ISOZYME POLYMORPHISM IN THE LEGUMINOUS GENUS *LEUCAENA*

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ABSTRACT

The leguminous genus *Leucaena* includes 15 species, several of which have become pantropical owing to their importance as fodder, fuelwood, shade and ornamental trees. In all present study, protocols for obtaining reproducible isozyme phenotypes in *Leucaena* were developed for six isozyme systems (ACO, ADH, IDH, MDH, PGI and PGI) using L-histidine and citric acid for running and tray buffers with cotyledon tissues. Standard descriptions of isozymic phenotypes were established using accession K997 of a local Hawaiian population *L. leucocephala* ssp. *leucocephala* as a control. Ontogenetic studies revealed that ADH phenotypes were developmentally variable. However, the phenotypes of other isozyme systems were not variable ontogenetically.

One hundred sixty-nine accessions of 12 *Leucaena* species, including 3 tetraploid species and 9 diploid species, were analyzed for six isozyme systems. Extensive polymorphism was observed among the species, and the weighted polymorphism (P_w) was estimated. The higher P_w values characterized outcrossing species and the lower P_ws were among self-pollinated species. Five of these isozyme systems (ACO, ADH, IDH, MDH and PGI) provided bands that were found useful in identifying taxa within the genus. These systems were also effective in detecting inter- and intra-specific hybrids. Three loci of ACO and two loci of

IDH in the sibcrosses of *L. lanceolata* (K10) were confirmed.

Seventy-nine accessions of "common" *L. leucocephala* ssp. *leucocephala* from tropical countries and thirty-six of "giant" *L. leucocephala* ssp. *glabrata*, mainly from Central America were tested for isozyme variation. The uniformity of isozymic expression of all "common" types suggests that they are offsprings of a "pure" line variety or a single tree. In contrast, the "giant" types of *L. leucocephala* ssp. *glabrata* were quite diverse. The "giant" type trees could be easily distinguished from the "common" in the ACO system. The ancestral relationship of *Leucaena* diploids and polyploids were analyzed using the electrophoresis technique. The results did not support the hypotheses that (1) *L. diversifolia* ssp. *diversifolia* (2n=104) is an autotetraploid from *L. diversifolia* ssp. *trichandra* (2n=52) and (2) *L. pallida* (2n=104) is an amphidiploid from *L. diversifolia* ssp. *trichandra* and *L. esculenta* (2n=52).

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Chapter 1

INTRODUCTION

The genus *Leucaena* Bentham became widely popularized after the UH "Hawaiian Giant" varieties were released to the world in the 1960's. The utilization of these "giant" trees as forage for animals, wood for fuel and industrial use, trees for reforestation and soil improvement in agroforestry systems has been reviewed by several authors, e. g., NAS (1984), Brewbaker and Hutton (1979) and Brewbaker (1987). Because of leucaena's economic importance in several developing tropical countries, efforts have been made to improve varieties through hybridization and selection, notably for high psyllid resistance, cold and acid soil tolerance (Brewbaker, 1987).

The genus *Leucaena* includes 13 widely recognized species (Brewbaker, 1987) and others still under consideration (Sorensson, 1991, pers. comm.). Most species of the genus possess economic promise, but only a few species have been directly exploited. Elucidation of basic genetics and evolution of any relationships in this genus will aid in designing breeding programs to optimally utilize its genetic resources. However, there is very limited evidence for genetic markers in *Leucaena*, and very few qualitative traits have been carried through to genetic analysis (Brewbaker, 1987).

Isoenzymes have provided a large number of genetic markers for other taxa (Tanksley and Orton, 1983), but their study in *Leucaena* has been limited. The objectives of this research were **1)** to determine suitable isozyme analytic procedures to test selected isozymes in Kreb's cycle and the glycolytic pathway by choosing different running and tray buffers; **2)** to conduct ontogenetic studies of different life-cycle stages and tissues of selected species; **3)** to survey isozymic polymorphism in *Leucaena* species and identify polymorphic gene loci using selected isozymes; **4)** to apply isozyme markers to test the following evolutionary hypotheses that **a)** *L. leucocephala* ssp. *leucocephala* "common" types are genetically one pure line, **b)** *L. leucocephala* ssp. *glabrata* Rose "giant" type is isozymically distinct from the "common" type, **c)** *L. diversifolia* ssp. *diversifolia* (2n=104) is an autotetraploid of *L. diversifolia* ssp. *trichandra* (2n=52), and **d)** *L. pallida* (2n=104) is an amphidiploid of *L. diversifolia* ssp. *trichandra* and *L. esculenta* (2n=52).

Chapter 2

LITERATURE REVIEW

The introduction of starch gel electrophoresis (Smithies, 1955) and the development of activity stains for the visualization of specific enzymes (Hunter and Markert, 1957) were major contributions to isozyme genetics in plant and animal research. Isozyme applications in plant research have been proposed and reviewed by several authors, e. g., Peirce and Brewbaker (1973) in horticultural science, Falkenhagen (1986) in forestry and Weeden (1989) in plant breeding. The main advantages of using isozymes as tools for plant breeding and genetic research are the following: **1)** the absence of significant physiological effects of most isozyme polymorphism, **2)** the lack of epistatic or pleiotropic interactions among different loci, **3)** the simple genetic basis of most variation, **4)** the codominant expression displayed by allelic products, and **5)** the consistency of expression on many enzymes irrespective of environmental conditions or tissue sampled (Weeden, 1989). Due to those advantages, isozymes have been used for the study of systematics (Gottlieb, 1977, 1981; Crawford, 1983) and developmental biology (Whitt, 1975), for estimating genetic diversity of the species (Peirce and Brewbaker, 1973; Weeden, 1989) and for elucidating the origin of polyploids (Gottlieb, 1977; Werth, 1989).

2.1 Developmental biology studies

Isozymes have long been applied to developmental biology studies. The main purpose of such studies is to understand the gene regulation at different times and in different tissues during plant development. For example, the basic peroxidase of corn is associated with cell-wall fractions of mature tissue (Brewbaker and Hasegawa, 1975). Alcohol dehydrogenase activities in several angiosperm species and conifers are highly developmental regulated during the seed germination (Harry et al. 1987). Although changes in isozyme expression may be useful in developmental genetic study, they can be a hindrance to plant breeders because uniform expression of isozymes throughout development allows screening for isozyme variants at diverse stages (Mowrey and Werner, 1990).

2.2 Genetic diversity and systematics

One of the applications of isozymes in plant breeding is to estimate the genetic diversity or heterozygosity in plant species (Peirce and Brewbaker, 1973, Weeden, 1989). This can aid plant breeders in choosing parental materials for breeding programs. This also can provide population geneticists and systematists with simple genetic markers necessary to analyze gene flow, differential selection pressure and genetic relationship among populations and taxa (Gottlieb, 1981). Measurements of genetic variability as

proposed by Brown and Weir (1983) include **1)** percentage of polymorphic loci, **2)** degree of heterozygosity, **3)** average number of alleles per locus and **4)** heterozygosity of allele frequencies. Allelic frequencies at 25 enzyme loci in seven species of *Prosopis* (Leguminosae: Mimosoideae) were used to estimate the genetic variability of the species (Saidman and Vilardi, 1987). Phenotypic variation of enzymes were also used to estimate polymorphism among *Beta vulgaris* populations (Nagamine et al. 1989). Studies of *Cucumis sativus* L. by Knerr et al. (1989), *Gymnaedenia conopsea* by Scacchi et al. (1989), and *Erythranthe* (Mimulus) by Vickery et al. (1989) also showed the practical application of isozymes to estimate genetic diversity.

The use of isozyme data for taxonomic classification was proposed by Sibley (1962), and an earlier review of isozymes for plant systematics was that of Gottlieb (1977). Details of the application of electrophoretic data for systematic studies were given by Crawford (1983). Carulli and Fairbrothers (1988) employed starch gel electrophoresis to assess systematic relationships among *Aeschynomene virginica*, *A. indica*, and *A. undis*. Allozyme data proved to be entirely consistent with the morphological systematics of these species. Hoffman and co-workers (1986) reported that their isozyme polymorphism survey in *Lens* (Leguminosae) was valuable in elucidating relationships between the taxa, as well as suggesting new hypothesis on the origin of the

cultivated lentil.

2.3 Origin of polyploid species

The determination of the ancestral parentage of present ploypoids could be made through a number of traditional evaluations including morphological similarities and chromosome pairing between the suggested ancestors and ploypoids. Electrophoretic evidence may also be used to help elucidate the derivation of polyploid species (Werth, 1989). Smith-Huerta (1986) reported that electrophoretic phenotypes of each tetraploid *Clarkia* polyploid was a simple combination of the electromorphs found in diploid progenitors with only two exceptions in *C. similis*, and the level of electrophoretic variability was strongly correlated with breeding system rather than to ploidy level. Studies of *Cucurbita* by Weeden (1984) and *Malus* by Chevreau (1985) also showed that polyploid species display nearly twice the number of isozymes characteristic of diploid species. Hutchinson and colleagues (1983) using 5 isozymes (acid phosphortase, esterase, leucine aminopeptidase, peroxidase and 6-phosphoglucinate dehydrogenase) found that *Avena barbata* was a diploidized tetraploid, i.e., a tetraploid in which pairing is preferential within each pair of homologues. Ranker and Haufler (1989) employed genetic evidence from electrophoresis and restriction site analyses of cpDNA to elucidate the origin of tetraploid *Hemionitis*

pinnatifida. Their data clearly supported the hypothesis that diploid *H. palmata* is one of tetraploid *H. pinnatifida*'s progenitors.

2.4 Isozymes of *Leucaena*

Electrophoresis as a tool for *Leucaena* systematic study was employed by Pan (1985). He examined five isozyme systems (leucine amiinopeptidase, acid phosphortase, malate dehydrogenase, esterase and peroxidas), of which only peroxidase gave clear results. The data could not conclusively support his thesis that *L. pallida* (2n=104) is an amphidiploid derived from *L. esculenta* (2n=52) and *L. diversifolia* ssp. *trichandra* (2n=52), even though many morphological characters and geographical distributions of *L. pallida* are intermediate between the latter two species (Pan 1985). Other early work at UH by Hamill (1965, unpublished) and Sakamoto (1971, unpublished) under Dr. Brewbaker used four isozymes of leucine aminopeptidase, esterase, peroxidase and amylase to explore the relationship among five *Leucaena* species. Schiffino-Wittmann and Schlegel (1991) also analyzed *L. leucocephala*, *L. diversifolia* and its hybrids (F1, F2 and F3) for 21 enzymes, and only esterase showed different band patterns for those tested materials.

Chapter 3

MATERIALS AND METHODS

3.1 Plant materials and sample preparation

3.1.1 Procedural modifications

A local Hawaiian population of *L. leucocephala* ssp. *leucocephala*, cv. K997, was selected for initial experiments to define suitable isozymic procedures because of its availability and suspected genetic uniformity (Brewbaker, pers. comm.). Seeds were scarified with sulfuric acid (12M) for 6 to 8 minutes, or treated with boiling water for 1 to 2 minutes, and washed liberally in tap water before soaking overnight. Seeds were then placed on moist germination paper in petri dishes at laboratory (temperature 25°C, light 14 hours). After seedling radicles had extended 1 cm, germinated seeds were placed in a holding dish in a cold room (4°C). This allowed all seedlings to be at the same stage of development during the sampling of cotyledons for isozyme analysis.

3.1.2 Ontogenetic study

For the ontogenetic study, 25 seeds for each accession of three taxa were used. These included *L. diversifolia* ssp. *diversifolia* (K946 and K947, 2n=104), *L. diversifolia* ssp. *trichandra* (K927, 2n=52), and *L. pulverulenta* (K957 and K958, 2n=56). Seeds were scarified with sulfuric acid or

boiling water, soaked overnight, sown into the dibble tubes with soilless medium in greenhouse at Waimanalo Research Station on the second day. After 10 days germination, the first isozyme survey was conducted. After two months, seedlings were transplanted into 4 x 6-inch pots with the same medium. Seedlings were fertilized biweekly with triple 19-19-19 NPK (Gaviota) and maintained for late sampling. Average heights of the three species were recorded before each isozyme analysis. The major stages and tissues of these *Leucaena* species for isozyme survey were cotyledon (10, 20 days), root and hypocotyl (20 days), and shoot and leaf (20, 60, 180 days).

3.1.3 Phenotypic variation in genus

Twelve *Leucaena* species (excluding *L. salvadorensis*, *L. multicapitula* and *L. sp.*) were selected from UH *Leucaena* germplasm collections to assess phenotypic variation of isozyme systems (Table 1). Many of these were from single trees as half-sib or selfed progenies; composites are annotated (c) in the table. Origins, locales, PI number and ID number of these accessions are listed in Appendix A. An attempt was made to select accessions from as wide a geographical range as possible within each species. Twenty five seed samples were taken from each accession for each of 12 species (approx. five accessions per species). Seeds were scarified and germinated as above. Cotyledon tissue

Table 1. *Leucaena* species, accession numbers and origins of accessions tested in this study

	Species	Subspecies*	Number of accessions	Accessions (c) if composite
1	*L. collinsii*	ssp. *collinsii*	1	K905
		ssp. *zacapana*	5	K911 K912 K913 K914 K917
2	*L. diversifolia*	ssp. *trichandra*	8	K408 K483 K821 K823 K907 K919 K927 K936
		ssp. *diversifolia*	7	K776c K782 K790 K796 K802 K946c K947c
3	*L. esculenta*	ssp. *esculenta*	3	K689 K948 K949
		ssp. *matudae*	1	K950
4	*L. greggii*		5	K854 K855c K858 K862c K956c
5	*L. lanceolata*	ssp. *lanceolata*	4	K10 K381c K401 K470
		ssp. *sousae*	1	K952
6	*L. leucocephala*	ssp. *leucocephala*	77	listed in Table 2
		ssp. *glabrata*	36	listed in Table 3
7	*L. macrophylla*	ssp. *macrophylla*	3	K836 K880 K955
		ssp. *nelsonii*	1	K902
8	*L. pallida*		4	K376c K806 K819 K953c
9	*L. pulverulenta*		3	K870 K957c K958c
10	*L. retusa*		4	K503 K506 K899 K900
11	*L. shannonii*	ssp. *magnifica*	1	K916
		ssp. *shannonii*	4	K741c K924 K925 K954c
12	*L. trichodes*		1	K903c
Total			169	

* subspecies designations follow these adopted by Sorensson (1992)

was examined for the study.

3.1.4 Evolutionary analysis

Evolutionary hypothesis tests were conducted for the isozyme systems on 77 accessions of *L. leucocephala* ssp. *leucocephala* ("common" types) from 29 countries worldwide (Table 2). In addition, 37 accessions of *L. leucocephala* ssp. *glabrata* ("giant" types) from 5 countries mainly in Central America were also tested (Table 3). Seed scarification and germination procedures were the same as above. Cotyledon tissue sampling procedures for these "common" and "giant" accessions were modified as follows. First, 5 seeds were sampled for each accession to determine if variation existed in the isozyme systems. If polymorphism was observed, at least 5 more seeds (per accession) were analyzed.

3.2 Electrophoresis

3.2.1 Starch gel preparation

A 12 % (w/v) starch gel of hydrolyzed potato starch (Sigma Chemicals) was used throughout this study. Forty five grams of the starch along with 375 ml of gel buffer solutions were poured into a 1000 ml side arm flask. The mixture was swirled vigorously by hand for one minute to avoid the formation of lumps. The flask was then heated over a gas burner (constantly swirling) until the solution

Table 2. Countries & regions, number of accessions tested in this study, number of accessions showing polymorphism and K numbers in *L. leucocephala* ssp. *leucocephala*

Countries and regions	No. of accessions		Accessions (*) if polymorphic				
	Total	Polymorphic					
American Somoa, USA	1	0	K85				
Australia	7	2	K31	K46*	K54	K55*	K56
			K57	K58			
Benin	1	0	K304				
Brazil	1	0	K49				
Fiji	2	0	K47	K84			
Ghana	1	0	K41				
Honduras	1	1	K12*				
India	1	0	K25				
Indonesia	1	0	K71	K83			
Ivory Coast	1	1	K62*				
Malaysia	1	1	K676*				
New Britain	1	0	K35				
New Caledonia	5	0	K36	K37	K38	K48	K61
Philippines	7	1	K21	K22*	K43	K50	K68
			K73	K677			
Rep. of S. Africa	1	1	K24*				
Sensgal	1	0	K2				
Sierra Leone	3	1	K42*	K89	K269		
Singapore	1	0	K70				
South African	2	0	K87	K88			
Sri Lanka	1	0	K40				
Tahati	1	0	K86				
Taiwan, ROC	9	5	K13	K60	K76	K77*	K78
			K79*	K80*	K81*	K82*	
Tanzania	2	0	K32	K115			
Thailand	14	4	K51	K313	K314	K315	K316
			K317*	K318	K319	K320	K321*
			K322	K323	K325*	K326*	
Hawaii, USA	7	3	K63*	K72*	K278	K279*	K281
			K341	K997c			
Uganda	1	0	K64				
Vietnam	2	0	K52	K53			
Virgin Islands, USA	1	0	K26				
Zaire	1	0	K23				
Total(countries)	29	29					
Total(accs.)	77	21					

Table 3. Countries & regions, number of accessions tested in this study, number of accessions showing polymorphism and K numbers in *L. leucocephala* ssp. *glabrata*

Countries and regoins	No. of accessions		Accessions (*) if polymorphic
	Total	Polymorphic	
El Salvador	5	4	K28* K217 K417* K418* K419*
Honduras	2	1	K29* K329
Mexico	26	20	K8* K132 K140* K358* K378* K395 K397* K452* K499* K517* K538c* K562c* K565 K584* K598* K607* K608* K614c* K617* K619 K633c* K636* K638 K664c* K665c*
New guinea	2	1	K1 K6*
Thailand	1	1	K678c*
Total(countries)	5	5	
Total(accs.)	36	27	

reached 75°C, and then subjected to a vacuum until small bubbles were evacuated. The solution was then poured into a plexiglass mold (dimensions: 18 x 13 x 1 cm). Any bubbles that formed while pouring the starch solution into the molds were removed with a spatula before the gel started to harden. The gel was allowed to cool for one hour at lab room temperature, and then further cooled by putting in a cold room (4°C) covered with polyethylene film to prevent desiccation for one hour prior to loading samples.

For the convenience of daily laboratory operation, normal procedure for preparing gels was modified. After gel solution was poured into gel mold, gel was kept for overnight for about 12 hours at lab temperature (25°C) before being placed in the cold room or refrigerator (4°C) on second day for hardening.

