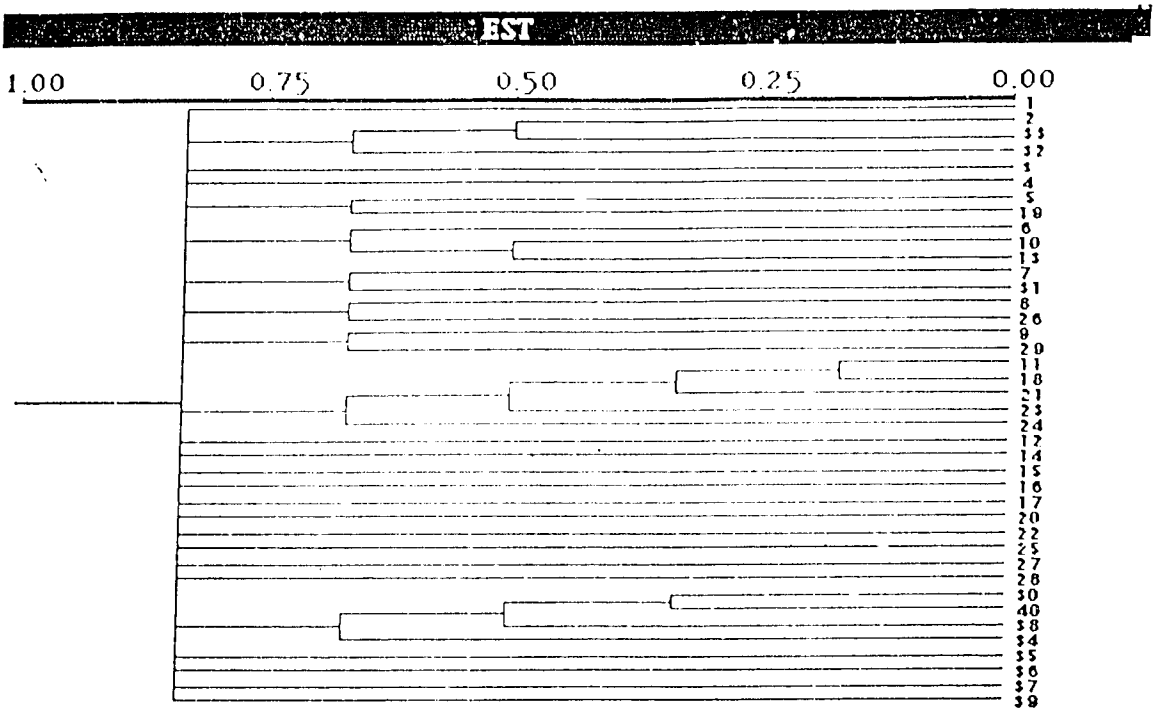

Figure 4: Phenogram of genetic similarity of *Minquartia* individuals, based on the 23 bands occurring in the system EST (UPGMA analysis). Numbers to the right of the branches correspond to the pattern number of the figure in appendix 2.

Hyeronima alchorneoides.

For this species, we carried out extensive searches for isozyme patterns that could distinguish male and female trees. We to date have been unable to distinguish sex patterns for this species. A second continuing goal of our research with this species, although beyond the AID PSTC project, is to assay the spatial and soil relationships of genetic variability within our study population, both with isozyme and with DNA techniques (work in progress).
Table 3 shows the electrophoretic conditions for the isozyme analysis of young leaves of *Hyeronima alchorneoides.*

Table 3: Electrophoresis conditions for the isozyme analysis of young leaves form *Hyeronima alchorneoides.*

Tissue Intermediate leaf	Extraction buffer # (1:2)	Electrode and gel buffer	voltage % Bis	% acrylamide
Isozyme system PILON				
Esterase (EST)	7	C	120	10%
Fluorescent Esterase (EST-F)	7	C	120	10%
Phosphogluco isomerase (PGI)	7	C	200	9%
Malic enzyme (MEZ)	9	C	320	9%
b-Galactosidase (b-Gal)	9	C	200	10%

C: Gel buffer: 9:{ 0.05 M TRIS base, 0.007 M Citric acid pH 8.3 }: 1{Electrode buffer}. Tank buffer: 0.04M LiOH. 0.19 M Boric acid pH 8.3
7: TRIS base 0.1 M, KCl 10 mM, MgCl2 0.05M, EDTA 1mM, BMPE 14 mM, Ascorbic acid 0.1M, PVP 0.20gr/ml.
9: 0.011M EDTA, 0.001M KCl, 0.02M MgCl2, 4% PVP, 0.2M Phosphate buffer pH 7.5, 1.7 mM Sodium metabisulfite, 0.027 mM NADP, 0.003 mM NAD, 0.003 DTT, 10% Glycerol.

Simarouba amara.

As for *Hyeronima*, our work on *Simarouba* with isozyme approaches, constitutes a post-PSTC phase of research and is on-going.
Table 4 shows the electrophoretic conditions for the isozyme analysis of young leaves of *Simarouba amara.*

Table 4: Electrophoresis conditions for the isozyme analysis of young leaves form *Simarouba amara*

Tissue Intermediate leaf		Extraction buffer # (1:2)	Electrode and gel buffer	voltage % Bis	% acrylamide
Isozyme system	ACEITUNO				
Esterasas (EST)		9	C	120	10 %

C: Gel buffer: 9:{ 0.05 M TRIS base, 0.007 M Citric acid pH 8.3 }: 1{Electrode buffer}. Tank buffer: 0.04M LiOH, 0.19 M Boric acid pH 8.3
7: TRIS base 0.1 M, KCl 10 mM, MgCl2 0.05M, EDTA 1mM, BMPE 14 mM, Ascorbic acid 0.1M, PVP 0.20gr/ml.
9: 0.011M EDTA, 0.001M KCl, 0.02M MgCl2, 4% PVP, 0.2M Phosphate buffer pH 7.5, 1.7 mM Sodium metabisulfite, 0.027 mM NADP, 0.003 mM NAD, 0.003 DTT, 10% Glycerol.