3.2.2 Protein extraction and gel loading

Proteins were extracted from cotyledon, hypocotyl, root tip, shoot and leaf of seedling and mature plant by grinding approximately 100 mg tissue (fresh weight) in 100-150 μl extraction buffer (Bousquet, 1987) as modified by Aradhya (1992) in Table 4. Tissue samples were fresh collected from seedlings or from mature plants and kept cold during the extraction procedure by placing a ceramic grinding block (12 wells, 8 x 11 x 0.8 cm) on ice. A paper wick (Beckman chromatography, 2 to 4 x 12 mm) was used to soak the liquid

Table 4. Recipe for 50 ml Extraction buffer (modification of Aradhya, 1992)

Concentration	Chemmicals	Quantities
0.1 M	Tris	0.606 g
0.2 M	Sucrose	3.423 g
0.5 mM	EDTA	0.008 g
5 mM	DTT (DL-dithiothreitol)	0.039 g
12 mM	L-cysteine hydrochloride	0.105 g
25 mM	L-ascorbic acid (not sodium)	0.220 g
1%	PEG (polyethyleneglycol 20,000)	0.500 g
	Distill water	50 ml
Adjust pH to 7.5 with HCl, then add		
0.1%	BSA (bovine serium albumine)	0.050 g
0.01 M	Sodium bisulfite	0.190 g
	Diethyldithiocarbamic acid	0.050 g
	2% Tween 80 (polyoxyethlene)	1 ml
10%	DMSO (dimethyl sulfoxide)	5 ml
	2-Mercaptoethanol	0.5 ml
	PVP-36 (polyvinyl-polypyrrolidone)	4.0 g

derived from macerated tissue combined with extraction buffer solution. The saturated wicks were blotted with paper and inserted into a slit 2.5 cm from the bottom or cathodal side of the chilled gel for anodal migrating enzymes. Usually, twenty five to thirty wicks were arranged into the gel slit. Once all samples were set, the gel was squeezed gently and covered with polyethylene film. A container with ice was placed on the top of the gel to ensure good contact between wicks and gel during electrophoresis and also to keep the gel cool. In order to save electrophoretic expenses, inserting up to 50 wicks into a gel slit was also tried.

3.2.3 Electrophoretic buffers and staining systems

Three selected buffer systems were tested: Tris/citric acid, Tris/citric acid/EDTA, and L-histidine/citric acid in Table 5. Ten enzyme systems were rested in combination with all 3 buffer systems. The recipes used for the selected ten enzyme staining solutions were derived from several authors (Cardy et al. 1980, Conkle et al. 1982, and Soltis et al. 1983). The ten selected isozyme systems with their characteristics are listed in Table 6, together with staining formulae of enzyme systems, stocks, and buffer solutions. Reduced quantities of ingredients such as NAD, NADP, MTT, PMS, and substrates were tested for the selected systems.

Table 5. Selected electrode and gel buffer systems (M/L)

Type	pH		Electrode	Gel	Reference
A	7.5	Tris	0.223	0.008	Solte, D. E.
		Citric acid	0.069	0.002	et al. 1982
B	7.1	Tris	0.1	0.1	Raelson, J. V.
		Citric acid	0.001	0.003	et al. 1989
		EDTA	0.001	0.001	
C	6.5	L-histidine	0.065	0.016	Cardy et al.
		Citric acid	0.012	0.002	1981

Table 6. Tested isozymes, EC desination, characteristics and formulae of enzyme systems, stocks, and buffer solutions for staining

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ACO (Aconitase EC 4.2.1.3, monomer)

Tris HCl 0.1 M (pH = 8.0)	45 ml
$MgCl_2$ (1%)	1 ml
NADP	1 ml
Cis-aconitic acid	40 ml
Isocitric dehydrogenase	3 units
PMS	1 ml
MTT	1 ml

ADH (Alcohol dehydrogenase EC 1.1.1.1, dimer)

Tris HCl 0.1 M (pH = 8.0)	45 ml
NAD	1 ml
PMS	1 ml
MTT	1 ml
Ethanol 95%	10 ml

GDH (Glutamate dehydrogenase EC 1.4.1.3, dimer)

Tris HCl 0.1 M (pH = 8.0)	45 ml
L-glutamicc acid	1 g
NAD	1 ml
PMS	1 ml
MTT	1 ml

G6PDH (glucose-6-phosphate dehydrogenase EC 1.1.1.49, dimer)

Tris HCl 0.1 M (pH = 8.0)	45 ml
$MgCl_2$ (1%)	1 ml
D-glucose-6-phosphate	20 mg
NAD	1 ml
PMS	1 ml
MTT	1 ml

IDH (Isocitrate dehydrogenase EC 1.1.1.42, dimer)

Tris HCl 0.1 M (pH = 8.0)	45 ml
DL isocitric acid	30 mg
$MgCl_2$ (1%)	1 ml
NADP	1 ml
PMS	1 ml
MTT	1 ml

(Table 6. cont.)

MDH (malate dehydrogenase EC 1.1.1.37, dimer)

Tris HCl 0.1 M (pH = 8.0)	45 ml
DL-malic acid (pH = 7.0)	3 ml
NAD	1 ml
PMS	1 ml
MTT	1 ml

6-PGDH (6-phosphoglucinate dehydrogenase EC 1.1.1.44, dimer)

Tris HCl 0.1 M (pH = 8.0)	45 ml
6-phosphoglucinic acid	10 mg
$MgCl_2$(1%)	1 ml
NADP	1 ml
PMS	1 ml
MTT	1 ml

PGI (phosphoglucose isomerase EC 5.3.1.9, dimer)

Tris HCl 0.1 M (pH = 8.0)	45 ml
D-fructose-6-phosphate	10 mg
$MgCl_2$ (1%)	1 ml
NADP	1 ml
PMS	1 ml
MTT	1 ml
G6pdh	10 units

PGM (phosphoglucomutase EC 2.7.5.1, monomer)

Tris HCl 0.1 M (pH = 8.0)	45 ml
D-glucose-1-phosphate	70 mg
$MgCl_2$ (1%)	1 ml
NADP	1 ml
PMS	1 ml
MTT	1 ml
G6pdh	10 units

SKDH (shikimic dehydrogenase EC 1.1.1.25, monomer)

Tris HCl 0.1 M (pH = 8.0)	45 ml
Shikimic acid	100 mg
NADH	1 ml
PMS	1 ml
MTT	1 ml

(Table 6. cont.)

Stock and buffer solutions:

NAD (B-nicotinamide adenine dinucleotide)		5 mg/ml water
NADP (B-nicotinamide adenine dinucleotide phosphate)		5 mg/ml water
PMS (phenazine methosulfate)		2 mg/ml water
MTT (3-(4,5,-dimethyl thiazolyl)-2,5-diphenyl tetrazolium bromide)		4 mg/ml water
G6PDH (glucose-6-phosphate dehydrogenase)		2 units ml
0.1 M Tris HCl buffer	Trizma base	12.1 g
	hydrochloride	x ml
	Distill water	1.0 liter
	pH = 8.0	
Malic acid solution	DL-malic acid	134.1 g
	NaOH	80.0 g
	Distill water	1.0 liter
	pH = 7.0	

3.2.4 Electrophoresis and enzyme activity staining

When sample loading was complete, the gel was taken to the cold room (temperature 4°C), and set on top of two plexiglass buffer tanks with dimensions of 23 x 6.5 x 4.5 cm. The tanks were attached to a DC power source (FB 105 electrophoresis system). The power current on the gel was monitored and adjusted to a constant 50 mA. After first hour electrophoresis, the wicks were removed, and the electrophoresis was resumed for 4 to 5 hours. Finally, the gel was removed and sliced into eight slices (1.2 mm in thickness), discarding the top and bottom slices. Each slice was placed in plastic box and ready for staining. Staining solutions were prepared fresh, poured over the slice and swirled gently to eliminate air bubbles trapped underneath. Gels were incubated in the dark (37°C) and observed every 15 to 30 minutes to follow the progress of staining. When optimum resolution was observed, the gels were rinsed in deionized water and bands were recorded. After that, gels were fixed for long term storage by using a solution (5:5:1 v/v/v) of methanol, deionized water, and glacial acetic acid. The gels were wrapped with polyethylene film and refrigerated.

3.3 Data analysis

Isozyme band patterns were recorded and the standard description of these isozyme systems for K997 was established. The relative migration rate of each band was calculated following the standard description. The number of bands, phenotypes, and the frequencies with which each phenotype and band appeared in each species were calculated for analysis. Phenotypic variation of all isozymes was assessed within taxon by the estimation of phenotypic polymorphism (P_j) following Kahler et al. (1980):

$$P_j = \sum_{i=1}^{n} p_i(1-p_i) = 1 - \sum_{i=1}^{n} p_i^2,$$

where p_i is the frequency of the ith phenotype and n is the number of phenotypes observed per isozyme system and accession. The weighted average of phenotypic polymorphism (P_w) over all observed isozymes is estimated by

$$P_w = \sum_{j=1}^{k} (1/N_j)P_j \; / \; \sum_{j=1}^{k} (1/N_j)$$

where N_j is the total number of phenotypes observed jth isozyme for k isozyme systems. Adjusted average phenotypes of 25 seed samples in each species for six isozyme systems were calculated by adding average phenotypes of each isozyme system among tested accessions.

Several segregating F1 populations of *L. lanceolata* (K10) were used to test isozyme inheritance by comparing observed segregation ratios with expected ratios using chi-square analysis.

Frequencies of electromorphic phenotypes in all tested accessions of diploid and tetraploid species were compared and analyzed by using NTSYS programs (Rohlf, 1987).

Chapter 4

EXPERIMENTAL RESULTS

4.1 Procedural modifications for the genus *Leucaena*

4.1.1 Isozyme and buffer experiment

Six of the selected ten isozymes produced clear consistent isozyme bands in L-histidine buffer system (Table 7) by assaying cotyledons of *L. leucocephala* ssp. *leucocephala* (K997). These were ACO, ADH, IDH, MDH, PGI and PGM (Figure 1). All subsequent research was confined to these six isozyme systems. However, it should not be interpreted that the other isozymes could not be made to produce useful band patterns by adjusting experimental conditions.

4.1.2 Modified procedures and reduced chemical use

The results of band resolution from gels made by two gel preparation methods were compared. The band resolution from modification was the same as or even more clear than from the gel made by the normal procedures. Normally, the gel solidification was only one hour at laboratory temperature (25°C) before putting gel into cold room or refrigerator (4°C) for hardening. The modified procedures extended solidification period to 12 hours at laboratory temperature.

The use of only one-half chemical content in staining

Table 7. Quality* of band in phenotypes for 10 isozyme systems using three buffer systems for the "common" type *L. leucocephala* ssp. *leucocephala* (K997)

Isozyme systems	Buffer systems**		
	A	B	C
ACO	2	1	3
ADH	1	1	3
Gdh	-	-	-
G6PDH	1	1	2
IDH	1	1	3
MDH	2	2	2
6-PGDH	1	1	1
PGI	2	3	3
PGM	3	2	3
SKDH	1	1	2

* "-" no staining band, "1" not clear band,
"2" good band not consiste "3" clear band consistently.

** A. Tris/citrate, B. Tris/citrate/EDTA
C. L-histidine/citrate

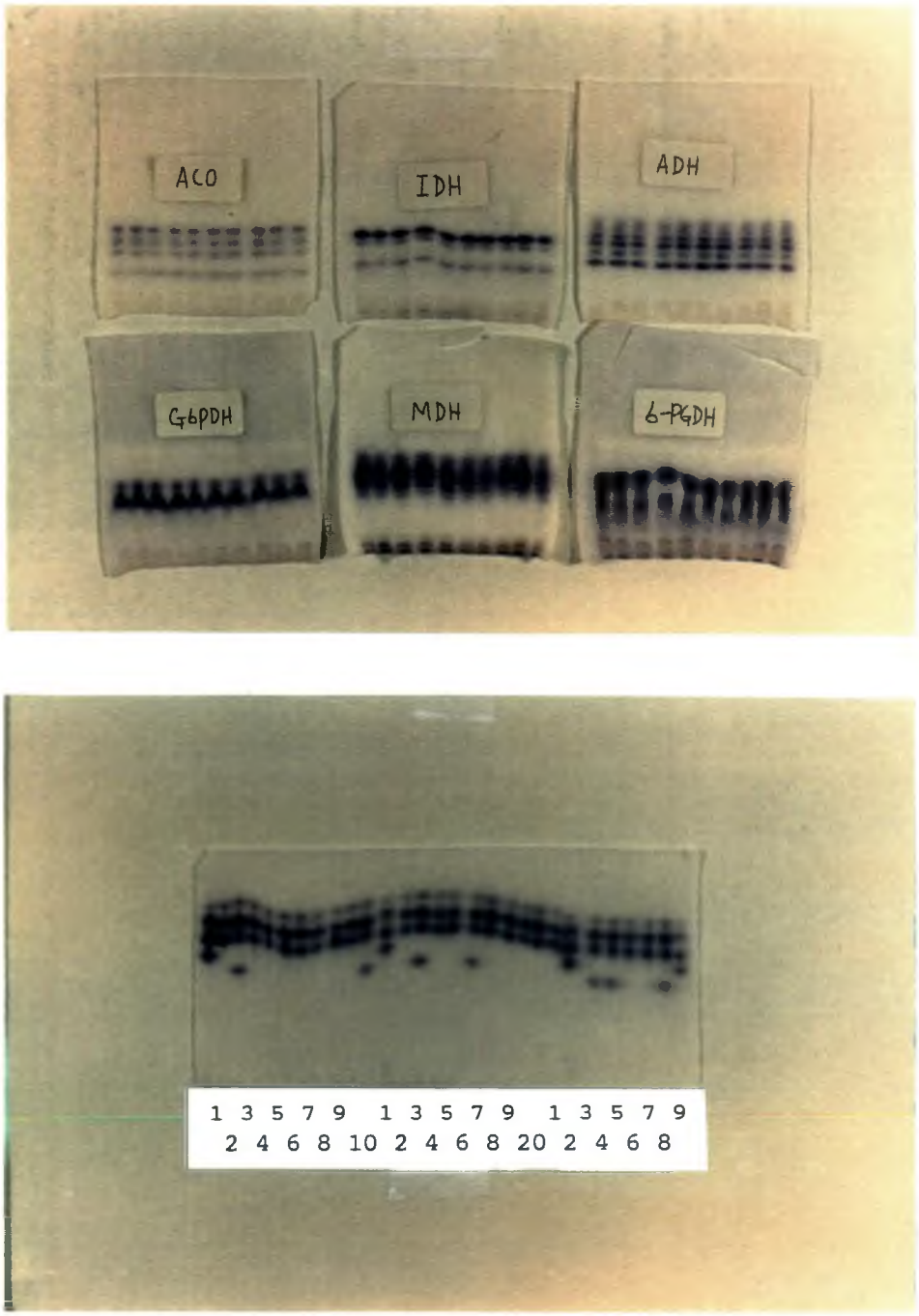


Figure 1. Banding patterns of **ACO, ADH, IDH, MDH, G6PDH,** and **6-PGDH** in *L. leucocephala* ssp. *leuccocephala* (K997, top), **PGM** in *L. diversifolia* ssp. *trichandra* (K483, bottom), and CK=K997 (line 1, 12, 23, and 29)

solutions (Cardy et al. 1980, Conkle et al. 1982 and Soltis et al. 1983) was tried. It was practical for these selected six isozyme systems if the gel was sliced only 1.2 mm in thickness. Putting more samples (up to 50 samples) into a gel set was also tried, and resolution of these isozyme bands was still clear and recordable.

4.1.3 Standard description of phenotypes for six isozymes

Cotyledons of one-week-old seedlings of K997 were used for the initial isozyme survey. No variation of band patterns for six isozyme systems (ACO, ADH, IDH, MDH, PGM and PGI) was found in K997 population (Figure 1). Standard marker bands were based on these studies. Four bands were found for ACO in K997. The fast moving band of ACO in K997 was designated as 100. The positions of slower bands were designated as percents of the fast band. In this way, the other three slow bands of ACO in K997 were 87, 73, and 56 (Figure 2). In the same manner, seven bands of ADH in K997 were designated 100, 93, 87, 80, 73, 67 and 56 (Figure 2). The three bands of IDH in K997 were 100, 90 and 69. The eight bands of MDH were 100, 93, 86, 80, 73, 66, 59 and 53. The four bands of PGM were 100, 93, 87 and 77, and the four bands of PGI were 100, 93, 85 and 76. In all subsequent surveys of other *Leucaena* species, band positions were described relative to the fastest moving band of K997 in

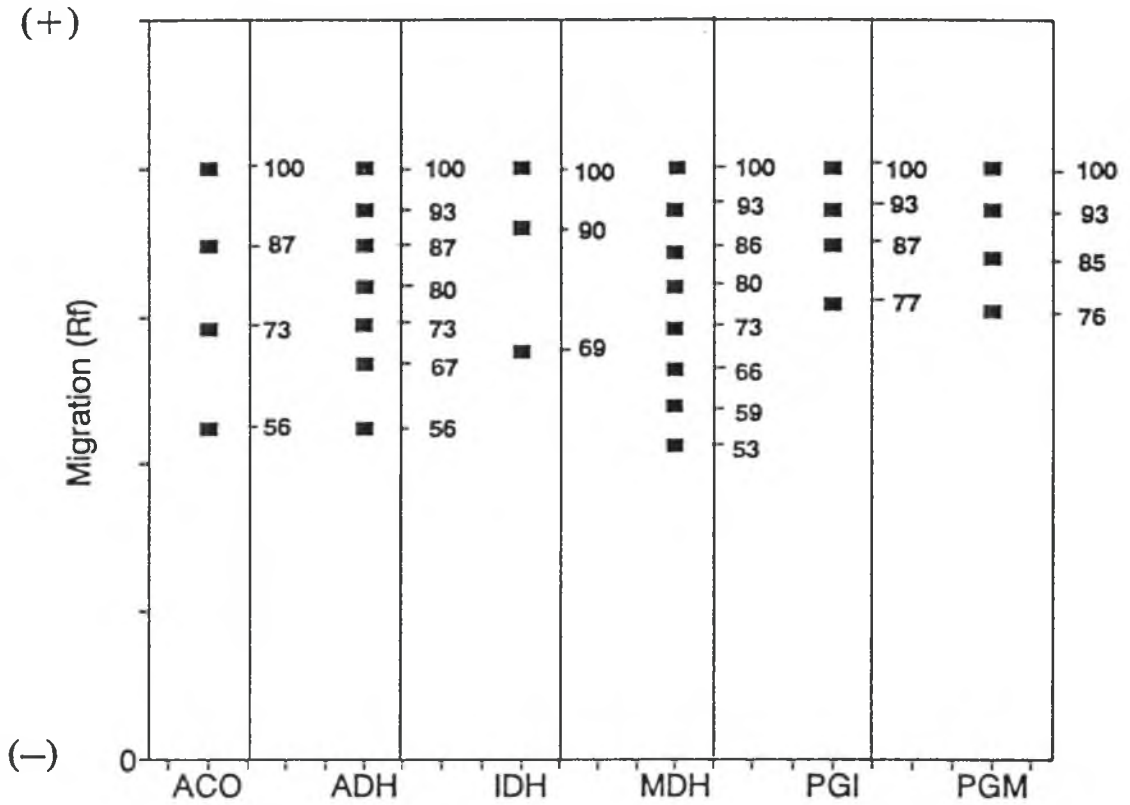


Figure 2. Schematic phenotypes of six enzyme systems in *L. leucocephala* ssp. *leucocephala* (K997)

each isozyme system as 100.

A standard description of isozymic bands is essential for electrophoretic analysis. K997 is a local Hawaiian "common" type of *Leucaena* and is highly homogeneous, a single self-pollinated variety. It was selected in our study as a control for all subsequent studies because it was highly uniform in cotyledon tissue for these isozyme systems.