2. DNA analysis

DNA extraction

Because of the presence of poly-phenolic compounds and polysaccharides, plant DNA extraction can be extremely difficult. We tested several published methods without success. We are now routinely using a modification of the method of Lodhi, M.A. et al. (1994). After an initial extraction in the presence of 2% CTAB in the presence of PVP, the DNA is precipitated at high NaCl concentration to inhibit polysaccharide co-precipitation.

This method allowed us to obtain clean DNA, amplifiable by PCR. The following table presents representative data for 6 *Hyeronima alchorneoides* individuals.

Tree I.D.	DNA concentration (ng/µl)	A_{260}/A_{280}	A_{260}/A_{230}
7002	336	1,75	1,41
7004	312	1,88	1,84
7006	234	1,84	1,77
7036	370	1,67	1,26
7069	211	1,97	2,34
7149	396	1,71	1,57

PCR amplification

For PCR amplification using arbitrary-sequence 10-mers, the following conditions were used:

2 µl	2,5 mM dNTP
2,5 µl	10x Universal Reaction Buffer
2,5 µl	40 mM $MgSO_4$
0,5 µl	30 µM Primer
10,5 µl	ddH_2O
3 µl	Taq polymerase
x µl	1 ng/µl ADN template

40 cycles of
- 20 seconds at 96°C
- 20 seconds at 30°C
- 1 minute at 72°C

Under these conditions, 10-15 bands are revealed with silver staining. These conditions are the result of extensive testing for optimum concentrations for DNA, Mg, Ca, dNTPs and primer. In cases where repeatability of the banding pattern is a problem, the protocol of Collins and Symons (1993) was used:

2 minutes at 95°C
39 cycle of:
- 1 min. at 95°C
- 10 sec. at 50°C
- 15 sec. at 45°C
- 20 sec. at 40°C
- 1 min. a 35°C
- 30 sec. at 45°C
- 1 min. 45 sec. at 72°C

5 min. at 72°C.

Amplification products were electrophoresed on mini-sub agarose gels, stained with ethidium bromide and photographed following standard procedures. Primers used were ten-mers from the Operon series C (OPC).
The conditions used guarantees that the amplification products patterns obtained from a given individual with a given primer are the same in independent amplification experiments. Figure 5 shows the results of four independent amplifications of *Hyeronima alchorneoides* DNA.

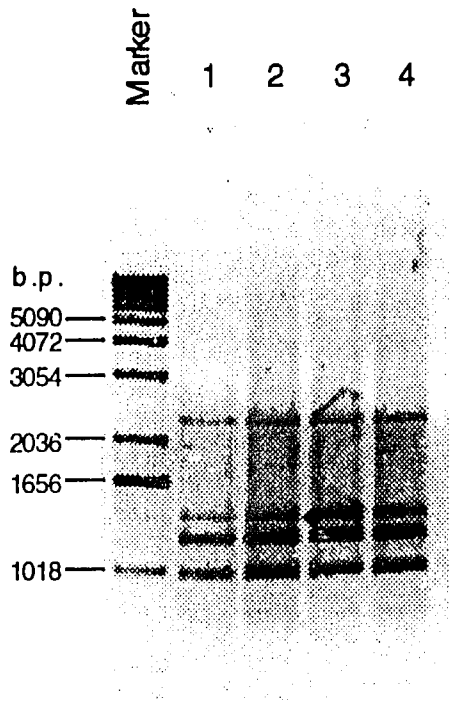

Figure 5: Reproducibility of the PCR amplification reaction. Four independent samples of *Hyeronima alchorneoides* DNA (lanes 1-4) were independently amplified in four PCR reactions. DNA concentration was 2ng/µl in the reaction mixture. Amplification products were electrophoresed in 2% agarose gels (8 x 10 cm) in 1x TBE buffer at 60 volts for 60 minutes. Gel stained with ethidium bromide. Primer used was OPC-07.

Results

A preliminary account of the results of the DNA analysis of the genetic variability of the three species under study is presented.

Twenty (20) primers from the Operon C series (OPC-1 to OPC-20) were tested for amplification using a sub-sample of 20 individual from each of the species under consideration. The following primers gave good amplification, and in **bold** are indicated those primers that revealed, in that sub-sample, the most polymorphic banding.

Minquartia guianensis and *Simarouba amara*	**01**	**02**	04	**05**	**06**	07	**08**	10	11	12	**14**	15	16	**19**	**20**
Hyeronima alchorneoides	**01**	**02**	04	**05**	**06**	07	**08**	10			**14**		16	**19**	**20**

Figure 6 shows the amplification results with primer OPC-05 for 18 individuals of *Minquartia guianensis* and 1 individual of *Simarouba amara*. Figure 7 shows the electrophoresis of PCR amplification products for 10 individuals of *Hyeronima alchorneoides* , 9 individuals of *Simarouba amara* and 18 individuals of *Minquartia guianensis* with primer OPC-06. Figure 8 shows the agarose gel electrophoresis of PCR amplification products wit primer OPC-05 of 10 individuals of *Hyeronima alchorneoides* and 9 individuals of *Simarouba amara.*

The polymorphic nature of the limited sampling presented is evident. It is also worth noting the different amplification patterns obtained with two different primers.

The number of DNA bands will be counted and evaluated for each gel according to Jeffreys *et al.*,1985, 1987 and Wetton *et al.*,1987.. A similarity index (D) is calculated to express the variation within population (Wetton *et al.* , 1987).

$$D = \frac{2 \ x \ N_{AB}}{N_A + N_B}$$

In which N_A and N_B are the number of fragments in individuals A and B and N_{AB} are the number of fragments shared by both. These measures are used to test differences among populations for level of variation using a T and T' multiple comparisons among means test (Sokal and Rohlf, 1981). The similarity index is also used to give a measure for the probability of two randomly taken individuals having the same total band pattern (D^N).

Besides calculating the similarity index, the variation within and between the populations is expressed as the identity within and between populations. This is a mesure of the chance of two amplified bands chosen from two plants (from the same or from two populations) being similar, as an average over all fragments. The differentiation between populations is expressed as the coefficient of relationship. which is comparable to an F_{ST}. In addition, the identities and distances between all populations within a species will be calculated.

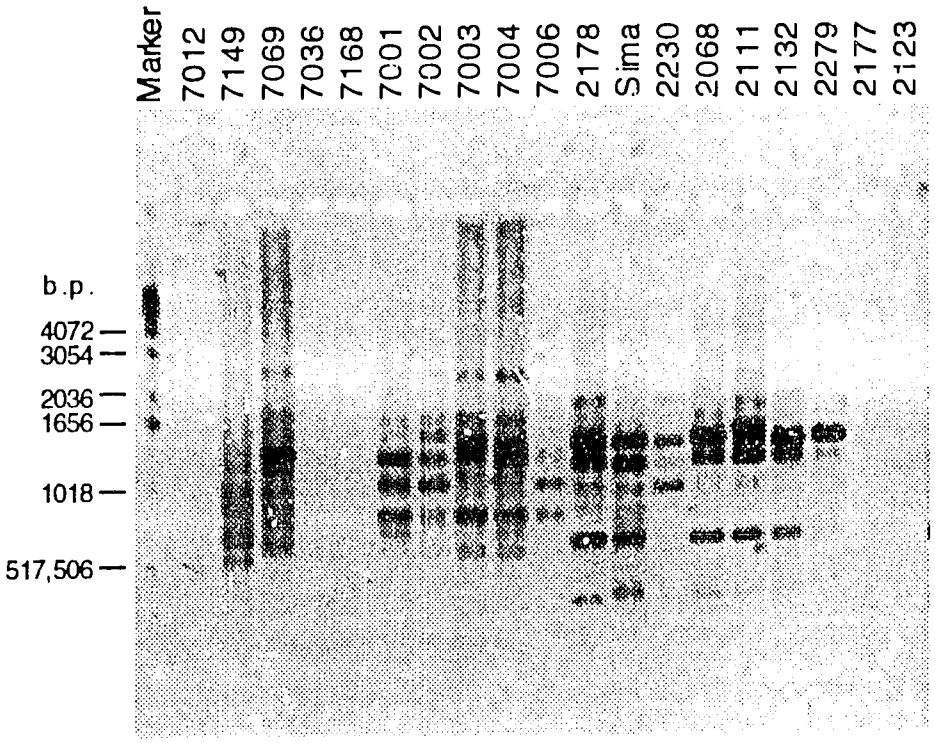

Figure 6: Agarose gel electrophoresis of PCR amplification products of DNA samples from *Hyeronima alchorneoides* (lanes 1-10) and *Simarouba amara* (lanes 11-19). Gel is 10 x 8 cm. 2% agarose in 1x TBE buffer, run at 60 volts for 90 minutes. Gel stained with ethidium bromide. Primer used is OPC-05.