4.2 Ontogenetic variation

4.2.1 Plant heights

Ontogenetic variations were observed in three selected *Leucaena* species--*L. diversifolia* ssp. *diversifolia* (2n=104), *L. diversifolia* ssp. *trichandra* (2n=52) and *L. pulverulenta* (2n=56). Seeds were sown in dibble tubes with soilless media in Dec. 1991 at Waimanalo greenhouse. Plant heights were measured before each isozymic analysis. *L. diversifolia* ssp. *trichandra* (2n=52) grew significantly faster than *L. diversifolia* ssp. *diversifolia* (2n=104) and *L. pulverulenta* after seedlings were 60 days old (Table 8).

4.2.2 Band patterns in different plant tissues

Five seedling tissues of three *Leucaena* species were tested for band pattern variations. None wwre observed in the different plant tissues of 20 day old seedlings for ACO, ADH, IDH, MDH, PGI and PGM (Figure 3).

Another type of variation was observed, however, in association with ontogenetic change. This type of variation was the intensity of staining in the different tissues (Figure 3). Bands of ACO, IDH, MDH, PGI and PGM in the tested species were less intense in roots and hypocotyl tissues compared with shoots and cotyledons of 20-day-old seedlings. Bands of ACO, IDH, MDH, PGI and PGM were more intense in shoots compared with fully open leaf from two- and six-month-old plants in the three tested species.

Table 8. Average heights of the three tested species in different stages

Species	K No.	Average Heights (cm)		
		15	60 (days)	180
L. diversifolia ssp. *trichandra*	K927	3	10	60 a
L. diversifolia ssp. *diversifolia*	K946	3	8	30 b
	K947	3	7	29 b
L. pulverulenta	K957	2.5	7	15 c
	K958	2.5	7	15 c

* Different letters show significant difference at P = 0.05.

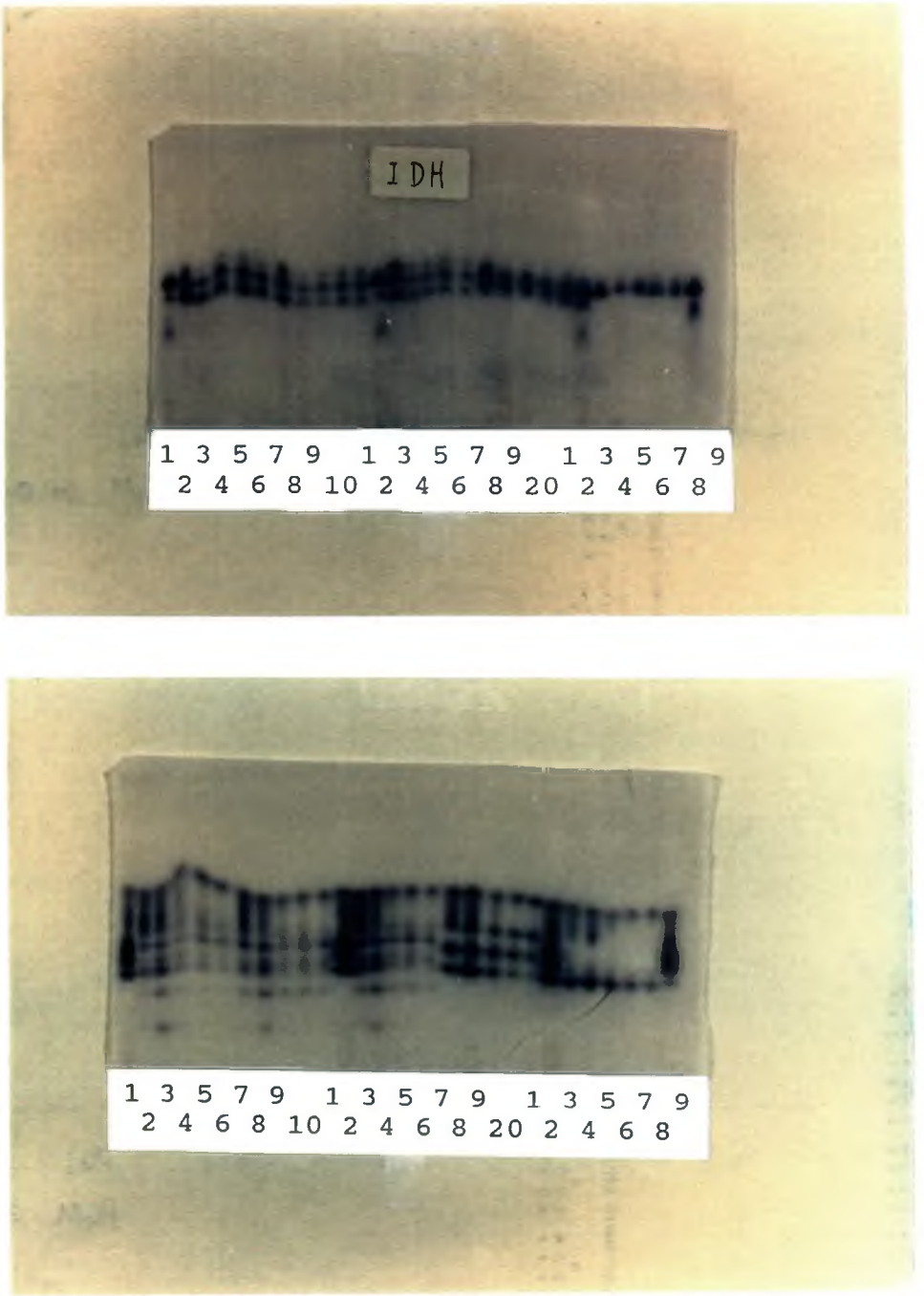


Figure 3. Phenotypes of **IDH** (top) and **PGI** (bottom) in shoots (line 2, 7, 13, 18 and 24), cotyledon (line 3, 8, 14, 19 and 25), hypocotyl (line 4, 9, 15, 20 and 26), up-root (line 5, 10, 16, 21 and 27) and root tips (line 6, 11, 17, 22 and 28) of K946 (line 2-6), K947 (line 7-11, 13-17) and K958 (line 18-22, 24-28), CK=K997 (line 1, 12, 23, and 29)

4.2.3 Band patterns in different plant stages

Seedlings ranging up to six-months in age of these three *Leucaena* species were assayed for the band pattern variation of six isozyme systems. Variations were not observed due to the age of plant for ACO, IDH, MDH, PGI and PGM. The only band pattern variation observed was that of ADH. ADH band pattern was changed in 20-day-old seedlings in all three tested species, and activity disappeared by 60 days.

Another type of variation observed in association with plant age was the intensity of bands. IDH bands were intense in the young shoot tissue of 60- and 180-day-old trees compared with the shoots of 20-day-old seedlings.

4.3 Isozymic variation among species and identification of polymorphic loci

In this study, 169 accessions from twelve *Leucaena* species were analyzed for six isozyme systems. Phenotypic variations, polymorphism, and specific bands for these *Leucaena* species were described. The species showing the highest phenotypic polymorphism (P_j and P_w) was *L. diversifolia* ssp. *trichandra* (section 4.3.3), while *L. leucocephala* ssp. *leucocephala* and *L. leucocephala* ssp. *glabrata* (section 4.4.2) showed the least phenotypic polymorphism. Phenotypes and their frequencies of *L. collinsii* and *L. diversifolia* ssp. *trichandra* are presented below. Finally, genetics of ACO and IDH variation in *L. lanceolata* (K10) is interpreted.

4.3.1 Phenotypic variation among species

4.3.1.1 Phenotypic variations in six isozyme systems

The mobilities of electromorphs in the twelve *Leucaena* species were described relative to the fastest migrating (most anodic) bands in the six isozyme systems of *L. leucocephala* ssp. *leucocephala*, K997, which were always set at a standard Rf 100. A total of 106 different electromorphs were found in the genus. The Rf value of different electromorphs, and the frequencies of each band observed in the tested *Leucaena* species are given in Table 9. It is clear that there was considerable variability

Table 9. Frequencies of different electromorphs in six isozyme systems observed in twelve Leucaena species

Isozyme systems	Bands	Rf	Leucaena species													
			COL	DIV4	DIV2	ESC	GRE	LAN	LEUc	LEUg	MAC	PAL	PUL	RET	SHAN	TRI
ACO	1	106	0	0	0.02	0.03	0	0	0	0	0	0	0	0	0.21	0.96
	2	103	0.27	0.01	0.50	0.39	0	0.07	0	0	0.06	0	0.13	0.55	0.01	0
	3	100	0.75	0.99	0.83	0	0.98	0.83	1	1	0.30	1	0.60	0.78	0.72	0
	4	97	0.18	0.03	0.14	0.73	0.98	0.15	0	0	0.21	0	0.59	0	0	0
	5	94	0	0	0	0.22	0	0.44	0	0	0.25	0	0.19	0.86	0.88	0.40
	6	90	0	0.99	0.46	0.75	0	0	0	0.26	0	1	0.32	0	0	0
	7	87	0.90	0.98	0.72	0.18	0	0.98	1	1	0.87	0	0.19	0.70	0.61	0.72
	8	84	0	0.99	0	0.71	1	0.49	0	0	0.25	0	0	0	0	0
	9	80	0.39	0	0.14	0	0	0.30	0.02	0.99	0.59	0.93	0.83	0.56	0.51	0.96
	10	73	0.70	0	0.68	0.33	0.98	0.46	1	1	0.47	0.68	0.97	0.25	0.39	0
	11	69	0	0	0	0.72	0	0	0	0	0	0.89	0	0.14	0.35	1
	12	66	0.66	0.10	0.17	0	0	0.08	0	0	0	0	0.2	0	0.19	0
	13	64	0	1	0.09	0	0	0.07	0	0	0.48	0	0	0.74	0	0
	14	56	0.01	0	0.12	0	0	0.44	1	1	0.06	0	0.13	0.26	0.41	0
	15	50	0	0	0	0	0	0.29	0	0	0	0	0	0	0	0
ADH	16	100	0	1	0	0	0.81	0	0.99	1	0	0	1	0	0.02	0
	17	93	0.05	1	0	0	1	0	0.99	1	0	0	1	0.14	0	0
	18	89	0	1	0.17	0	0	0	0	0	0	0	0	0	0	0
	19	87	0.46	0	0.01	0	1	1	0.99	1	1	1	1	0.86	0.61	0
	20	85	0	1	0.83	0.76	0	0	0	0	0	0	0	0	0	0
	21	80	0.8	1	0.97	0	1	1	0.99	1	0.25	1	1	0.01	1	0.88
	22	78	0	0	0	0.95	0	0	0	0	0	0	0	0	0	0
	23	73	0.99	0	0.09	0.59	1	1	1	1	1	1	1	1	0.99	1
	24	71	0	1	0.83	0.14	0	0	0	0	0	0	0	0	0	0
	25	67	0.58	0	0.06	0.82	1	0	1	1	0.72	1	0.87	0.05	0.80	1
	26	65	0	0.42	0.25	0	0	0	0	0	0	0	0	0	0	0
	27	62	0	0	0	0.20	0	0	0	0	0	0	0	0	0.20	0
	28	60	0.70	0	0.08	0	0	0.07	0	0	0	0.09	0	0	0.11	0
	29	56	0.26	0.42	0.31	0.77	1	1	1	1	1	0	1	0.86	0.98	1
	30	49	0.10	0	0	0	0	0	0	0	0	0	0	0	0.07	0
	31	42	0.10	0	0	0	0	0	0	0	0	0	0	0.14	0.16	0
IDH	32	116	0	0	0	0	0	0	0	0	0	0	0	0	0.098	0
	33	112	0	0	0	0	0	0	0	0	0	0	0	0	0.869	0.88
	34	110	0	0.49	0	0	0	0	0	0	0	1	0	0	0.107	0
	35	108	0	0	0.78	0	0	0	0	0	1	0	0	0	0.033	0
	36	105	0	0	0	0.22	0	0	0	0	0	0.307	0	0	0	0
	37	104	0.99	0	0.16	0	0	0	0	0	0.25	0	0	0	0	0
	38	103	0	0	0	0	1	0	0	0	0	0	0.32	0.954	0.098	0
	39	100	0.04	0.57	0.8	0.56	0	0.07	1	0.96	1	1	0.653	0	0.664	0
	40	98	0	0	0	0	0	0	0	0	0	0	0	0	0.033	0
	41	95	0	0	0.19	0.56	1	0.26	0.07	0.54	0.18	0.307	0.747	0.908	0	0.12

(Table 9. cont.)

Isozymes systems	Bands		Rf	Leucaena species												
				COL	DIV4	DIV2	ESC	GRE	LAN	LEUc	LEUg	MAC	PAL	PUL	RET	SHAN	TRI
IDH	42	11	93	0	0.02	0	0	0	0.34	0	0	0	0	0	0	0	1
	43	12	90	0.96	1	0.37	1	0	1	0.99	0.97	0.93	1	0.41	0	0.20	0
	44	13	87	0	0	0	0	1	0.21	0	0	0	0	0.25	0.70	0.80	0
	45	14	85	0.28	0	0.78	0.70	0	0	0	0	0	0	0.01	0	0	0
	46	15	80	0	0	0	0.52	0	0	0	0	0	0	0	0.01	0	0
	47	16	78	0	0.14	0.24	0	0	0	0	0	0	0	0	0	0	0
	48	17	74	0.20	0	0	0	0	0	0	0	0	0	0	0	0	1
	49	18	69	0.04	0.43	0.12	0	0	0.03	0.94	0.54	0.02	0.33	0	0	0	0
	50	19	62	0	0	0	0	0	0	0	0	0	0	0	0	0.20	0
	51	20	51	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	52	21	47	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0
	53	22	33	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0
MDH	54	1	107	0	0	0	0	0	0	0	0	1	0	0	0	1	1
	55	2	100	1	1	0	1	1	0.53	1	1	1	1	1	1	0	0
	56	3	96	0	0	0.85	0	0	0	0	0	0.13	0	0	0	0	0
	57	4	93	1	0	0.20	1	1	0	1	1	1	1	1	1	0.20	0
	58	5	90	0	0	0	0	0	0	0	0	0	0	0	0	0.80	1
	59	6	86	1	1	0.91	1	1	0.77	1	1	0	1	1	1	1	1
	60	7	80	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	61	8	73	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	62	9	66	1	1	1	1	1	1	1	1	0.87	1	1	1	1	1
	63	10	59	1	1	0.09	0	1	0.47	1	1	0.25	1	1	1	1	0.92
	64	11	53	1	1	0.09	0	0.72	0.47	1	1	0.05	1	0	0	0.32	0.92
	65	12	47	0.03	0	0.15	0	0	0	0	0	0	0	1	1	0	0.08
	66	13	25	0	0	0	0	0.28	0	0	0	0	0	0	0	0	0
PGI	67	1	123	0	0	0	0.14	0	0	0	0	0	1	0	0	0	0
	68	2	111	0	0	0	0	0	0	0	0	0	1	0.33	0	0.13	0
	69	3	108	0	0	0	1	0	0	0	0	0	0	0	0	0.11	0
	70	4	100	1	0	0.8	0.48	1	0.50	1	1	0.82	1	0	1	0.69	0
	71	5	93	0	1	0.26	1	0	0.51	1	1	0	0	0.64	0	0.39	1
	72	6	87	0.98	0	0.58	0.27	0	0.83	1	1	0.82	1	0.31	0	0.58	1
	73	7	81	0	0	0	0	0.93	0.09	0	0	0	0	0	0	0.03	0
	74	8	79	0	0.97	0	0	0	0	0	0	0	0	0	0	0	0
	75	9	77	0.88	0.94	0.63	0.65	0.95	0.72	1	1	1	0.31	1	0	0.97	0
	76	10	75	0	0.03	0	0	0	0	0	0	0	0	0	0.29	0	0
	77	11	71	0	0	0	0	0.31	0	0	0	0	0	0	0.57	0.05	0
	78	12	69	0.10	0	0.71	0	0	0.35	0.07	0.65	0	0.31	0	0	0.24	0
	79	13	66	0	0	0	0	0	0	0	0	0	0	0	0.45	0	0
	80	14	61	0.02	0.95	0.26	0	0	0.21	0	0	0	0.31	0	0	0.40	0
	81	15	53	0	0	0.61	0	0	0	0	0	0	0	0	0.37	0.06	0
	82	16	45	0	0	0	0	0	0	0	0	0	0	0	0.08	0.07	0
	83	17	34	0	1	0.09	0	0.16	0	0	0	0	0	0	0.08	0	0

(Table 9. cont.)

Isozyme systems	Bands	Rf	Leucaena species												
			COL	DIV4	DIV2	ESC	GRE	LAN	LEUc	LEUg	MAC	PAL	PUL	RET	SHAN	TRI
PGI	84	18 30	0	0	0	0	0	0	0	0	0	0	0	0.73	0	0
	85	19 25	0	0	0.13	0	0.72	0	0	0	0	0	0	0.24	0	0
	86	20 15	0	0	0	0	0.96	0	0	0	0	0	0	0	0	0
	87	21 5	0	0	0	0	0	0	0	0	0	0	0	0.40	0	0
PGM	88	1 112	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0
	89	2 106	0	0	0	0	0	0.07	0.03	0.34	0.29	0	0	0	0.37	0
	90	3 103	0.02	0	0.20	0	0.20	0.14	0	0	0	0	0	0	0.16	0
	91	4 100	0.78	0.86	0.65	0.35	0	0.78	0.98	0.97	0.61	0.15	0	0	0.63	1
	92	5 96	0	0	0	0.49	0.45	0	0	0	0.15	0.99	0	0.84	0	0
	93	6 93	1	0.98	1	0	0	0.97	1	1	0.96	0	0.28	0	0.85	1
	94	7 90	0	0	0	0.29	0.91	0	0	0	0	1	0	0.29	0.85	0
	95	8 85	1	0.71	0.71	0.14	0	1	1	1	0.78	0	1	0	0.37	0
	96	9 83	0	0	0	0.75	0	0	0	0	0	1	0	0	0.15	0
	97	10 80	0	0	0	0	0	0	0	0	0	0	0	0.97	0.15	0
	98	11 76	0.04	0.98	0.10	1	0	0.19	1	1	0.70	0.76	1	0	0.44	0.56
	99	12 71	0	0	0	0	0	0.15	0	0	0	0	0	0.61	0	0
	100	13 69	0	0.02	0	0.24	0.96	0	0	0.03	0.42	0.61	0	0	0	0
	101	14 67	0.04	0	0.18	0	0	0.31	0	0	0	0	0.56	0.03	0	0.8
	102	15 61	0.16	0	0	0	0.55	0	0	0	0.04	0	0	0.14	0	0
	103	16 54	0	0	0	0	0.28	0	0	0	0	0	0	0	0	0
	104	17 47	0	0	0	0	0.12	0	0	0	0	0	0	1	0	0
	105	18 44	0	0	0	0.16	0	0	0	0	0	0	0	0	0	0
	106	19 40	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0

among species. Every species showed unique bands in the six isozyme systems, although the frequency of unique bands was not very high in most cases.