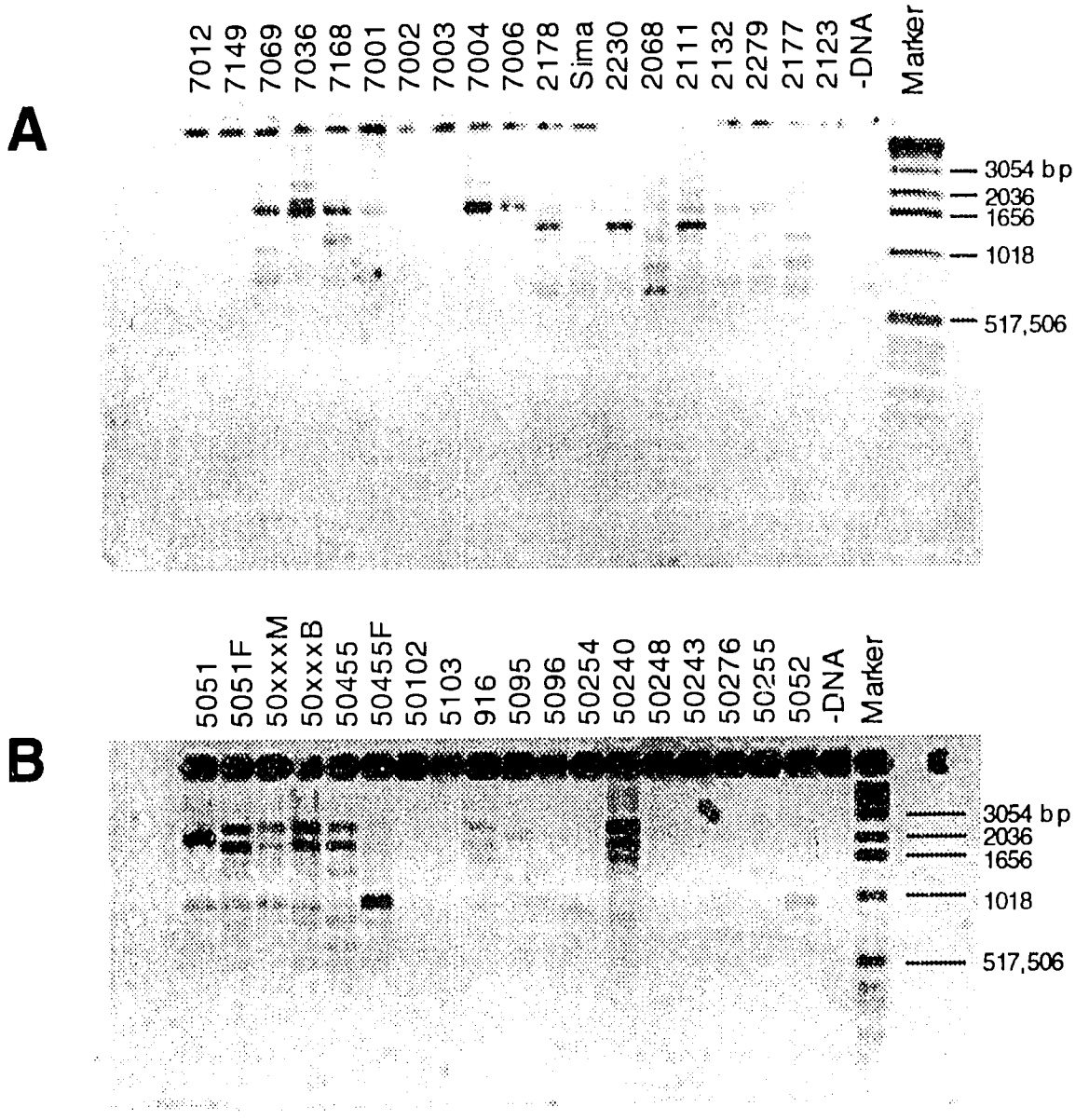

Figure 7: Agarose gel electrophoresis of PCR amplification products of DNA samples from *Hyeronima alchorneoides* (lanes 1-10) and *Simarouba amara* (lanes 11-19) (Panel A) and *Minquartia guianensis* (Panel B). Gel is 10 x 15 cm. 2% agarose in 1x TBE buffer, run at 60 volts for 90 minutes. Gel stained with ethidium bromide. Primer used is OPC-06.

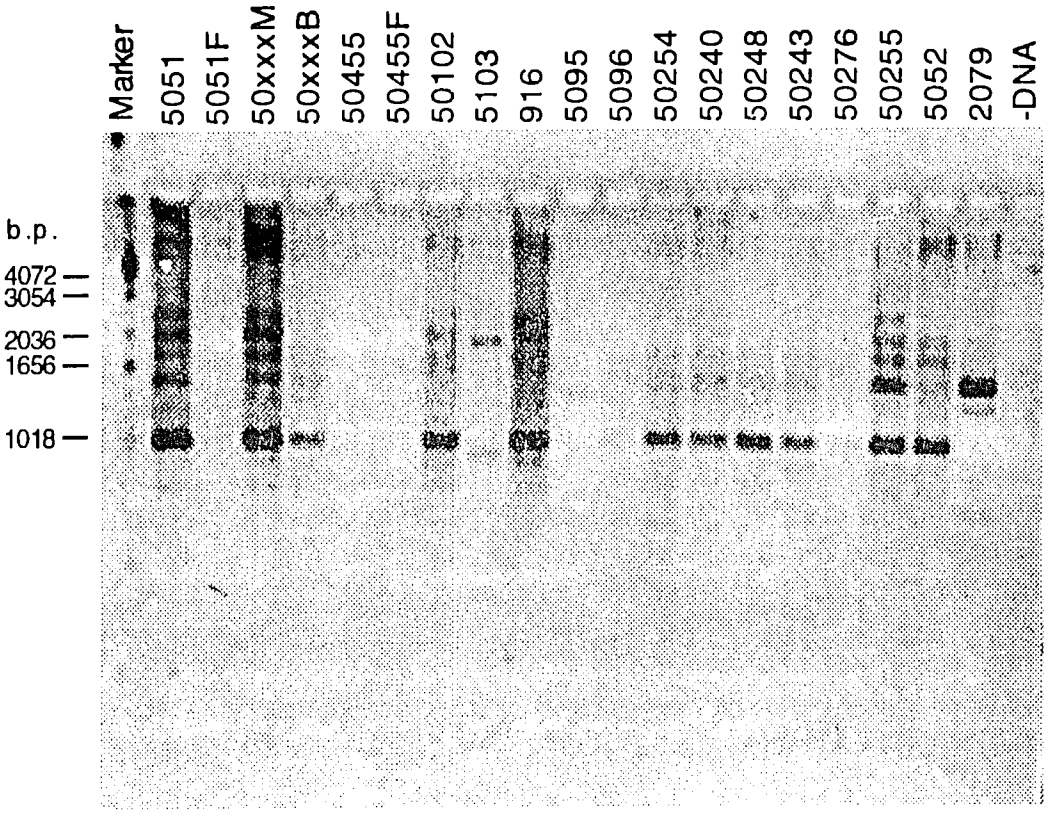

Figure 8: Agarose gel electrophoresis of PCR amplification products of DNA samples from *Minquartia guianensis* (lanes 1-18) and *Simarouba amara* (lane19). Gel is 10 x 8 cm. 2% agarose in 1x TBE buffer, run at 60 volts for 90 minutes. Gel stained with ethidium bromide. Primer used is OPC-05.

III. Impact, relevance, and Technology Transfer

The study and assessment of genetic variability will be crucial for the development of new strategies for germplasm conservation and plant improvement. Extensive discussion are under way with the Costa Rican Nation Biodiversity Institute (INBio) to apply the DNA fingerprinting technologies developed in this project to the analysis of variability on several plant and insect species. Several researchers from other groups of the University of Costa Rica (both CIBCM and the School of Biology) have been trained on DNA fingerprinting. They are applying the technology to other plant and animal systems: *Bactris* (peach-palm, pejibaye), wild beans (*Phaseolus lunatus*) and several bees species. An extensive evaluation of *musa* collections are under way. It is important to consider the feasibility of larger scale trials, considering populations of the tree species analyzed, outside the La Selva Biological Station.

A fully equipped laboratory is available at the CIBCM for the analysis of genetic variation. This equipment will be used on other projects, and new scientists will be trained on the developed technologies. Being CIBCM in a university environment, continued use of the facilities and equipment is granted.

IV. Project Activities/Outputs

a. This is a list of meetings attended for entire project

PSTC/CDR networking meeting on tree crops. BOSTID/US-AID, E.A.R.T.H., Costa Rica. Molecular genetic analysis of valuable native timber species. Clark, D. A., Macaya, G., Schaal, B. A. and Sittenfeld, A. April 2-8, 1992

Macaya, Villalobos, Paz, August 10-21, 1992.Theoretical and Practical Course "Frontiers in genome research: Genome structure and disease" UNESCO/ICRO, CIBCM, UCR.

Macaya, Villalobos, Paz, Schaal. November 23-December 12, 1992. "Caracterización bioquímica y molecular de genomas de plantas: su aplicación en evolución y biología de la conservación", Red Latinoamericana de Botánica, Escuela de Biología, CIBCM, Universidad de Costa Rica.

Macaya, Villalobos, Paz, April 18-23, 1994. Workshop on Open questions in molecular evolution. International Society of Molecular Evolution, Sloan Foundation, ICGEB, IUBMB, COGENE/ICSU, CONICIT. Guanacaste, Costa Rica.

Villalobos, May 23-25, 1994 - participated in the Second DNA Fingerprinting Workshop, a theoretical and practical course on RFLP, microsatellite, RAPD and DAF techniques. University of Tennessee, Knoxville.

Villalobos, May 25-28, 1994 - participated in the Fifth Gatlinburg Symposium, University of Tennessee, Knoxville.

b.- Training

Training of Heidy Villalobos (HV) took place from May 15 to June 15, 1994 in two stages: First at the University of Tennessee at Knoxville, in the laboratory of Dr. Peter Gresshoff, and then Washington University, St. Louis, in the laboratory of Dr. Barbara A. Schaal. In Dr. Peter Gresshoff's laboratory 45 samples from the three tree populations under study were analyzed by PCR amplification using arbitrary sequence primers (8-mers). Several clear and well-defined polymorphisms were detected with a set of primers. They are now being systematically tested in the lab in Costa Rica. At the same time, the silver-staining techniques were improved with the assistance of G. Caetano-Anollés. This visit to Knoxville came as an outcome of Dr. Gresshoff's visit to Costa Rica under a project to develop DNA probes for genetic analysis funded by the International Atomic Energy Agency.
The two weeks in Dr. Schaal's laboratory, from June 1-15, were spent analyzing the said samples with RAPD fingerprinting (10-mers). Several clear and well defined polymorphisms were observed. They are now being systematically tested in the laboratory at San José. Also, samples were tested for PCR amplification of specific mitochondrial sequences.

c. - Publications:

Paz-Perafán, G. B. (1996) Análisis de isoenzimas para determinar la variabilidad genética en *Minquartia guianiensis* (Manú). M. Sc. Thesis, Universidad de Costa Rica.

V. Project Productivity

Most of the original goals proposed for the project were accomplished. Specifically, the technological development and transfer to implement a laboratory fully equipped for the analysis of genetic variation was achieved. Furthermore, personnel was trained on the latest technologies and analytical procedures. Difficulties implementing the techniques, mainly DNA extraction procedures, delayed the project, and the final analysis of genetic variability, as revealed by DNA fingerprinting, will be a post-project activity.

VI. Future Work

A second continuing goal of our research with *Hyeronima*, is to assay the spatial and soil relationships of genetic variability within our study population, both with isozyme and with DNA techniques (work in progress). As for *Hyeronima*, our work on *Simarouba* with isozyme approaches, constitutes a post-PSTC phase of research and is on-going.

For the DNA analysis, we will analyze the results of PCR amplifications using the 8 selected primers for the Operon C series on 130 *Minquartia*, 84 *Hyeronima* and 60 *Simarouba* individuals. Also, 50 individuals from each species will be PCR amplified using 5 primers for the Operon AC series.

Octamers will be tested as primers for PCR amplification, in an effort to reveal more variability.

The technology developed on the project will be applied to other plant species. Preliminary results are available on the DNA fingerprinting analysis of a *Musa* germplasm collection (work in progress).