The total numbers of observed isozymic phenotypes of each isozyme system in each species are summarized in Table 10. ACO and PGM showed more phenotypes than the other isozyme systems, averaging 16.1 and 13.2. ADH and MDH showed few phenotypes, averaging 4.6 and 2.0. Due to the different number of accessions tested in each species, the total number of observed phenotypes in each species could not be compared directly. However, the average number of phenotypes in these isozyme systems when adjusted for a sample size of 25 individuals showed that fewer phenotypes were found among self-pollinating species DIV4, LEUc and LEUg. It clearly indicated that selfing species showed less isozymic variation.

The total numbers of different electromorphs (different bands) observed in each isozyme system in each species are summarized in Table 11. ACO and MDH showed the most different bands, averaging 8.1 and 7.9 respectively. Among twelve species, SHAN and DIV2 showed the most electromorphs of six isozymes (56 and 55, respectively, vs. an average of 41.6).

The numbers of bands per phenotype in six isozyme systems varied from 1 to 9 (Table 12). Number of phenotypes observed in six isozyme systems and frequencies of

Table 10. Observed number of phenotypes in each isozyme system in twelve *Leucaena* species

Species	No. of accs.	No. of plants	Isozymes						Adjusted average phenotypes*
			ACO	ADH	IDH	MDH	PGI	PGM	
COL	6	125	20	14	10	2	6	11	22.60
DIV4	7	175	6	2	5	1	4	6	8.57
DIV2	8	180	41	9	24	4	25	18	23.75
ESC	5	79	16	12	15	1	8	20	29.11
GRE	5	103	4	2	1	2	9	24	19.43
LAN	5	107	24	2	7	3	14	16	23.83
LEUc	77	650	2	1	3	1	2	3	6.49
LEUg	36	360	3	1	4	1	2	4	8.11
MAC.	4	100	14	3	4	4	2	21	16.50
PAL	3	75	6	2	4	1	2	6	13.00
PUL	3	75	20	2	8	1	4	3	15.33
RET	4	87	31	4	9	1	9	14	24.43
SHAN	5	122	31	9	5	3	15	36	23.77
TRI	1	25	8	2	2	3	1	3	19.00
Total	169	2263	226	65	101	28	103	185	
Average			16.14	4.64	7.21	2.00	7.36	13.21	18.14

* Adjusted average number of phenotypes in all systems, based on 25 samples per accession

Table 11. Observed number of bands of each isozyme system in twelve *Leucaena* species

Species	No. of accs.	No. of plants	Isozymes						Total
			ACO	ADH	IDH	MDH	PGI	PGM	
COL	6	125	8	9	9	6	5	7	44
DIV4	7	175	8	8	6	7	6	5	40
DIV2	8	180	12	10	8	9	10	6	55
ESC	5	79	9	7	6	6	6	8	42
GRE	5	103	4	7	3	9	7	7	37
LAN	5	107	12	6	6	7	7	8	46
LEUc	77	650	4	7	4	8	5	5	33
LEUg	36	360	7	7	4	8	5	6	37
MAC.	4	100	10	5	6	9	3	8	41
PAL	3	75	5	5	6	8	7	6	37
PUL	3	75	10	7	6	8	4	4	39
RET	4	87	9	7	7	8	9	8	48
SHAN	5	122	10	9	8	9	10	10	56
TRI	1	25	5	4	4	9	2	4	28
Total	169	2263	113	98	83	111	86	92	
Average			8.07	7.00	5.93	7.93	6.14	6.57	41.64

Table 12. Observed range in number of isozyme bands per phenotype in six isozyme systems in twelve *Leucaena* species

Species	No. of Accs.	No. of plants	Isozymes					
			ACO	ADH	IDH	MDH	PGI	PGM
COL	6	125	3-6	3-6	2-6	5-6	2-5	2-5
DIV4	7	175	3-6	6-8	2-4	7	4-5	2-5
DIV2	8	180	3-6	3-7	1-6	5-6	3-7	1-5
ESC	5	79	3-6	3-6	1-6	6	2-5	2-6
GRE	5	103	3-4	6-7	3	8-9	1-6	1-7
LAN	5	107	3-6	5-6	1-3	5-7	1-5	3-5
LEUc	77	650	4	7	3	8	4-5	4-5
LEUg	37	370	4-6	7	3	8	4-5	4-5
MAC	4	100	3-6	3-4	3-4	7-8	1-3	1-6
PAL	3	75	3-5	4-5	3-6	8	4-7	3-6
PUL	3	75	2-5	6-7	1-3	8	1-3	2-4
RET	4	87	3-6	3-4	1-4	8	3-7	2-6
SHAN	5	122	2-6	4-7	3	7-8	2-7	3-6
TRI	1	25	3-5	3-4	3	6-9	2	3-4

phenotypes in each tested *Leucaena* species are summarized in Appendix B. Based on phenotypes, and assumptions about the number of polymerism of active isozyme molecules, it is estimated that the following numbers of loci, respectively, govern the phenotypes for the five isozymes: ACO, 3; ADH, 3; IDH, 1-2; PGI, 1-2; PGM, 3.

4.3.1.2 Statistics of phenotypic polymorphism

One hundred sixty-nine accessions of twelve *Leucaena* species (excluding *L. salvadorensis*, *L. multicapitula* and *L. sp.*) were tested in this study for the six isozyme systems. Isozymic polymorphisms were observed in all twelve species. Only accessions in the self-pollinating species *L. leucocephala* and *L. diversifolia* ssp. *diversifolia* accessions did not show polymorphism. Seventy-five percent of tested *L. leucocephala* ssp. *leucocephala* did not show polymorphism in these isozyme systems. Twenty-five percent of tested *L. leucocephala* ssp. *glabrata* did not show polymorphism either. One of the seven tested *L. diversifolia* ssp. *diversifolia*, K802, did not show polymorphism either.

Almost all tested accessions from outcrossing species showed polymorphisms in these isozyme systems, and they are presumed to be heritable. Genetic interpretation of polymorphisms could not be made criticaally, however, without progeny testing. The magnitude of variation in

isozyme phenotypes was expressed by the estimation of phenotypic polymorphism (P_j) and weighted polymorphism (P_w) of Kahler et al. (1980). A summary of the average P_j and weighted P_w of six isozymes for tested accessions is provided in Table 13. The degree of polymorphism varied greatly between different isozyme systems. The average P_js of ACO and PGM were the highest. The average P_js of PGI and IDH were also high in comparison with that of ADH. The average P_j of MDH was the lowest.

The lowest levels of phenotypic polymorphism (P_j and P_w) were for self-pollinating species, *L. diversifolia* ssp. *diversifolia* and the two subspecies of *L. leucocephala*. The "common" *L. leucocephala* showed the lowest P_w (0.01). All outcrossing species showed very high levels of polymorphism. *L. diversifolia* ssp. *trichandra*, *L. trichodes*, and *L. collinsii* showed highest P_ws, 0.28, 0.25 and 0.23 respectively. The average weighted P_w across all species was 0.15.

4.3.1.3 Specific bands for identifying species

All isozyme systems showed considerable variability in the genus, and each species showed unique bands and phenotypes. Unique bands that could be used to identify individual species are summarized in Table 14. ACO, IDH, and MDH were found useful to be able to identify eight species. If IDH and MDH were combined with ADH and PGI, the

Table 13. Averages and the weighted polymorphism (P_j and P_w) of six isozyme systems in *Leucaena* species*

Species	No. of accs.	No. of plants	Isozymes						AVG	Weighted values
			ACO	ADH	IDH	MDH	PGI	PGM	P_j	P_w
COL	6	125	0.55	0.32	0.44	0.07	0.33	0.45	0.34	0.23
DIV2	8	180	0.67	0.35	0.48	0.07	0.54	0.63	0.47	0.28
DIV4	7	175	0.17	0.14	0.16	0.00	0.14	0.11	0.12	0.08
ESC	4	79	0.49	0.53	0.51	0.00	0.49	0.55	0.43	0.14
GRE	5	103	0.10	0.19	0.00	0.28	0.48	0.74	0.30	0.18
LAN	5	107	0.69	0.09	0.34	0.00	0.60	0.57	0.38	0.17
LEUc	77	650	0.03	0.00	0.04	0.00	0.05	0.02	0.02	0.01
LEUg	36	360	0.05	0.00	0.23	0.00	0.19	0.23	0.12	0.07
MAC	4	100	0.50	0.05	0.26	0.20	0.10	0.69	0.30	0.17
PAL	3	75	0.48	0.13	0.32	0.00	0.05	0.50	0.25	0.12
PUL	3	75	0.72	0.16	0.38	0.00	0.14	0.23	0.27	0.12
RET	4	87	0.76	0.08	0.40	0.00	0.42	0.62	0.38	0.12
SHAN	5	122	0.56	0.26	0.16	0.00	0.59	0.78	0.39	0.18
TRI	1	25	0.77	0.21	0.21	0.56	0.00	0.64	0.40	0.25
Average			0.47	0.18	0.28	0.09	0.30	0.48	0.30	0.15

* See the text for a description of models to calculate P_j and P_w

Table 14. Specific bands (Rf) identified in *Leucaena* species

Species	Isozymes				
	ACO	ADH	IDH	MDH	PGI
COL			51		
DIV2				96	
DIV4	90,87,84				
ESC				59*,53*	
GRE			103,95,87		15
LAN		100*,93*		93*,90*	100
LEUc	100,87.73 56				
LEUg	100,87,80 73,56				
MAC				106,86*	
PAL	90	100*,93* 56*			
PUL		100		53*,47	
RET		100*		53*,47	
SHAN				106,86	
TRI			93,74		

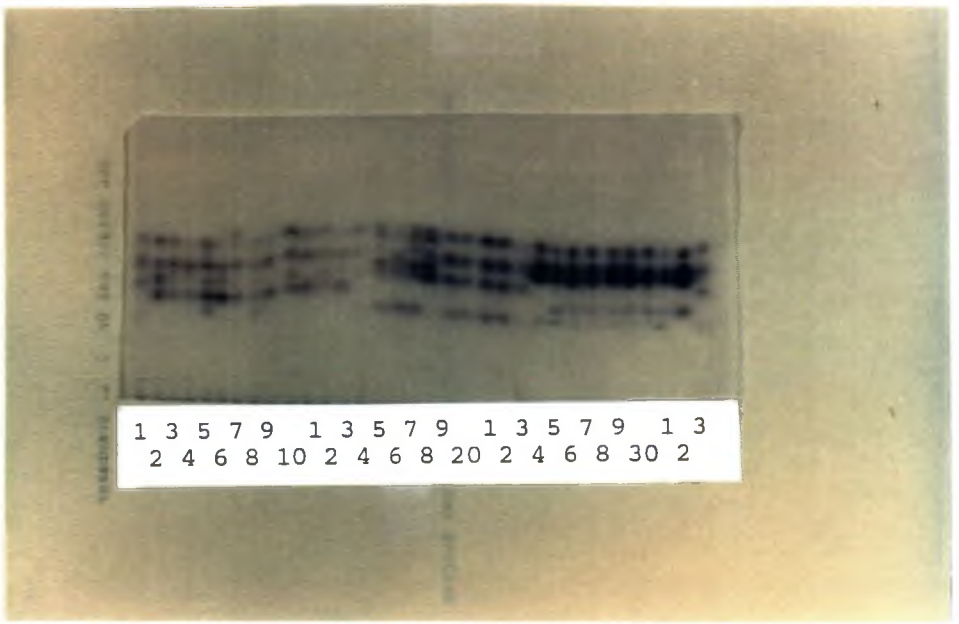
* band missing in the indicated isozyme systems

other species also could be identified by the presence or absence of specific isozyme bands.

Examples of species-specific bands include three close ACO bands of Rf 90, 87 and 84 of *L. diversifolia* ssp. *diversifolia* that were absent in all other species. The absence of MDH bands of Rf 59 and 53 was unique in *L. esculenta*. The combination of the presence of band Rf 90 in ACO and the absence of bands Rf 100 and 93 in ADH was characteristic of *L. pallida*. Specific bands could also be used to identify the species, subspecies, and interspecific hybrids in the seedling stages, notably for the subspecies like the "common" and "giant" *L. leucocephala*, and diploid and tetraploid *L. diversifolia*.

4.3.1.4 Characterizing interspecific hybrids

F1 hybrid seeds from *L. leucocephala* ssp. *glabrata* (K636) and *L. diversifolia* ssp. *diversifolia* (K156), *L. salvadorensis* (K746) and *L. retusa* (K502), *L. retusa* (K502) and *L. shannonii* (K769) and parent tree shoots were tested for the characterization of F1 hybrids. Phenotypes of IDH, PGI and ACO in F1 seedling cotyledons clearly showed differences compared to the two parents (Figure 4). Two types of differences were observed. In the first, the F1 combinations (e.g., K636 X K156) showed both parental bands (e.g., ACO Rf 56 and 64) as a probable result of co-dominant alleles. The second tyoe of difference, consisting of the



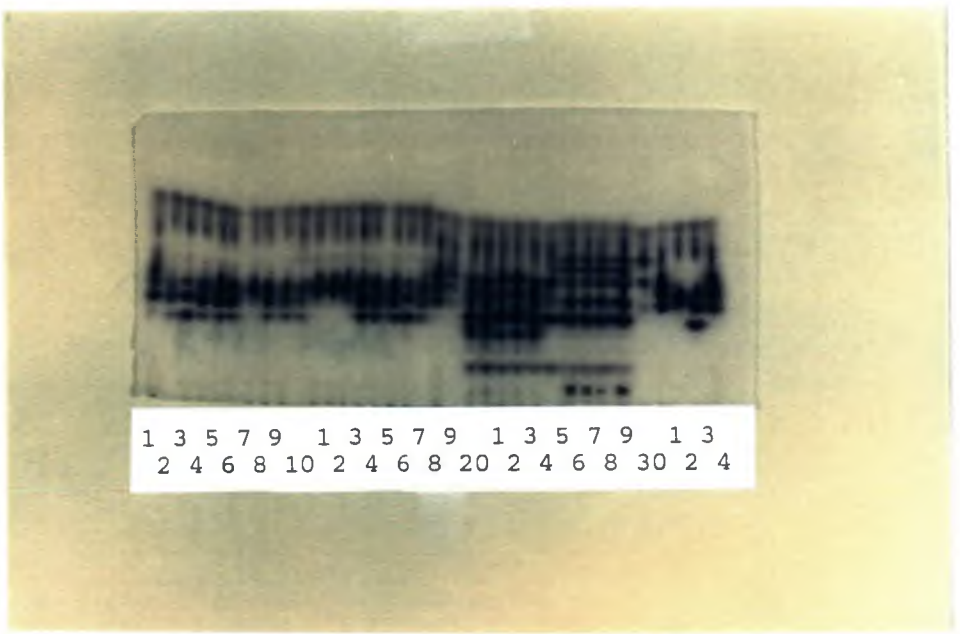


Figure 4. Banding patterns of **ACO** (top) in K636 (line 24), K156 (line 32) and their F1 progenies (line 25-32), CK=K997 (line 1, 12, 23 and 33)

Figure 5. Banding patterns of **PGI** (bottom) in K746 (line 20), K502 (line 26), K769 (line 31) and their F1 progenies (K746 X K502, line 21-25, K502 X K769, line 27-30), CK=K997 (line 1, 19 and 34)

creation of new bands which were not present in either two parents, (e.g., F1 of K746 X K502 and of K502 X K769) was probably the result of heterozygosity involving polymeric isozymes (Figure 5).

4.3.2 Phenotypic variations of isozymes in *L. collinsii*

4.3.2.1 Phenotypic variations in six isozymes

Six *L. collinsii* accessions were surveyed, five from Guatemala (latitude 14.36' to 14.56'N and longitude 89.31' to 90.47'W) and one from Mexico. These were quite diverse isozymically, and results of the separate analysis are disccussed below.

ACO. Isozyme phenotypes of ACO in six tested *L. collinsii* accessions showed a high degree of intraspecific variability. Seven different bands (with Rf values from 56 to 103), and twenty distinct phenotypes were found (Figure 6). Phenotypes had from three to six bands. The most common phenotypes were Types 1, 2, and 3, which comprised more than 50 percent of phenotypes observed (Figure 6 and Table 15). Although the phenotypes were quite complex, it was concluded that three loci controlled these polymorphism. The putative Aco1 locus included three different bands 103, 100 and 97. Aco2 included three bands 87, 80 and 73. Aco3 also included three bands 73, 66 and 56 (Figure 6). All three loci showed polymorphism in their isozymic expression.

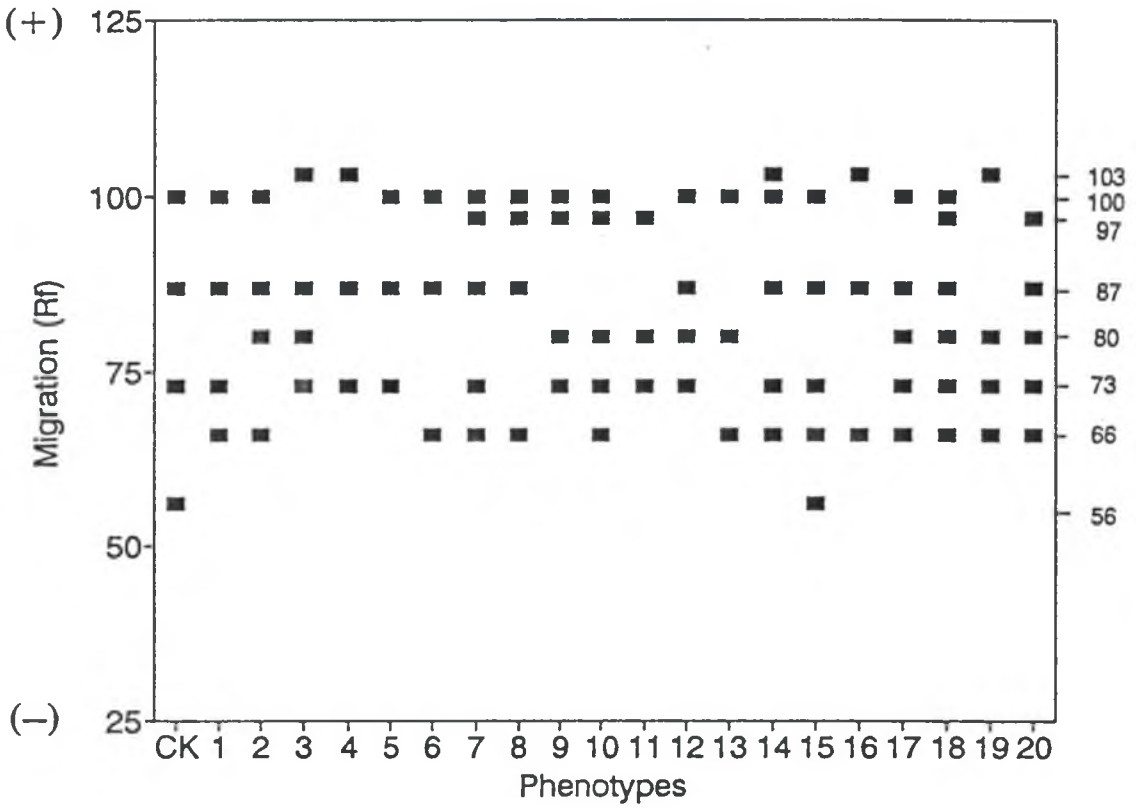


Figure 6. Schematic phenotypes of ACO in *L. collinsii*, CK = K997

Table 15. Frequencies of six isozyme phenotypes in *L. collinsii*

Phenotypes	Accessions						Total	Frequency %
	K905	K911	K912	K913	K914	K917		
ACO								
1	19	3	5				27	21.60
2		17				9	26	20.80
3				15			15	12.00
4				10			10	8.00
5	4				2	3	9	7.20
6		1					1	0.80
7					5		5	4.00
8		1			4		5	4.00
9					4		4	3.20
10					3		3	2.40
11					3		3	2.40
12	1				1		2	1.60
13		2					2	1.60
14	1	1	5				7	5.60
15			1				1	0.80
16			1				1	0.80
17					1		1	0.80
18					1		1	0.80
19					1		1	0.80
20					1		1	0.80
ADH								
1					26	12	38	30.40
2	24			8			32	25.60
3				17			17	13.60
4		11	4				15	12.00
5		5					5	4.00
6			5				5	4.00
7		4					4	3.20
8			3				3	2.40
9	1						1	0.80
10		1					1	0.80
11		1					1	0.80
12		1					1	0.80
13		1					1	0.80
14		1					1	0.80
MDH								
1	25	25	8	25	26	12	121	96.80
2			4				4	3.20

(Table 15. cont.)

Phenotypes	Accessions						Total	Frequency %
	K905	K911	K912	K913	K914	K917		
IDH								
1	7		2	19	24		52	52.00
2	15		2				17	17.00
3			5	2		9	16	16.00
4				3			3	3.00
5						3	3	3.00
6	3						3	3.00
7					2		2	2.00
8			2				2	2.00
9			1				1	1.00
10				1			1	1.00
PGI								
1	25	16	6	18	19	12	96	76.80
2		9			5		14	11.20
3			3	6	1		10	8.00
4			1	1			2	1.60
5			2				2	1.60
6				1			1	0.80
PGM								
1	18		7	5	20	11	61	61.00
2	5		1	2	6	1	15	15.00
3				12			12	12.00
4				3			3	3.00
5			2				2	2.00
6	1		1				2	2.00
7				1			1	1.00
8				1			1	1.00
9				1			1	1.00
10			1				1	1.00
11	1						1	1.00
Total samples	25	25	12	25	26	12	100	

K913 from El Barreal, Dept. of Guatemala (14.50'N and 90.04'W) showed the unique phenotypes (types 3 and 4) different from the other accessions in *L. collinsii* (Table 15).

ADH. Phenotypes of ADH in the six accessions were also quite diverse. Nine different bands (with Rf ranging from 42 to 93), and fourteen phenotypes were found in this system (Figure 7). Phenotypes had from three to six bands. The most common phenotypes were types 1 and 2 (Table 15). Based on the migrations of the nine different bands and the dimeric nature of ADH. It can be assumed that ADH isozymic bands in *L. collinsii* were controlled by three loci. The first two loci Adh1 and Adh2 determine the five fast-moving bands from Rf value 67 to 93. The third putative locus determines the four slow moving bands from Rf value 42 to 60 (Figure 7).

MDH. Phenotypes of MDH in the *L. collinsii* accessions were quite uniform. Only two phenotypes were found involving six different bands with Rf values ranging from 47 to 80 (Figure 7). Nearly all *L. collinsii* showed the type 1 phenotype (Table 15).

IDH. *L. collinsii* accessions were also diverse in IDH isozymic expression. Nine different migration bands (with Rf values from 33 to 103), and ten phenotypes were found. Each phenotype had from two to six bands (Figure 8). The most common phenotype was type 1 (Table 15). Because of the

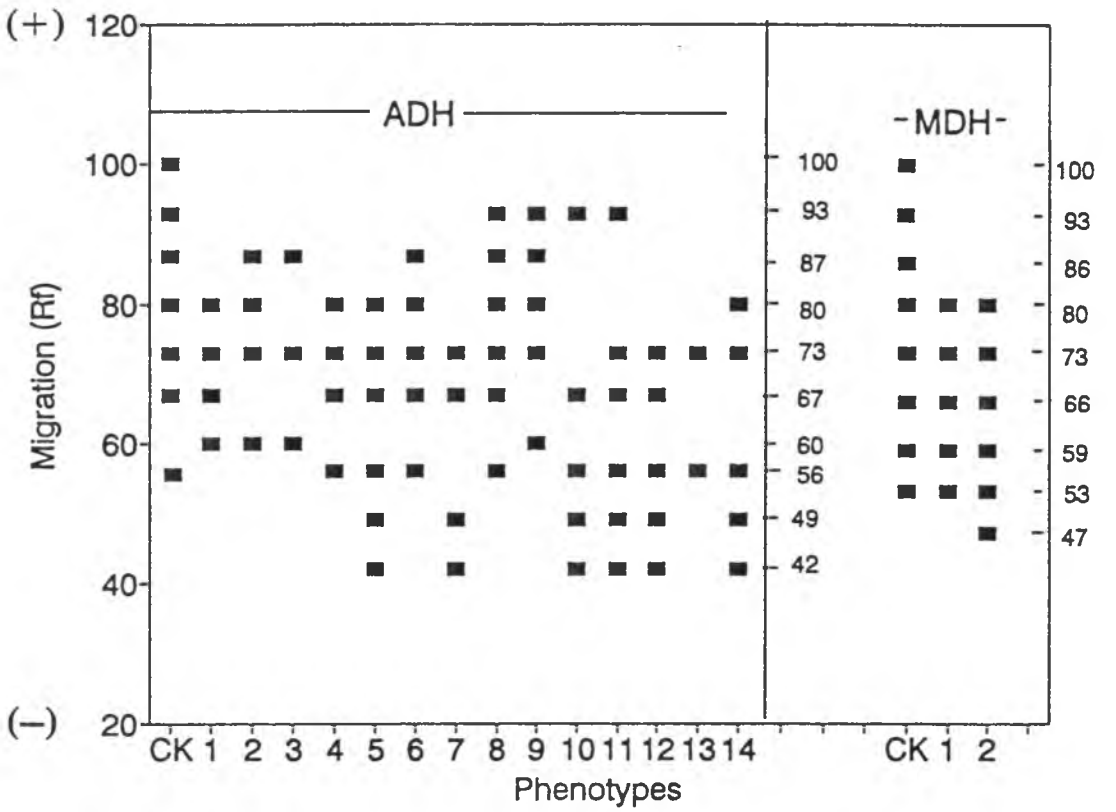


Figure 7. Schematic phenotypes of ADH and MDH in *L. collinsii*, CK = K997

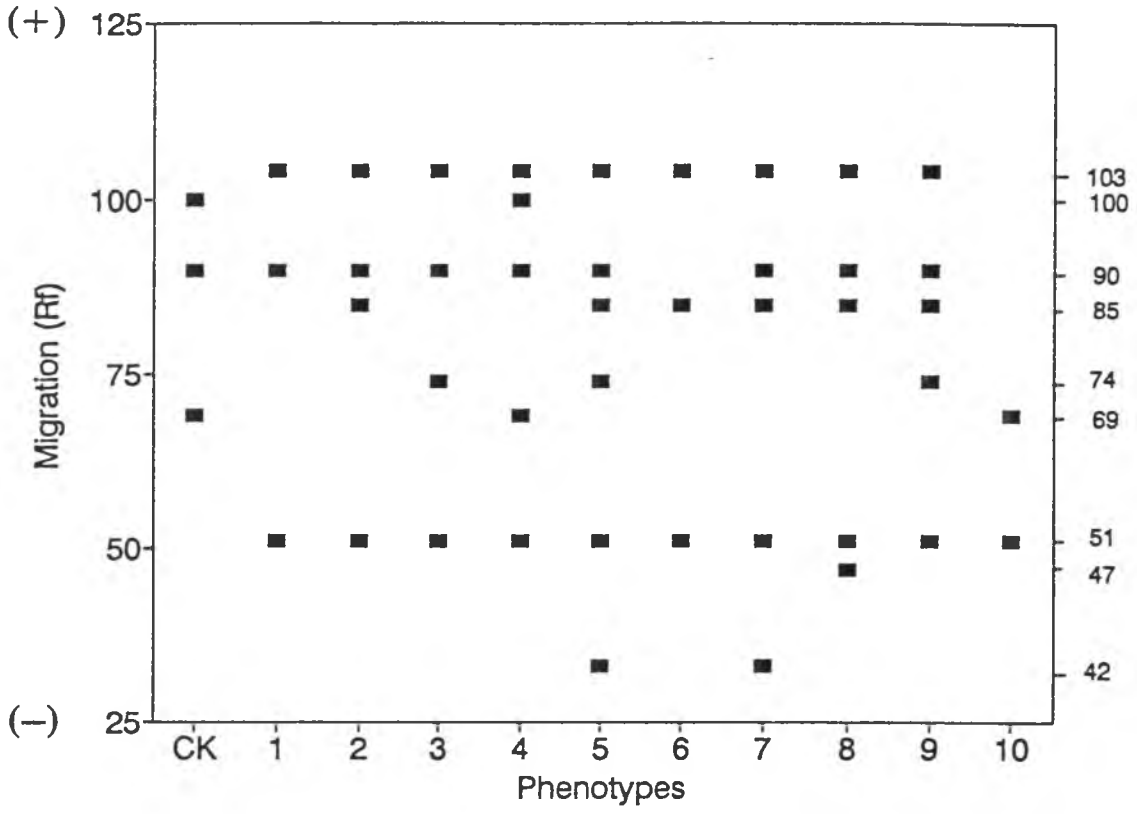


Figure 8. Schematic phenotypes of **IDH** in *L. collinsii*, CK = K997

dimeric nature of IDH, two loci were sufficient to explain IDH polymorphism in banding patterns in *L. collinsii*. A specific band (Rf=51) for *L. collinsii* was found in all plants of six accessions tested.

PGI. Five different bands (with Rf values from 61 to 100) and six phenotypes were found in the PGI system. Each phenotype had from two to five bands. The most common phenotype (comprised 77%) was the type 1 (Figure 9 and Table 15). All six accessions of this species segregated this common phenotype, and band Rf 100 characterized all plants.

PGM. Phenotypes of PGM in the *L. collinsii* accessions were also quite diverse. Seven different migration bands (with Rf values from 61 to 103) and eleven phenotypes were observed (Figure 10). Each phenotype had from two to five bands. The most common phenotypes were types 1, 2 and 3, comprising 88 % of all tested individuals (Figure 10 and Table 15). Bands of Rf 93 and 85 were present in all plants. Based on the observed phenotypes and its monomeric nature, three loci could be postulated for PGM system.

4.3.2.2 Phenotypic polymorphism and similarities among accessions

Phenotypic variation in *L. collinsii* accessions was estimated by phenotypic polymorphism values (P_j) and weighted values (P_w) following methodology of Kahler et al (1980). The values for six isozymes in six *L. collinsii*

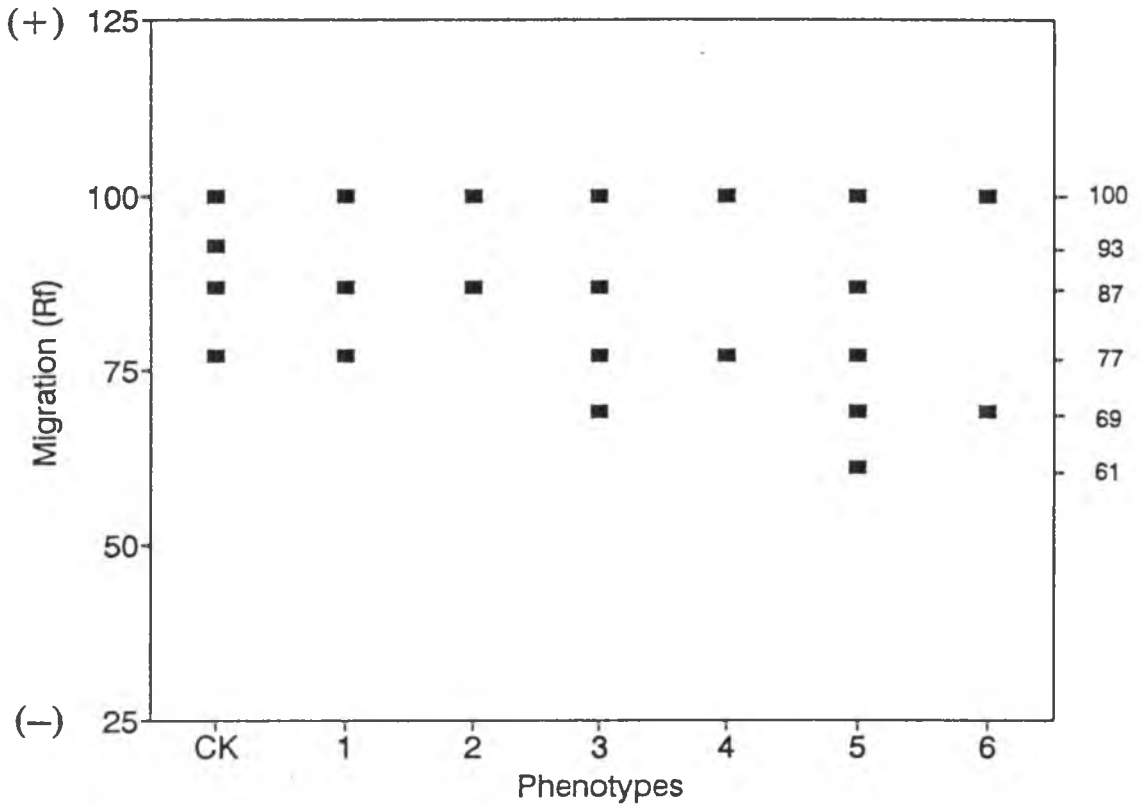


Figure 9. Schematic phenotypes of PGI in *L. collinsii*, CK = K997

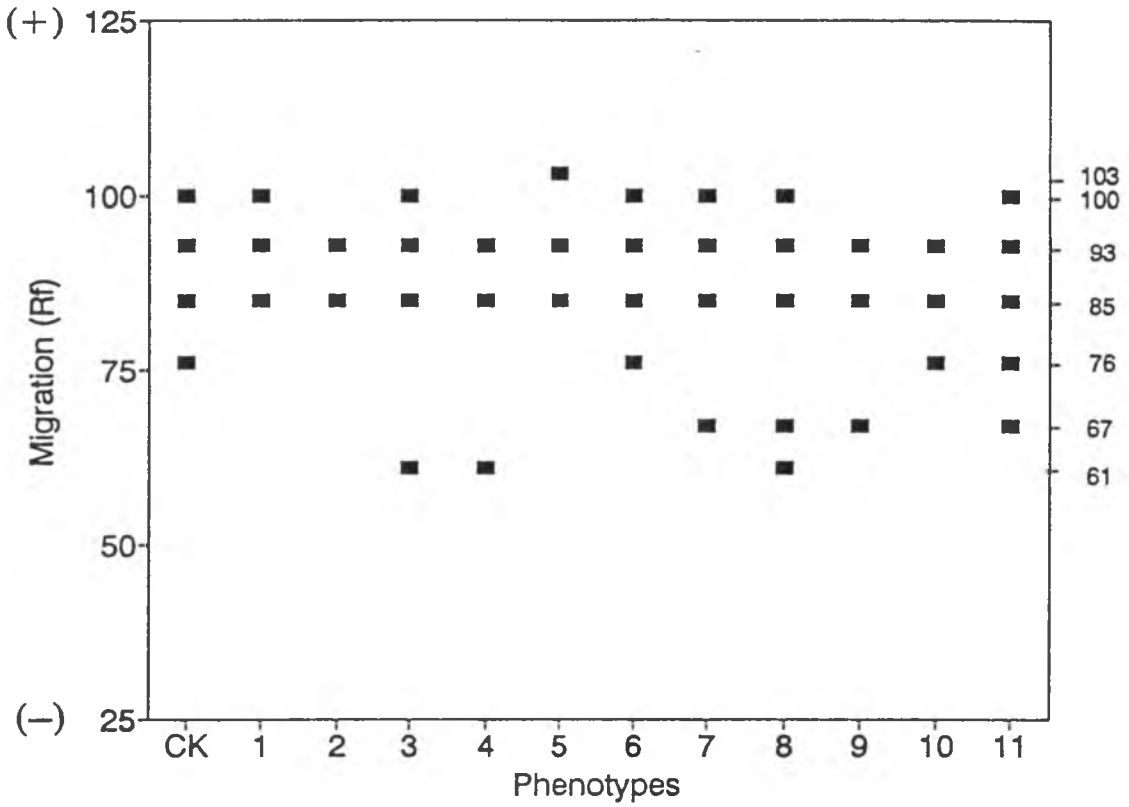


Figure 10. Schematic phenotypes of PGM in *L. collinsii*, CK = K997

Table 16. Phenotypic polymorphism values (P_j) of *L. collinsii* over isozymes and accessions, with averages and weighted values (P_j and P_w)

Accessions	Isozymes						Average P_j	Weighted value P_w
	ACO	ADH	IDH	MDH	PGI	PGM		
K905	0.39	0.08	0.55	0.00	0.00	0.44	0.24	0.12
K911	0.51	0.73		0.00	0.46			
K912	0.64	0.65	0.74	0.44	0.65	0.61	0.62	0.55
K913	0.48	0.44	0.40	0.00	0.46	0.70	0.41	0.24
K914	0.88	0.00	0.14	0.00	0.38	0.36	0.29	0.16
K917	0.38	0.00	0.38	0.00	0.00	0.15	0.15	0.07
Average	0.55	0.32	0.44	0.07	0.33	0.45	0.34	0.23
Percentage*	100	67	100	83	67	100		
Total**	20	14	10	2	6	11		

* The percent (%) of accessions phenotypically polymorphic per isozyme
** The total number of phenotypes observed in each isozyme system

accessions are summarized in Table 16. Among these accessions, the average P_j was the highest in ACO. The average P_js of PGM and IDH were also high in comparison with those of PGI and ADH. The average P_j was the lowest in MDH. It could be seen that the degree of polymorphism varied between different isozymes in *L. collinsii*. The weighted average P_ws among accessions were quite different, ranging from 0.07 to 0.55. The average P_w for these six accessions was 0.23.

Phenotypic similarity among *L. collinsii* accessions was computed for the average taxonomic distance coefficient by use of NTSYS program (Rolhf, 1987). The phenotypic distance coefficients from these accessions varied from 0.19 to 0.27 (Table 17).

The phenotypic distance matrix among all pairwise comparisons was subjected to a cluster analysis with UPGMA algorithm. The cluster analysis for five *L. collinsii* accessions resulted in two groups. The Mexican subspecies *L. collinsii ssp. collinsii* (K905) and the Guatemalan *L. collinsii ssp. zacapana* (K912, K913, K914 and K917) could not be separated by this analysis. A dendrogram of phenotypic dissimilarity among the 5 accessions suggested the rather close relationship of accessions of K914 from Zacapa and K917 from Chiquimula state in Guatemala (Figure 11).