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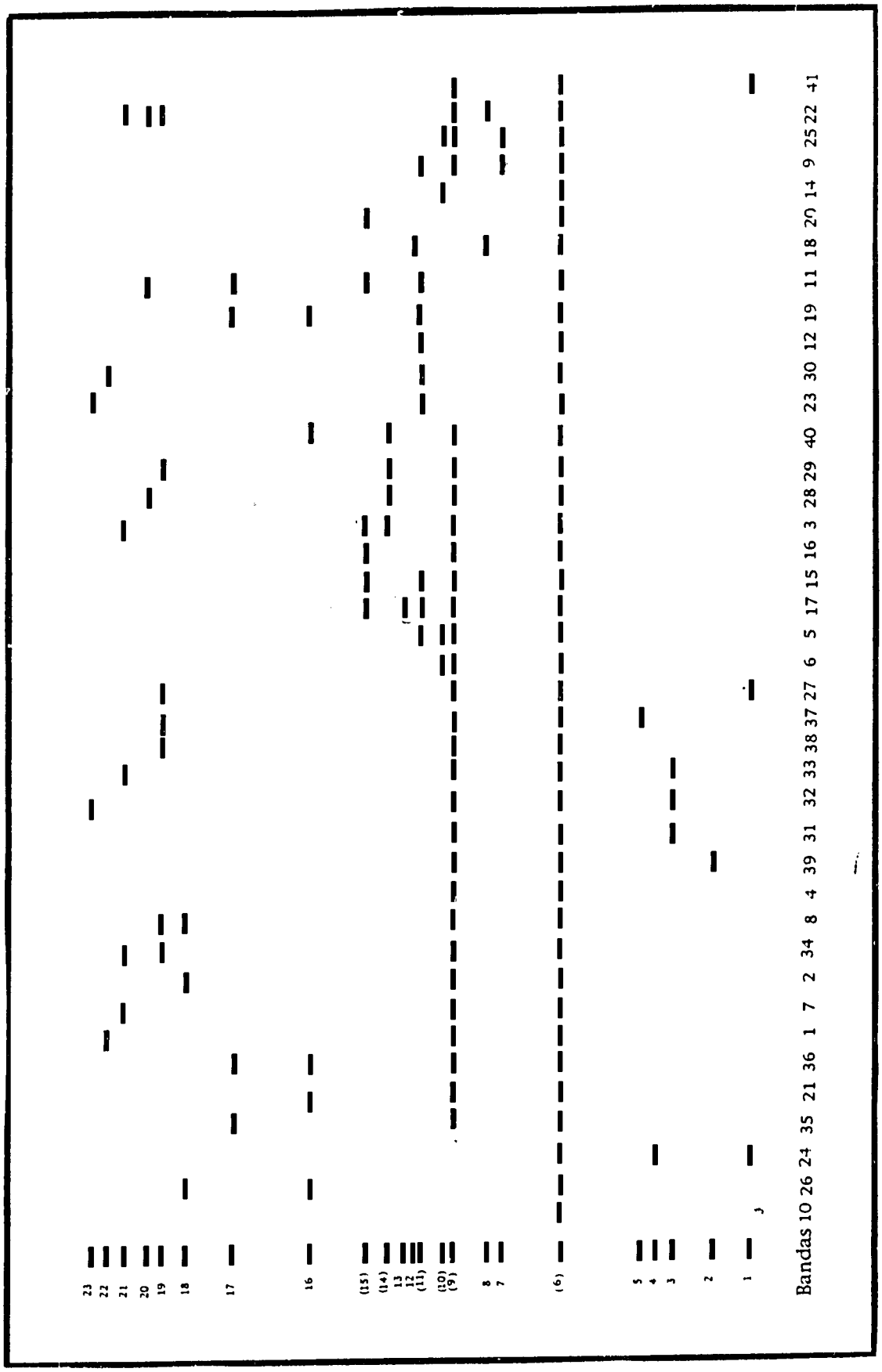
Appendix 1: Listing of the sampled individuals of *Minquartia*, with location, size and soil data