Table 17. Phenotypic distance coefficients (dissimilarity) among five acccessions of *L. collinsii*

Accessions	K905	K912	K913	K914	K917
K905	0.00				
K912	0.20	0.00			
K913	0.22	0.22	0.00		
K914	0.24	0.22	0.22	0.00	
K917	0.26	0.23	0.27	0.19	0.00

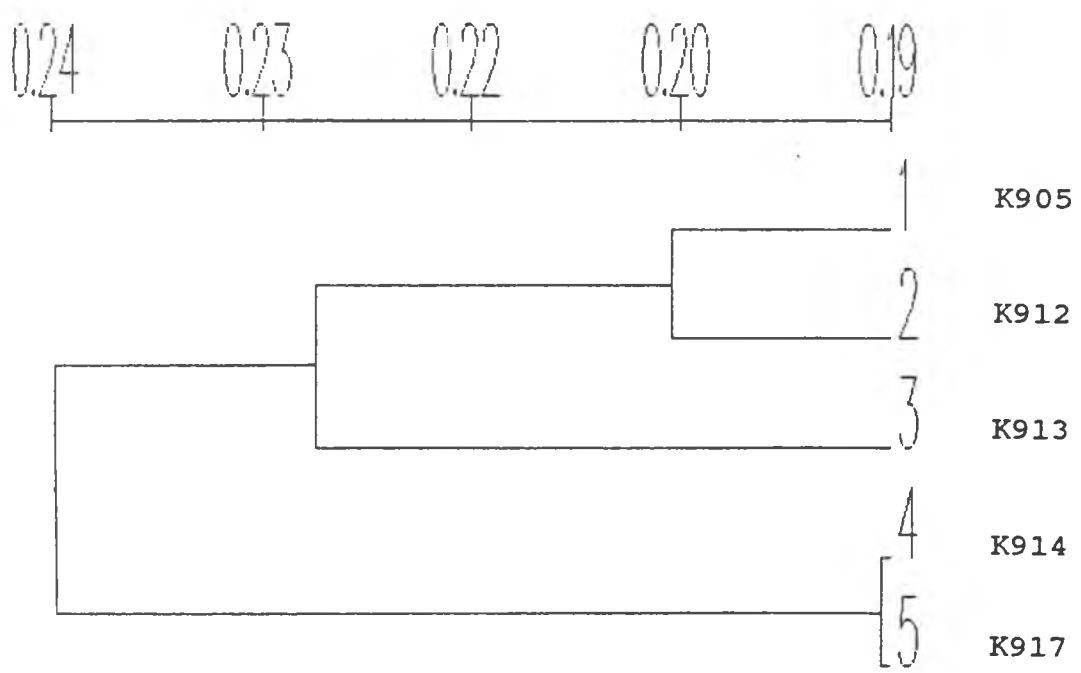


Figure 11. Cluster analysis dendrogram of six enzyme phenotypes in *L. collinsii*

4.3.3 Phenotypic variation in isozymes of *L. diversifolia* ssp. *trichandra*

4.3.3.1 Phenotypic variations in six isozymes

Eight accessions of *L. diversifolia* ssp. *trichandra* were analyzed for six isozyme systems. Four were from Guatemala, two from Honduras and two from Mexico, with latitudes ranging from 14.01' to 17.20'N and longitudes from 86.21' to 96.55'W. Phenotypes were very diverse, as were morphological characters among these accessions. Each accession showed specific phenotypes in ACO and PGI. A specific band of MDH, Rf 96, could be used as a marker for the identification of this species. Details of the isozyme analysis are presented below.

ACO. Phenotypes of ACO in the eight *L. diversifolia* ssp. *trichandra* accessions were very diverse. Eleven different bands (with Rf value ranging from 56 to 106) created forty one different phenotypes (Figure 12). Each phenotype had from three to six bands. Each accession showed its unique phenotypes. Three loci could be assigned to these different migration bands, as was true for *L. collinsii*. The putative Aco1 in *L. diversifolia* ssp. *trichandra* including 4 fast-moving bands with Rf values of 97, 100, 103 and 106. Aco2 included 4 middle bands of Rf 80, 84, 87 and 90. Aco3 included 4 slow moving bands of Rf 56, 64, 66 and 73. No common phenotypes were observed (Table 18).

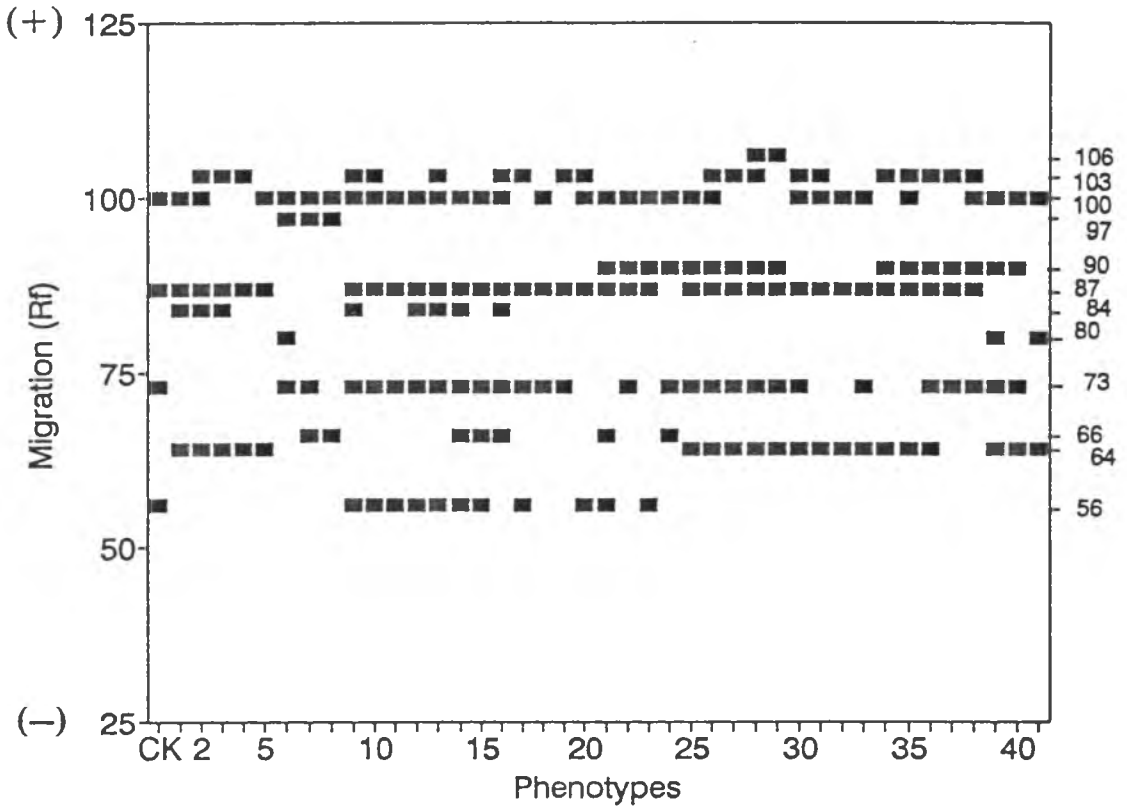


Figure 12. Schematic phenotypes of **ACO** in *L. diversifolia* ssp. *trichandra* (2n=52), CK = K997

Table 18. Frequencies of six isozyme phenotypes in *L. diversifolia* ssp. *trichandra*

Phenotypes	Accessions								Total	Frequency %
	K408	K483	K821	K823	K907	K919	K927	K936		
ACO										
1	8								8	4.44
2	7								7	3.89
3	2								2	1.11
4	2								2	1.11
5	1								1	0.56
6		4							4	2.22
7		13							13	7.22
8		8							8	4.44
9				1					1	0.56
10				3					3	1.67
11				2					2	1.11
12				5					5	2.78
13				1					1	0.56
14				3					3	1.67
15				2					2	1.11
16				3					3	1.67
17			2						2	1.11
18			5						5	2.78
19			1						1	0.56
20			1						1	0.56
21			1						1	0.56
22			8				1		9	5.00
23			1						1	0.56
24			1						1	0.56
25					2	1			3	1.67
26					16		3		19	10.56
27					3				3	1.67
28					3				3	1.67
29					1				1	0.56
30						6			6	3.33
31						8			8	4.44
32						4			4	2.22
33						2			2	1.11
34						3	6		9	5.00
35						1	1		2	1.11
36							5		5	2.78
37							2		2	1.11
38							2		2	1.11
39								20	20	11.11
40								3	3	1.67
41								2	2	1.11

(Table 18. cont.)

Phenotypes	Accessions								Total	Frequency %
	K408	K483	K821	K823	K907	K919	K927	K936		
ADH										
1	15	19	11	20		4	19	25	113	61.10
2	5	6	14			10	6		41	22.20
3					1				1	0.54
4					13				13	7.03
5					2				2	1.08
6					4	6			10	5.41
7						5			5	2.70
MDH										
1	20	25			25	25	25		120	70.60
2			9						9	5.29
3			16						16	9.41
4								25	25	14.70
IDH										
1	11								11	5.95
2	2	19		14	12	22		23	92	49.7
3	6				3				9	4.86
4	1								1	0.54
5		1							1	0.54
6		2		2				1	5	2.7
7		2						1	3	1.62
8		1							1	0.54
9			10						10	5.41
10			7						7	3.78
11			5						5	2.7
12			1						1	0.54
13			1						1	0.54
14			1						1	0.54
15				2					2	1.08
16				2					2	1.08
17					10				10	5.41
18						3			3	1.62
19							8		8	4.32
20							8		8	4.32
21							1		1	0.54
22							1		1	0.54
23							1		1	0.54
24							1		1	0.54

(Table 18. cont.)

Phenotypes	Accessions								Total	Frequency %
	K408	K483	K821	K823	K907	K919	K927	K936		
PGI										
1	15								15	8.33
2	5			4					9	5.00
3		14							14	7.78
4		4							4	2.22
5		4							4	2.22
6		2							2	1.11
7			4			5	21		30	16.70
8			17						17	9.44
9			4						4	2.22
10				12					12	6.67
11				4					4	2.22
12					11	1			12	6.67
13					5				5	2.78
14					9	3			12	6.67
15						9			9	5.00
16						2			2	1.11
17						1			1	0.56
18						1			1	0.56
19						2			2	1.11
20							4		4	2.22
21								10	10	5.56
22								3	3	1.67
23								2	2	1.11
24								1	1	0.56
25								1	1	0.56
PGM										
1	6		2			11		5	24	13.00
2	11	18	1	3		4	2	13	52	28.10
3	1								1	0.54
4	2				2				4	2.16
5		7				5		2	14	7.57
6			7	3			10		20	10.80
7			13			4			17	9.19
8			2						2	1.08
9				9				2	11	5.95
10				1					1	0.54
11				4					4	2.16
12					9				9	4.86
13					3				3	1.62
14					1				1	0.54
15					3				3	1.62
16					2	1	13		16	8.65
17								2	2	1.08
18								1	1	0.54
Total samples	20	25	25	20	20	25	25	25	185	

ADH. ADH isozymic phenotypes of the eight accessions were quite uniform. Ten different bands (with Rf ranging from 56 to 89) and seven phenotypes were observed. Most accessions, however, showed either type 1 and 2. K907 from Guatemala showed quite different phenotypes (types 3, 4, 5, and 6) compared with all others (Figure 13 and Table 18).

MDH. Phenotypes of MDH among the accessions were quite uniform. Nine bands (with Rf ranging from 47 to 96) and four phenotypes were found (Figure 13). However, 71 % of all samples from the accessions showed only type 1 (Table 18). K821 from Mexico showed different phenotypes (types 2 and 3) from others. A specific MDH band at Rf 96 was found to be unique in comparing *L. diversifolia* ssp. *trichandra* with MDH bands of other species.

IDH. Phenotypes of the IDH system were also very diverse in the diploid *L. diversifolia* accessions. Eight different bands (with Rf ranging from 56 to 110) and twenty four phenotypes were found (Figure 14). Each phenotype had from two to five bands. The most common phenotype was type 2. K821 from Mexico and K927 from Guatemala showed phenotypes uniquely different from all others (Table 18).

PGI. These diploid *L. diversifolia* accessions were also very diverse in the PGI isozyme system. Ten bands (with Rf ranging from 25 to 100) and twenty five phenotypes were found (Figure 15). Each phenotype had from three to seven bands. As with ACO, each accession showed its unique

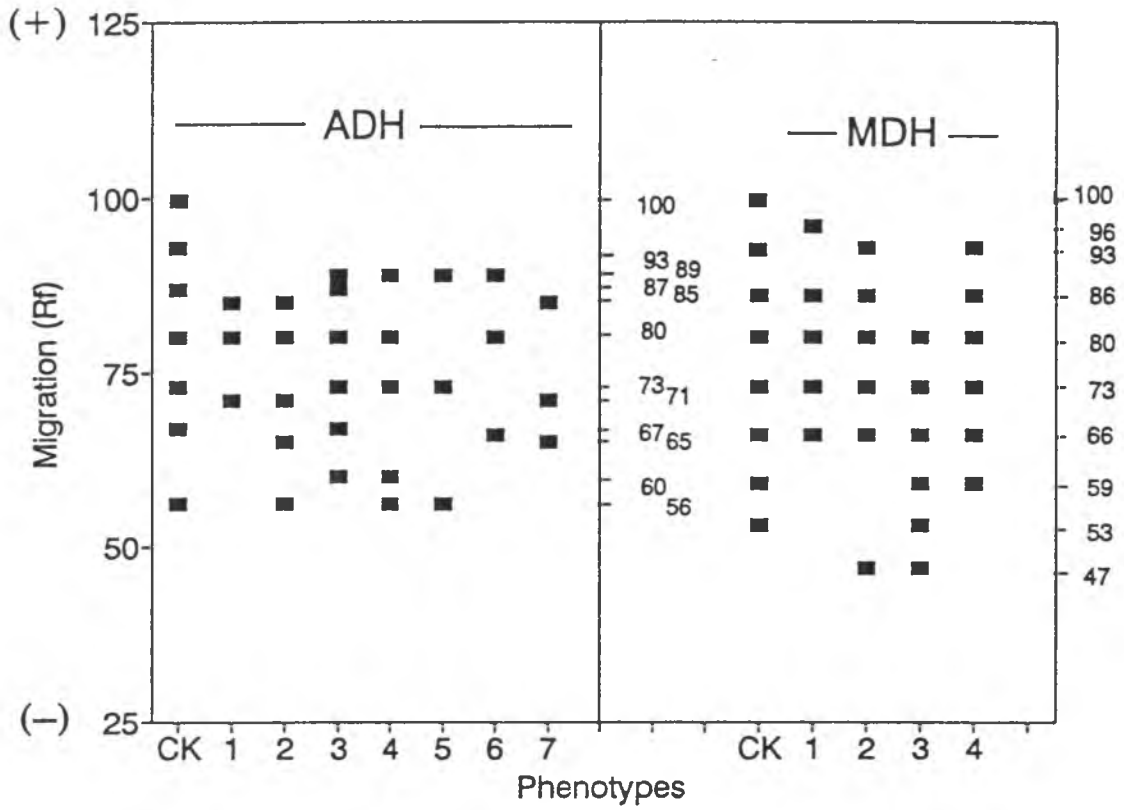


Figure 13. Schematic phenotypes of ADH and MDH in *L. diversifolia* ssp. *trichandra* (2n=52), CK = K997

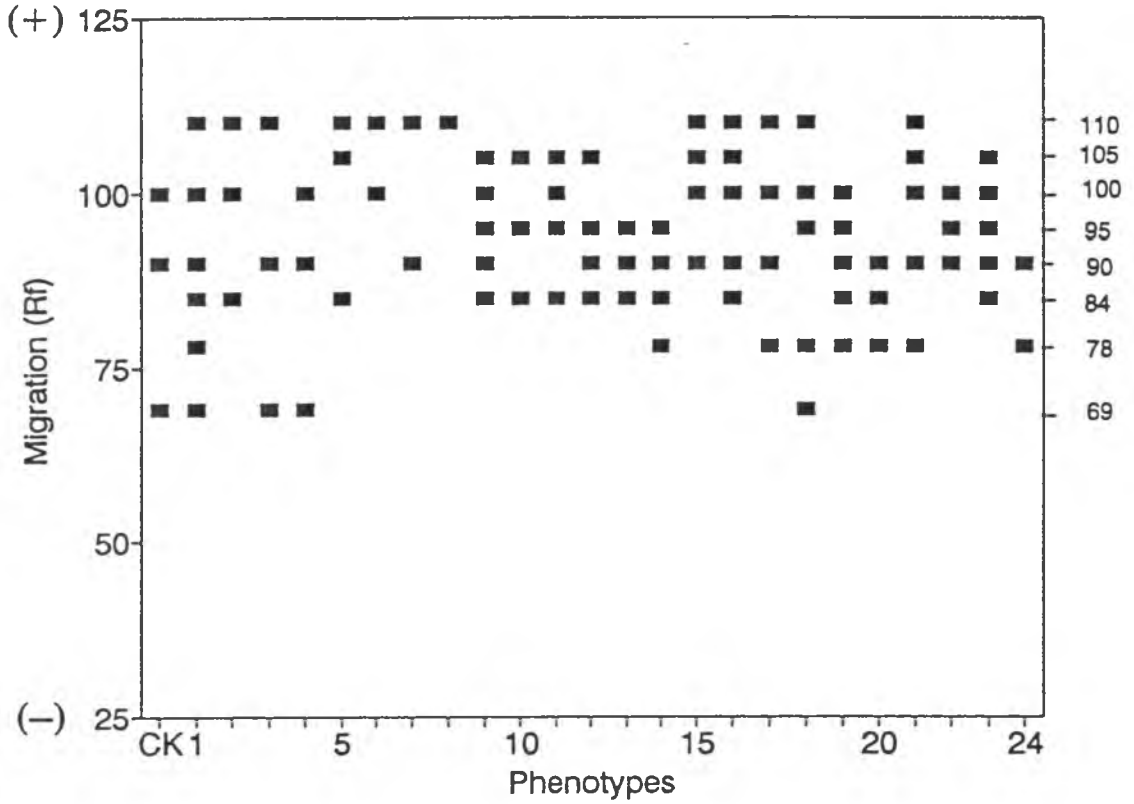


Figure 14. Schematic phenotypes of IDH in *L. diversifolia* ssp. *trichandra* (2n=52), CK = K997

specific phenotypes different from others (Figure 15 and Table 18). No phenotypes occured with high frequency.

PGM. Phenotypes of PGM in the accessions were also very diverse. Six bands (with Rf ranging from 67 to 104) and eighteen phenotypes were found (Figure 16). The number of bands in each phenotype ranged from one to five. Almost all accessions showed the type 2 phenotype except for K907 (Table 18).

4.3.3.2 Phenotypic polymorphism and similarities among accessions

Phenotypic variation of six isozymes in eight *L. diversifolia* ssp. *trichandra* (2n=52) accessions was estimated by phenotypic polymorphism values P_j and P_w. The average P_js and P_ws of six isozymes for eight accessions are presented in Table 19. The highest average P_js among the accessions were of ACO and PGM, and the lowest average P_j was of MDH. The degree of polymorphism varied between different isozyme systems, as with P_js in *L. collinsii* (Table 15). The weighted average P_ws among eight accessions were also quite different, ranging from 0.14 to 0.52. The weighted average P_w across the accessions was 0.28, highest among the tested *Leucaena* species.