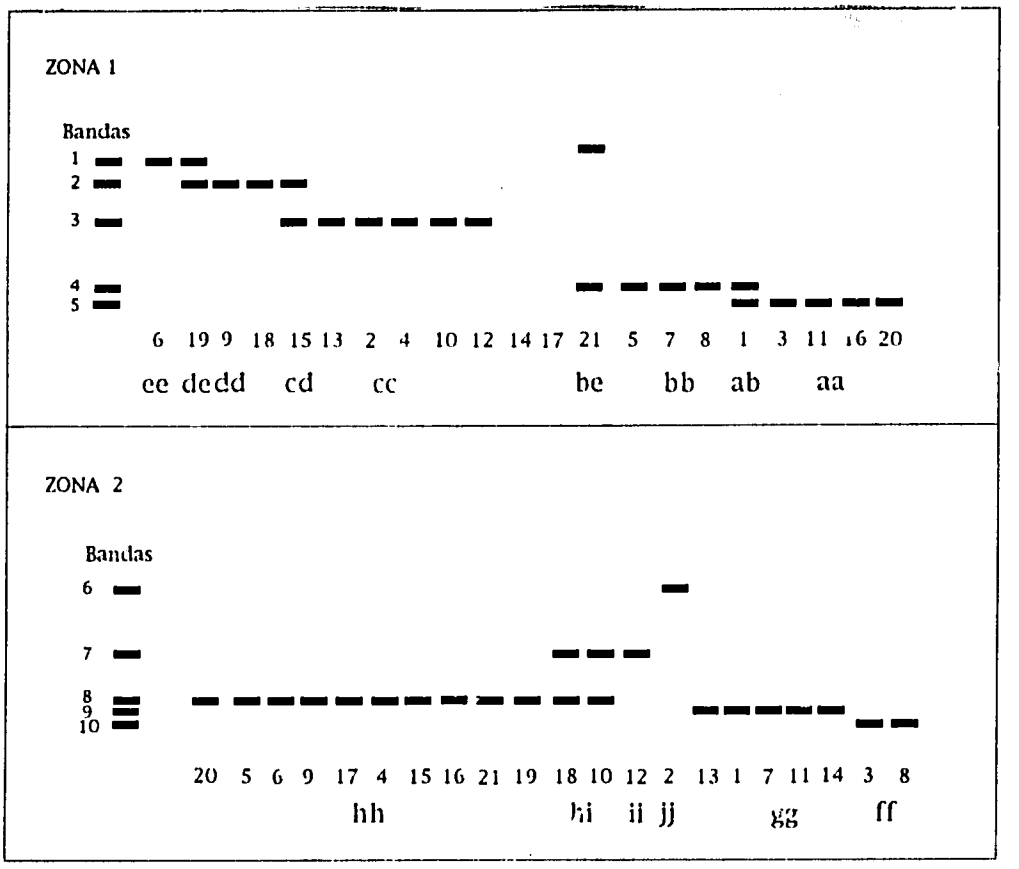
ID	X	Y	SOIL	SIZE	CAMINO		DIA94
50313	10074.284	9340.251	ALUVION	1	CES	800	47
50115	11516.370	9030.017	ALUVION	1	CCL	300	50
50100	9916.505	9245.733	ALUVION	1	CEN	700	50
50318	11783.080	8701.604	ALUVION	1	SHO	448	54
50138	11276.310	8738.459	ALUVION	1	CCL	710	58
50139	11279.643	8743.850	ALUVION	1	CCL	710	58
5062	10077.774	9127.958	ALUVION	1	SURA	1050	63
5096	9887.452	9414.261	ALUVION	1	CEN	525	63
50320	11541.635	9012.882	ALUVION	1	CCL	300	65
50319	10758.908	8720.987	ALUVION	1	SHO	448	75
5093	10001.950	9584.119	ALUVION	1	CEN	250	84
5098	9945.342	9271.345	ALUVION	2	CEN	667	101
50118	11367.689	8904.437	ALUVION	2	CCL	450	104
50160	11310.756	8662.734	ALUVION	2	CCL	710	107
50107	10139.097	9355.498	ALUVION	2	CES	720	117
50108	10098.783	9404.540	ALUVION	2	CES	750	138
5063	10060.569	9133.079	ALUVION	2	SURA	1043	143
50122	11275.907	8987.724	ALUVION	2	CCL	510	153
5094	9988.300	9468.138	ALUVION	2	CEN	300	158
5067	10120.712	9360.271	ALUVION	2	CES	746	184
5095	9898.818	9389.602	ALUVION	2	CEN	558	194
50119	11349.379	8964.405	ALUVION	3	CCL	450	214
5097	9884.490	9419.255	ALUVION	3	CEN	525	223
50147	11259.785	8714.363	ALUVION	3	CCL	710	261
50452	11528.664	8720.837	ALUVION	3	CCL	650	288
50326	11297.031	8806.669	ALUVION	3	CCL	650	327
5074	11917.037	8750.448	ALUVION	3	SSE	650	347
5081	11826.355	8799.031	ALUVION	3	SHO	365	371
50392	11462.708	8717.491	ALUVION	3	CCL	650	415
5086	9918.599	9287.338	ALUVION	3	CEN	667	459
5002	12308.111	8188.835	ALUVION	3	SSE	1540	516
50391	11501.227	8750.415	ALUVION	3	CCL	650	542
50295	10339.374	9605.660	ALUVION	3	AREAI		575
50155	11248.928	8712.253	ALUVION	3	CCL	775	212
5050	11250.800	7149.814	ULTISOL	1	CC	1780	41
5055	11325.207	6605.629	ULTISOL	1	CC	2401	45
50250	11186.752	7125.731	ULTISOL	1	CC	1875	47
50248	11269.593	7187.426	ULTISOL	1	CC	1739	50
50255	11246.507	7196.855	ULTISOL	1	CC	1750	50
50254	11246.817	7190.204	ULTISOL	1	CC	1750	59
50246	11192.831	6960.542	ULTISOL	1	CC	2005	65
50240	11246.386	6796.124	ULTISOL	1	CC	2185	70
50238	11284.804	6665.263	ULTISOL	1	CC	2335	84
50251	11180.645	7133.389	ULTISOL	1	CC	1875	92
50243	11215.047	7007.103	ULTISOL	1	CC	1965	97
50237	11324.572	6610.484	ULTISOL	2	CC	2400	105
5053	11301.061	6669.619	ULTISOL	2	CC	2335	110
50367	11281.886	6935.551	ULTISOL	2	CC	2010	126
50368	11150.000	7210.600	ULTISOL	2	CC	1875	142
50244	11272.497	7192.008	ULTISOL	2	CC	1735	143
50256	11261.854	7220.494	ULTISOL	2	CC	1735	157
50252	11191.085	7130.097	ULTISOL	2	CC	1875	166
50249	11260.643	7195.915	ULTISOL	2	CC	1735	173
50245	11290.966	7222.196	ULTISOL	2	CC	1695	185
50258	11241.015	7226.096	ULTISOL	3	CC	1735	234
50403	11288.348	6795.759	ULTISOL	3	CC	2187	246
50241	11213.294	6899.242	ULTISOL	3	CC	2070	265
50459	11303.317	7190.885	ULTISOL	3	CC	1720	267