The phenotypic similarity among *L. diversifolia* ssp. *trichandra* accessions was computed for the average taxonomic distance coefficient by use of NTSYS program. The

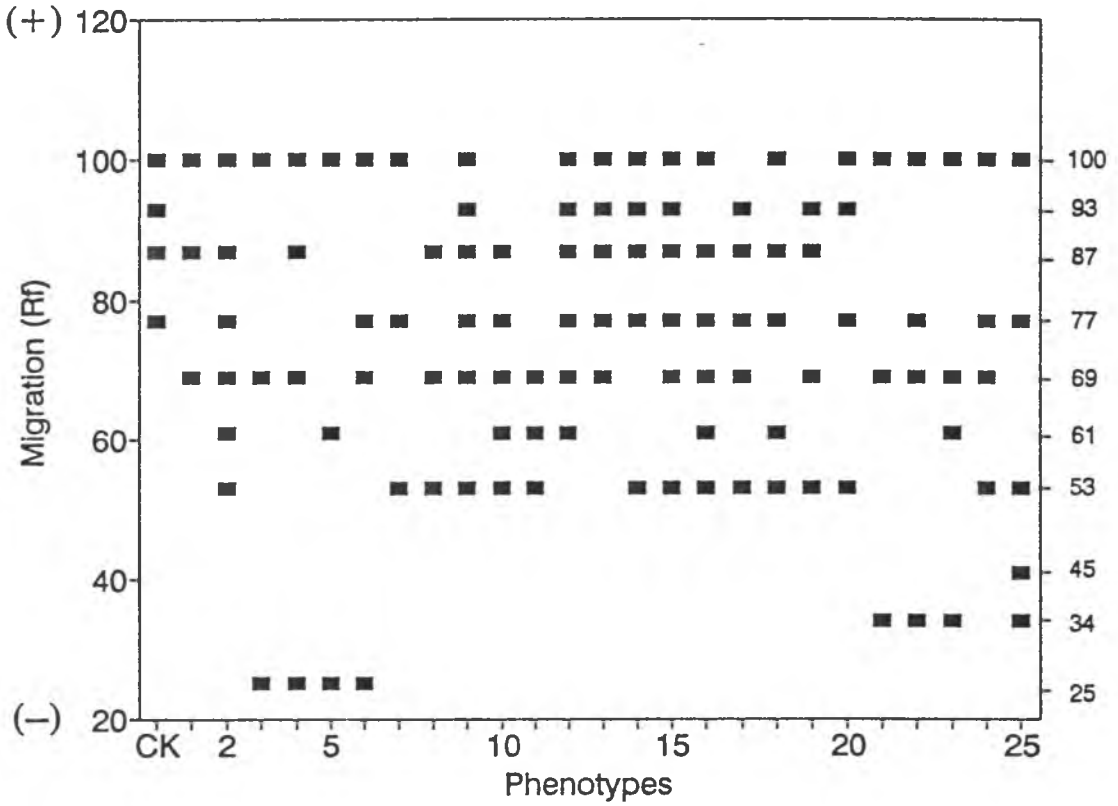


Figure 15. Schematic phenotypes of PGI in *L. diversifolia* ssp. *trichandra* (2n=52), CK = K997

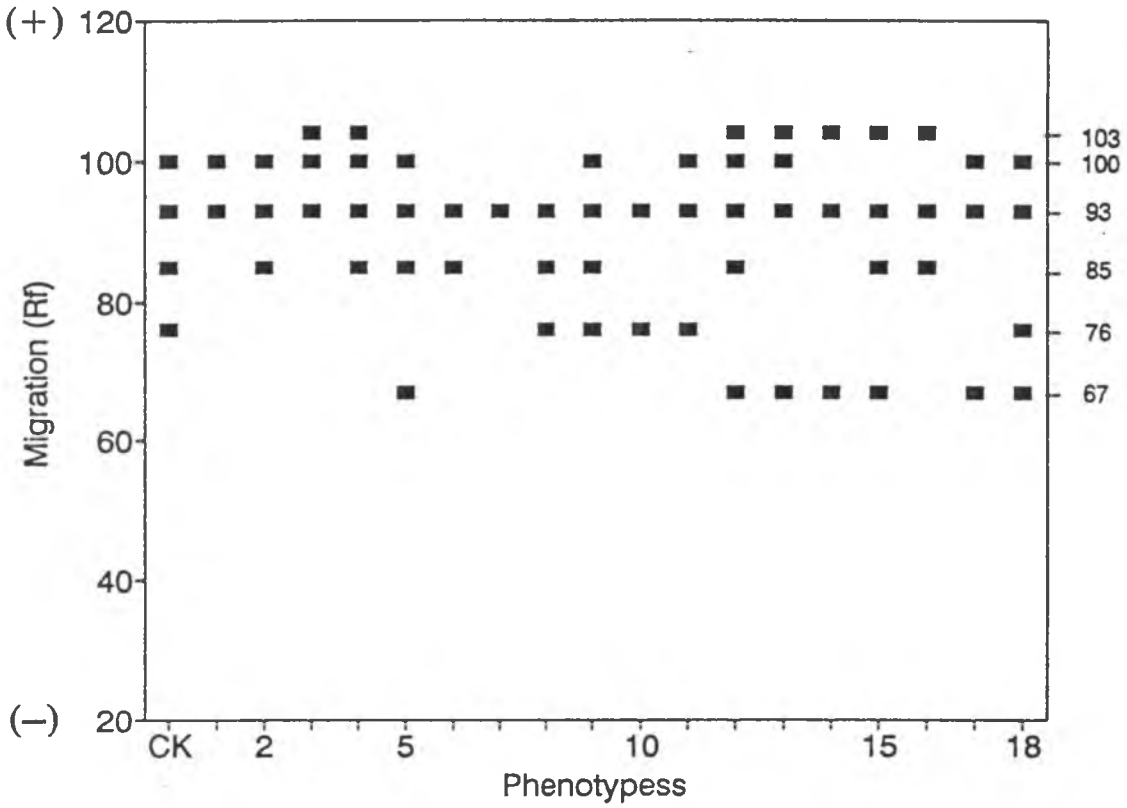


Figure 16. Schematic phenotypes of **PGM** in *L. diversifolia* ssp. *trichandra* (2n=52), CK = K997

Table 19. Phenotypic polymorphism values (P_j) of *L. diversifolia* ssp. *trichandra* over isozymes and accessions, with averages and weighted values (P_j and P_w)

Accessions	Isozymes						Average P_j value	Weighted P_w
	ACO	ADH	MDH	IDH	PGI	PGM		
K408	0.70	0.38	0.00	0.60	0.38	0.60	0.44	0.25
K483	0.60	0.36	0.00	0.41	0.60	0.40	0.40	0.23
K821	0.76	0.49	0.46	0.72	0.49	0.64	0.59	0.52
K823	0.85	0.00		0.48	0.56	0.71	0.52	0.20
K907	0.55	0.53	0.00	0.60	0.64	0.73	0.51	0.31
K919	0.79	0.72	0.00	0.21	0.78	0.71	0.54	0.34
K927	0.80	0.36	0.00	0.67	0.27	0.56	0.44	0.25
K936	0.34	0.00	0.00	0.15	0.60	0.67	0.29	0.14
Mean	0.67	0.35	0.07	0.48	0.54	0.63	0.47	0.28
Percentage*	100	75	14	100	100	100		
Total**	41	9	4	24	25	18		

* The percent (%) of accessions phenotypically polymorphic per isozyme
** The total number of phenotypess observed in each isozyme system

coefficients from the eight diploid *diversifolia* accessions varied from 0.13 to 0.22 (Table 20).

The phenotypic distance coefficient matrix among the accessions was subjected to a cluster analysis with UPGMA algorithm. The result are presented in Figure 17. The cluster dendrogram clearly shows that accessions from Honduras K483 and K936 are quite close, however, accessions K821 and K823 from Mexico are divergent from each others.

4.3.4 Identification of polymorphic gene loci

Genetic loci governing ACO and IDH polymorphism in *L. lanceolata* (K10) were identified by comparing the observed ratios in F1 hybrids with expected ratios by chi-square analysis.

ACO. Three zones of activity, Aco1, Aco2 and Aco3, were polymorphic on gels of *L. lanceolata* (Figure 18). At Aco1, parent 1 (K10-1) was homozygous for the fast band Rf 100 and parent 2 (K10-2) homozygous for the slow band Rf 94; they are interpreted as two alleles of a single locus. At Aco2, parent 1 was heterozygous showing bands at Rf 87 and Rf 80 and parent 2 was homozygous only for fast band Rf 87. At Aco3, parent 1 was homozygous only for fast band Rf 73, and parent 2 was heterozygous showing bands at Rf 73 and Rf 66 (Figure 18). Three plants in line 13, 22 and 25 showing homogenous in Aco1 were selfing plants, which were confirmed by homogenous expression in Aco3 band (Rf 73, line 13) and

Table 20. Phenotypic distance coefficients (dissimilarity) among eight accessions of *L. diversifolia* ssp. *trichandra*

Acccessions	K408	K483	K821	K823	K907	K919	K927	K936
K408	0.00							
K483	0.15	0.00						
K821	0.20	0.21	0.00					
K823	0.16	0.14	0.20	0.00				
K907	0.19	0.18	0.21	0.18	0.00			
K919	0.16	0.14	0.19	0.15	0.15	0.00		
K927	0.17	0.17	0.19	0.16	0.18	0.16	0.00	
K936	0.17	0.13	0.22	0.13	0.19	0.15	0.18	0.00

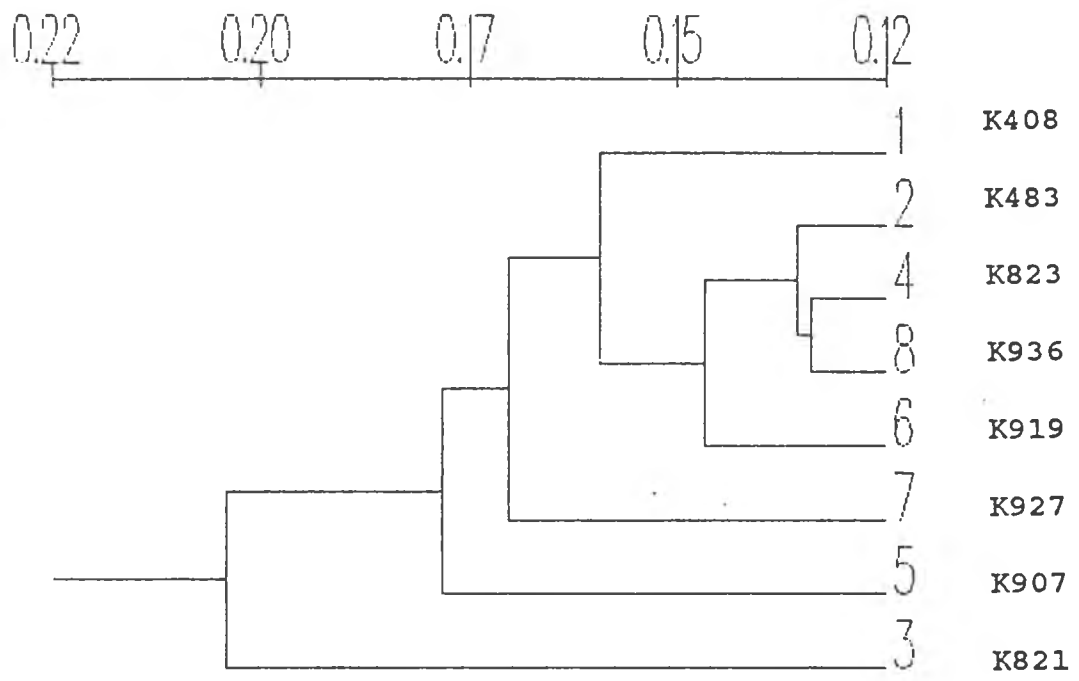


Figure 17. Cluster analysis dendrogram of six enzyme phenotypes in *L. diversifolia* ssp. *trichandra* (2n=52)

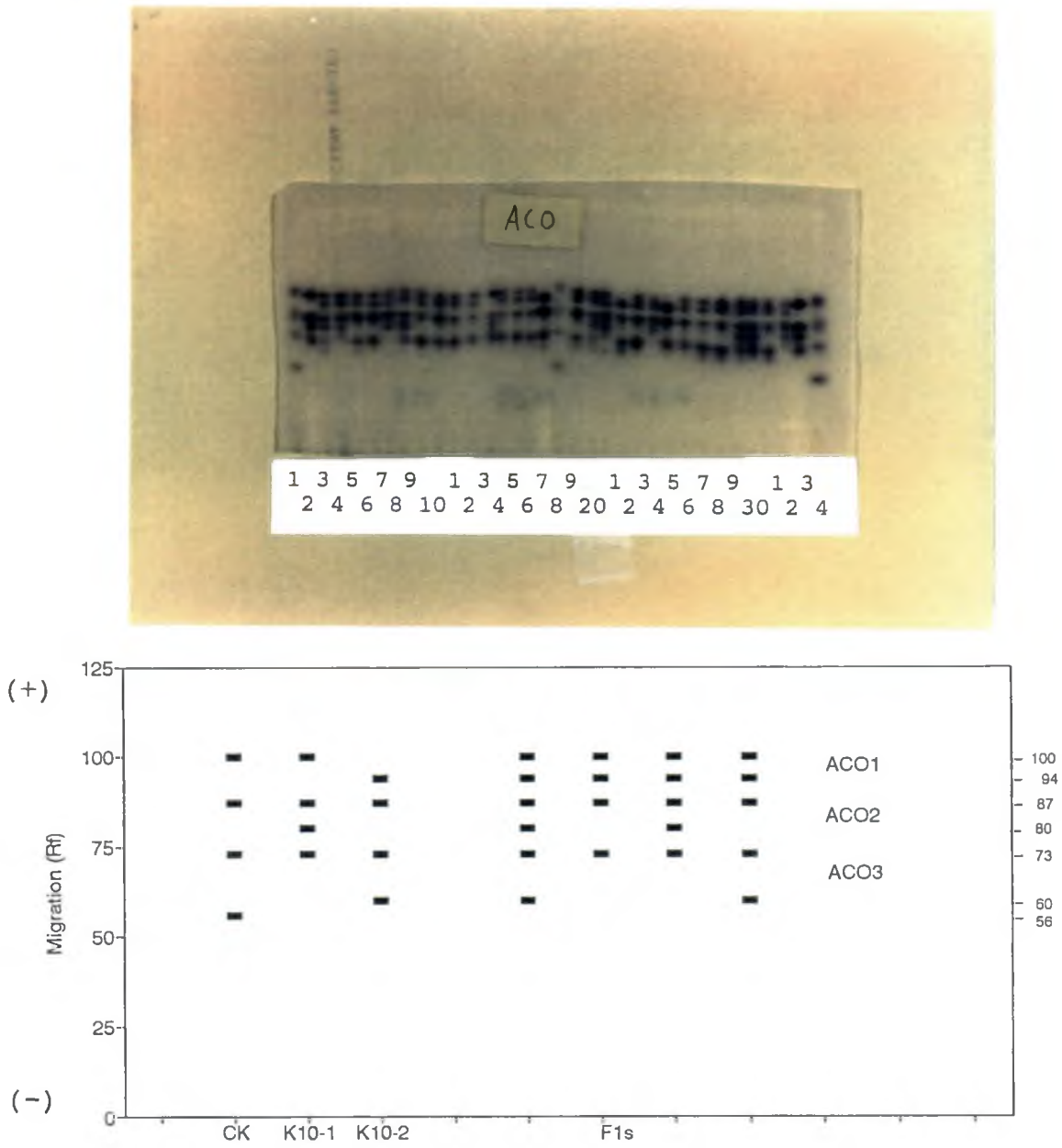


Figure 18. **ACO** phenotypes of parents (K10-1, line 2, 33, and K10-2, line 17) and their F1 progenies (line 3-16 and 19-32) of *L. lanceolata*, CK = K997 (line 1, 18 and 34), and its schematic phenotypes

Aco2 band (Rf 87, line 22 and 25). Forty-one F1 seedlings from both reciprocal crosses segregated in a 1:1 ratio at both Aco2 and Aco3 respectively and all were heterozygous for Aco1 (Table 21). Three loci also segregated in a 1:1:1:1 ratio independently with X^2 = 1.05 and P = 0.70-0.90 (Table 22). Segregation of these three loci showed no linkage among them.

IDH. Idh1 and Idh2 were identified in *L. lanceolata* (K10). At Idh1, both parents were homozygous, but at Idh2, parent 1 was heterozygous for the two bands at Rf 90 and Rf 87 and parent 2 was homozygous only showing fast band Rf 90. F1 from both reciprocal crosses segregated in a 1:1 ratio at Idh2 (Figure 19, Table 21).