50404	11339.782	6804.337	ULTISOL	3	CC	2187	268
50526	11267.155	6670.323	ULTISOL	3	CC	2335	288
50405	11316.930	6785.794	ULTISOL	3	CC	2187	321
50239	11252.048	6698.090	ULTISOL	3	CC	2275	332
50462	11272.279	7018.593	ULTISOL	3	CC	2010	335
50247	11229.990	6956.572	ULTISOL	3	CC	2010	338
50474	11313.019	6855.729	ULTISOL	3	CC	2187	342
50463	11258.207	7016.112	ULTISOL	3	CC	2010	344
50424	11308.436	7215.218	ULTISOL	3	CC	1642	348
50259	11287.025	7227.824	ULTISOL	3	CC	1691	354
5054	11319.739	6645.330	ULTISOL	3	CC	2360	364
50455	11297.058	6836.364	ULTISOL	3	CC	2187	379
50417	11181.754	7256.879	ULTISOL	3	CC	1750	395
50416	11305.235	6993.052	ULTISOL	3	CC	2010	433
50257	11255.471	7212.887	ULTISOL	3	CC	1735	444
5051	11208.431	6960.542	ULTISOL	3	CC	2005	464
50236	11356.655	6706.206	ULTISOL	3	CC	2360	548
50473	11320.348	6850.003	ULTISOL	3	CC	2187	579
5052	11238.773	6784.548	ULTISOL	3	CC	2193	592
50187	10936.686	8795.674	ARBOLEDA	1	CCC	780	52
50209	10635.514	8744.870	ARBOLEDA	1	SSO	250	55
5068	10765.168	8806.372	ARBOLEDA	1	SSO	38	59
50184	10880.962	8805.711	ARBOLEDA	1	CCC	750	59
5012	10903.872	8842.351	ARBOLEDA	1	CCC	705	62
50348	10456.740	8848.604	ARBOLEDA	1	SSO	605	63
50388	10571.595	8810.851	ARBOLEDA	1	SSO	220	66
50191	10924.838	8818.113	ARBOLEDA	1	CCC	780	68
50206	10881.910	8766.859	ARBOLEDA	1	CCC	780	69
50219	10518.582	8371.592	ARBOLEDA	1	SSO	760	77
5016	10864.203	8778.942	ARBOLEDA	1	CCC	780	88
50217	10603.367	8674.642	ARBOLEDA	2	SSO	330	113
5022	10917.885	8732.036	ARBOLEDA	2	CCC	1000	114
5020	10911.621	8743.150	ARBOLEDA	2	CCC	1000	117
5013	10966.414	8838.744	ARBOLEDA	2	CCC	705	147
50342	10803.510	9029.877	ARBOLEDA	2	CCC	600	159
50389	10583.737	8805.284	ARBOLEDA	3	SSO	220	215
50518	10601.571	8838.003	ARBOLEDA	3	SSO	220	216
5011	10975.522	8857.672	ARBOLEDA	3	CCC	663	219
50401	10488.380	8618.370	ARBOLEDA	3	SSO	490	222
50454	10491.363	8583.166	ARBOLEDA	3	SSO	490	233
50343	10921.008	8766.330	ARBOLEDA	3	CCC	780	264
50430	10631.192	8645.033	ARBOLEDA	3	SSO	340	272
50390	10665.871	8773.901	ARBOLEDA	3	SSO	200	276
50472	10501.505	8366.206	ARBOLEDA	3	SSO	700	277
50215	10617.621	8685.613	ARBOLEDA	3	SSO	320	286
50398	11027.447	8918.924	ARBOLEDA	3	CCL	635	339
50428	10558.472	8456.416	ARBOLEDA	3	SSO	172	348
50453	10707.709	8580.870	ARBOLEDA	3	SSO	172	370
5023	10914.755	8771.042	ARBOLEDA	3	CCC	780	376
50471	10503.760	8368.711	ARBOLEDA	3	SSO	700	382
50429	10590.723	8399.352	ARBOLEDA	3	SSO	172	397
5008	11002.881	8941.199	ARBOLEDA	3	CCC	663	504
5018	10877.325	8802.383	ARBOLEDA	3	CCC	750	660

Appendix 2: Diagram of the 40 enzymatic patterns displayed by *Minquartia* for esterases (EST)



Appendix 3: Diagram of the 21 enzymatic patterns displayed by *Minquartia* for the system PGI.



FINANCIAL STATUS REPORT

(Short Form)

1. Federal Agency and Organizational Element to Which Report is Submitted	2. Federal Grant or Other Identifying Number Assigned By Federal Agency	OMB Approval No.	Page	of
U.S.A.I.D.	PSTC Proposal No. 10-105 DHR-5600-G-00-1013-00	0348-0039	1	1 pages

3. Recipient Organization (Name and complete address, including ZIP code)

Duke University, Office of Sponsored Programs
705 Broad Street, Box 90491, Room 01
Durham, North Carolina 27708-0491

4. Employer Identification Number	5. Recipient Account # or Identifying #	6. Final Report	7. Basis
1560532129A1	343-9835-85500	[X] YES [ ] NO	[ ] Cash [X] Accrual

8. Funding/Grant Period (See Instructions) From: (Month, Day, Year)	TO: (Month, Day, Year)	9. Period Covered by this Report From: (Month, Day, Year)	TO: (Month, Day, Year)
05/24/91	11/23/95	06/01/95	11/23/95

10. Transactions:	I Previously Reported	II This Period	III Cumulative
a. Total outlays	144,623.46	8,809.10	153,432.56
b. Recipient share of outlays	3,147.26	267.30	3,434.56
c. Federal share of outlays	141,476.20	8,521.80	149,998.0
d. Total unliquidated obligations			0.0
e. Recipient share of unliquidated obligations			0.0
f. Federal share of unliquidated obligations			0.0
g. Total Federal share (Sum of lines c and f)			149,998.0
h. Total Federal funds authorized for this funding period			149,998.
i. Unobligated balance of Federal funds (Line h minus line g)			0

11. Indirect Expense	Type of Rate [ ] Provisional [X] Predetermined [ ] Final [ ] Fixed			
	b. Rate	c. Base	d. Total Amount	e. Federal Share
	29 % MTDC	$ 6,031.92	$ 2,777.18	$ 2,489.8

12. Remarks: Attach any explanations deemed necessary or information required by Federal sponsoring agency in compliance with governing legislation.

13. Certification: I certify to the best of my knowledge and belief that this report is correct and complete and that all outlays and unliquidated obligations are for the purposes set forth in the award documents.

Type or Printed Name and Title	Telephone (Area code, number and extension)
Tom M. Davis Director-Sponsored Programs	(919) 684-5442
Signature of Authorized Certifying Official Tom Davis	Date Report Submitted 1/8/96

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