Table 21. Segregation of ACO and IDH phenotypes in the progenies of *L. lanceolata* crosses (K10-1 X K10-2)

Locus	Phenotypes	No. of seedlings	Expected ratio	X^2	P
Aco1	FF/SS	41	--	--	1.00
Aco2	SS/FS	41	1 : 1	0.10	0.70-0.90
Aco3	FF/FS	41	1 : 1	0.40	0.50-0.70
Idh1	FF/FF	41	--	--	1.00
Idh2	FS/FF	41	1 : 1	1.56	0.20-0.30

Table 22. Segregation of Aco1, Aco2 and Aco3 phenotypes in the progenies of *L. lanceolata* crosses (K10-1 x K10-2)

	No. of seedlings with phenotypes				
Aco	1 2 3 FSFSFS	1 2 3 FSFFFF	1 2 3 FSFSFF	1 2 3 FSFFFS	Total
Observed	9	13	10	9	41
Expected	10.25	10.25	10.25	10.25	
Obs. - Exp.	-1.25	2.75	-0.25	-1.25	
$(Obs.-Exp.)^2/Exp.$	0.15	0.74	0.01	0.15	
X^2					1.05
Probability	0.70 - 0.90				

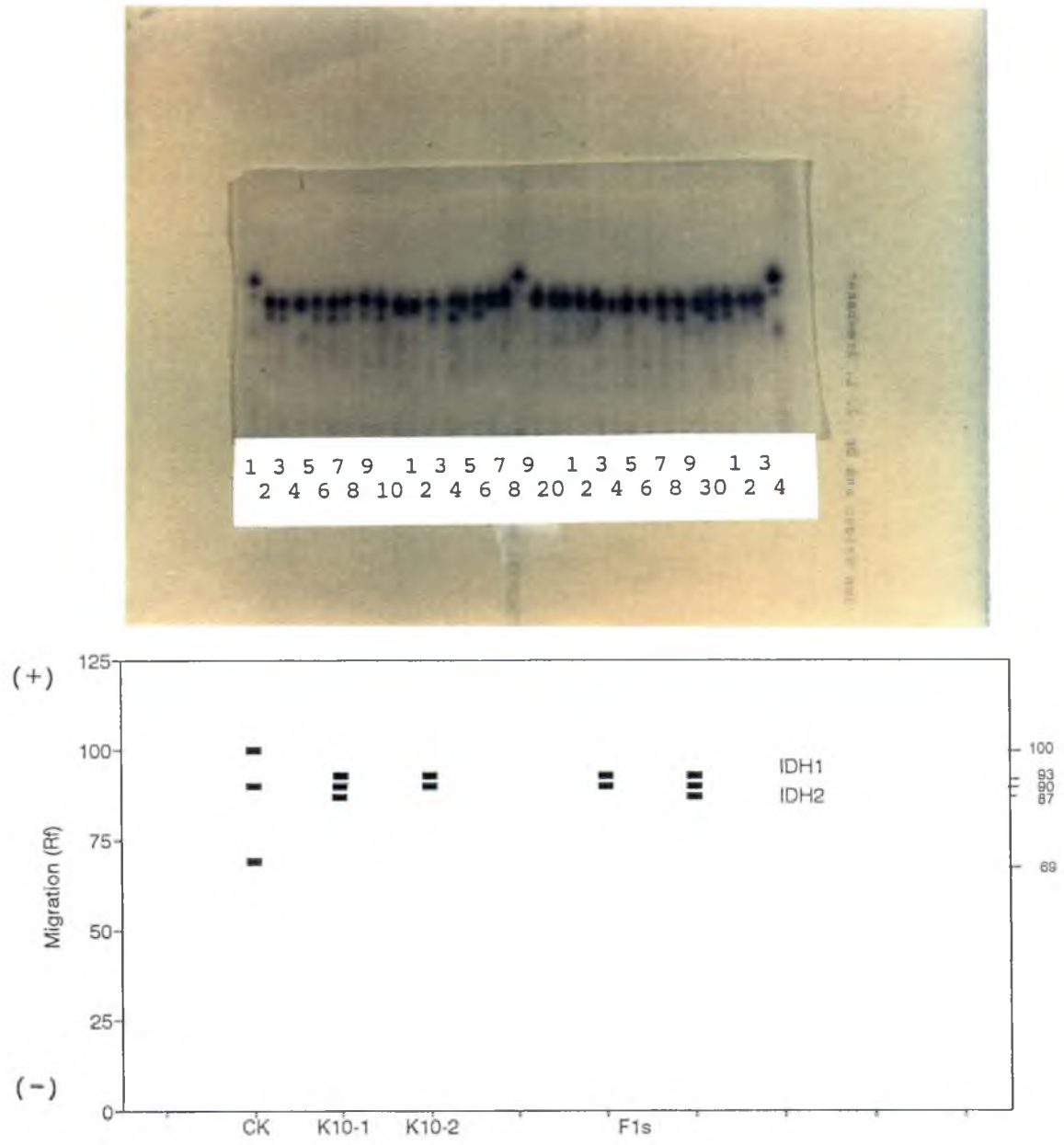


Figure 19. **IDH** phenotypes of parents (K10-1, line 2, 33, and K10-2, line 17) and their F1 progenies (line 3-16 and 19-32) of *L. lanceolata*, CK = K997 (line 1, 18 and 34), and its schematic phenotypes

4.4 Evolutionary considerations

4.4.1 The "common" vs. the "giant" *L. leucocephala*

Are all *L. leucocephala ssp. leucocephala* "common" types genetically one pure line ? Can *L. leucocephala ssp. glabrata* "giant" be distinguished isozymically from the "common" types ? In order to answer these questions and understand the genetic diversity of "common" *L. leucocephala* in the world, seventy-seven accessions of the "common" *L. leucocephala* were tested for the six isozyme systems. In addition, thirty-six accessions of the "giant" *L. leucocephala* mostly from Central America were also tested. Isozymic phenotypes and frequencies of the phenotypes for each accession, phenotypic polymorphism (P_j), and the weighted polymorphism (P_w) in the two *L. leucocephala* subspecies are presented below.

4.4.1.1 Phenotypic variations of the "common" varieties

Twelve phenotypes for the six isozyme systems were found in the 77 accessions of "common" *L. leucocephala* (Figure 20). Frequencies of the phenotypes for each accession are presented in Appendix C. Number of phenotypes observed in each isozyme system, P_js and P_ws are summarized in Table 23. Two of the six isozyme systems, ADH and MDH, did not show polymorphism in these accessions. The average P_js of the other isozymes were also low in comparison with

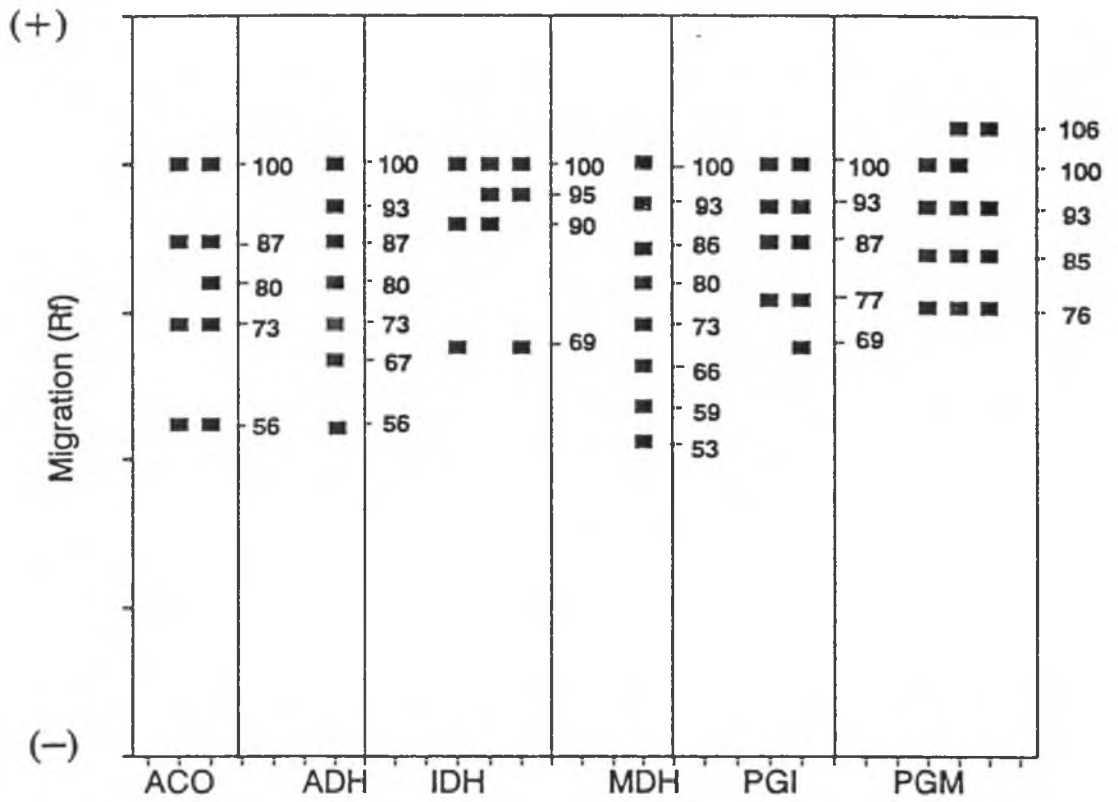


Figure 20. Schematic phenotypes of six enzyme systems in *L. leucocephala* ssp. *leucocephala*

Table 23. Phenotypic polymorphism values (P_j) of *L. leucocephala* ssp. *leucocephala* over isozymes and accessions, with averages and weighted values (P_j and P_w)

Accessions	Isozymes						AVG P_j	Weighted value P_w
	ACO	ADH	IDH	MDH	PGI	PGM		
K2	0	0	0	0	0	0	0	0
K13	0	0	0	0	0	0	0	0
K21	0	0	0	0	0	0	0	0
K22	0	0	0	0	0.31	0.65	0.16	0.10
K23	0	0	0	0	0	0	0	0
K24	0	0	0	0	0.38	0.21	0.10	0.07
K25	0	0	0	0	0	0	0	0
K26	0	0	0	0	0	0	0	0
K31	0	0	0	0	0	0	0	0
K32	0	0	0	0	0	0	0	0
K35	0	0	0	0	0	0	0	0
K36	0	0	0	0	0	0	0	0
K37	0	0	0	0	0	0	0	0
K38	0	0	0	0	0	0	0	0
K40	0	0	0	0	0	0	0	0
K41	0	0	0	0	0	0	0	0
K42	0	0	0	0	0.50	0	0.08	0.07
K43	0	0	0	0	0	0	0	0
K46	0	0	0	0	0.49	0	0.08	0.07
K47	0	0	0	0	0	0	0	0
K48	0	0	0	0	0	0	0	0
K49	0	0	0	0	0	0	0	0
K50	0	0	0	0	0	0	0	0
K51	0	0	0	0	0	0	0	0
K52	0	0	0	0	0	0	0	0
K53	0	0	0	0	0	0	0	0
K54	0	0	0	0	0	0	0	0
K55	0	0	0.47	0	0.21	0.11	0.13	0.04
K56	0	0	0	0	0	0	0	0
K57	0	0	0	0	0	0	0	0
K58	0	0	0	0	0	0	0	0
K60	0	0	0	0	0	0	0	0
K61	0	0	0	0	0	0	0	0
K62	0	0	0	0	0.32	0	0.05	0.04
K63	0	0	0.18	0	0.32	0.62	0.19	0.10
K64	0	0	0	0	0	0	0	0
K68	0	0	0	0	0	0	0	0
K70	0	0	0	0	0	0	0	0
K71	0	0	0	0	0	0	0	0
K72	0	0	0.18	0	0	0	0.03	0.02
K73	0	0	0	0	0	0	0	0
K76	0	0	0	0	0	0	0	0
K77	0.84	0	0.18	0	0	0	0.17	0.11
K78	0	0	0	0	0	0	0	0

(Table 23. cont.)

Accessions	Isozymes						AVG P_j	Weighted value P_w
	ACO	ADH	IDH	MDH	PGI	PGM		
K79	0.84	0	0.18	0	0	0	0.17	0.11
K80	0.64	0	0.42	0	0	0	0.18	0.09
K81	0.36	0	0.42	0	0	0	0.13	0.05
K82	0	0	0.18	0	0	0	0.03	0.02
K83	0	0	0	0	0	0	0	0
K84	0	0	0	0	0	0	0	0
K85	0	0	0	0	0	0	0	0
K86	0	0	0	0	0	0	0	0
K87	0	0	0	0	0	0	0	0
K88	0	0	0	0	0	0	0	0
K89	0	0	0	0	0	0	0	0
K109	0	0	0	0	0	0	0	0
K115	0	0	0	0	0	0	0	0
K269	0	0	0	0	0	0	0	0
K278	0	0	0	0	0	0	0	0
K279	0	0	0	0	0	0.18	0.03	0.02
K281	0	0	0	0	0	0	0	0
K304	0	0	0	0	0	0	0	0
K313	0	0	0	0	0	0	0	0
K314	0	0	0	0	0	0	0	0
K315	0	0	0	0	0	0	0	0
K316	0	0	0	0	0	0	0	0
K317	0	0	0.42	0	0	0	0.07	0.05
K319	0	0	0	0	0	0	0	0
K320	0	0	0	0	0	0	0	0
K321	0	0	0.18	0	0	0	0.03	0.02
K322	0	0	0	0	0	0	0	0
K323	0	0	0	0	0	0	0	0
K325	0	0	0	0	0.50	0	0.08	0.07
K326	0	0	0	0	0.50	0	0.08	0.07
K341	0	0	0	0	0	0	0	0
K676	0	0	0	0	0.48	0	0.08	0.07
K677	0	0	0	0	0	0	0	0
K997c	0	0	0	0	0	0	0	0
Average	0.03	0.00	0.04	0.00	0.05	0.02	0.02	0.01
Percentage*	5.19	0.00	12.99	0.00	12.99	6.49		
Total**	2	1	3	1	2	3		

* The percent (%) of accessions phenotypically polymorphic per enzyme
** The total number of phenotypes observed in each enzyme system

the P_js of the other species. The "common" *L. leucocephala* accessions showed high uniformity in isozymic expression. Among 77 accessions of the "common" type, fifty nine did not show polymorphism in six isozyme systems. The P_ws for the "common" *L. leucocephala* accessions ranged from 0 to 0.11. The average P_w for the species was 0.01, which was the lowest among twelve tested *Leucaena* species.

4.4.1.2 Phenotypic variations of the "giant" varieties

Fifteen phenotypes for the six isozyme systems were found in 36 accessions of the "giant" *L. leucocephala* (Figure 21). Frequencies of the phenotypes for each accession are showed in Appendix D. The number of phenotypes observed in each isozyme system, P_js and P_ws is presented in Table 24. Two isozyme systems, ADH and MDH, did not show polymorphism, as with the "common" types. However, the "giant" accessions showed one more phenotype for each of ACO, IDH and PGM systems. The average P_js of the other isozymes were higher than those of the "common" types, but still lower than those of the other species. The "giant" accessions showed more polymorphism in comparison with the "common" types. Of 36 accessions tested of the "giant" type, twenty seven showed polymorphism. The average P_ws of these accessions ranged from 0 to 0.16, and average 0.07 across accessions. This value was very similar to that

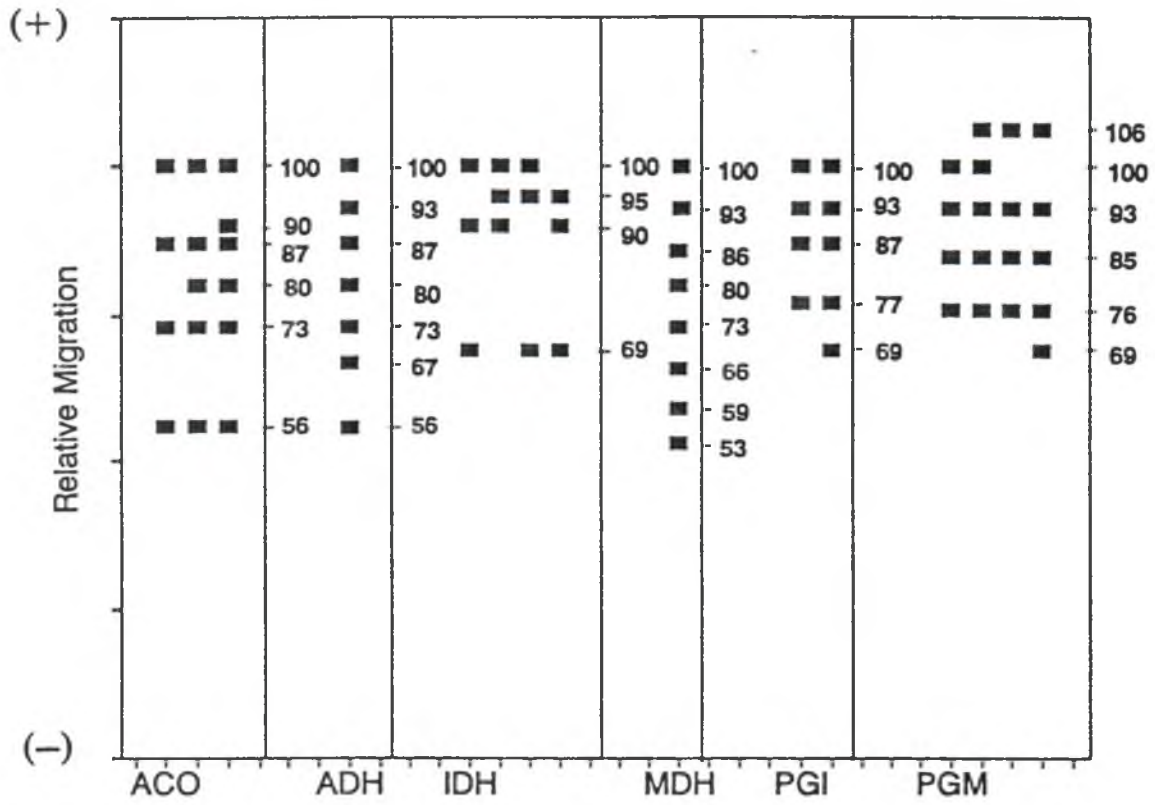


Figure 21. Schematic phenotypes of six enzyme systems in *L. leucocephala* ssp. *glabrata*

Table 24. Phenotypic polymorphism values (P_j) of *L. leucocephala* ssp. *glabrata* over isozymes and accessions, with averages and weighted values (P_j and P_w)

Accessions	Isozymes						AVG	Weighted
	ACO	ADH	IDH	MDH	PGI	PGM	P_j	value P_w
K1	0	0	0	0	0	0	0	0
K6	0.48	0	0.58	0	0.48	0	0.26	0.16
K8	0	0	0	0	0	0.46	0.08	0.03
K28	0.18	0	0	0	0	0.48	0.11	0.05
K29	0	0	0	0	0	0.32	0.05	0.02
K132	0	0	0	0	0	0	0	0
K140	0	0	0	0	0.48	0	0.08	0.07
K217	0	0	0	0	0	0	0	0
K329	0	0	0	0	0	0	0	0
K358	0	0	0.18	0	0.42	0	0.10	0.08
K378	0	0	0.46	0	0.50	0	0.16	0.11
K395	0	0	0	0	0	0	0	0
K397	0.34	0	0.50	0	0.49	0	0.22	0.14
K417	0	0	0.48	0	0.00	0.32	0.13	0.06
K418	0	0	0	0	0	0.32	0.05	0.02
K419	0	0	0.34	0	0	0.46	0.13	0.06
K452	0	0	0.58	0	0.42	0.18	0.20	0.12
K499	0.28	0	0.44	0	0	0.61	0.22	0.11
K517	0.18	0	0	0	0.32	0.18	0.11	0.08
K538c	0	0	0.42	0	0.42	0.66	0.25	0.14
K562c	0	0	0.50	0	0.50	0	0.17	0.11
K565	0	0	0	0	0	0	0	0
K584	0	0	0.46	0	0	0.34	0.13	0.06
K598c	0	0	0.34	0	0.32	0	0.11	0.07
K607c	0	0	0.42	0	0.50	0	0.15	0.11
K608	0	0	0	0	0.32	0.62	0.16	0.09
K614c	0	0	0.32	0	0	0.56	0.15	0.07
K617	0	0	0.70	0	0.48	0	0.20	0.12
K619	0	0	0	0	0	0	0	0
K633c	0	0	0.42	0	0.48	0.58	0.25	0.15
K636	0.18	0	0.42	0	0.32	0.56	0.25	0.14
K638	0	0	0	0	0	0	0	0
K662	0	0	0	0	0	0.18	0.03	0.01
K664c	0.18	0	0.50	0	0	0.50	0.20	0.09
K665c	0	0	0.18	0	0	0.50	0.11	0.05
K678c	0	0	0	0	0.48	0.32	0.13	0.10
Mean	0.05	0	0.23	0	0.19	0.23	0.12	0.07
Percentage *	19.44	0	50.00	0	44.44	52.78		
Total **	3	1	4	1	2	4		

* The percent (%) of accessions phenotypically polymorphic per enzyme
** The total number of phenotypes observed in each enzyme system

0.07 across accessions. This value was very similar to that of *L. diversifolia* ssp. *diversifolia* (2n=104, self-pollinated).

4.4.1.3 Characteristic band associated with the "giant" type of *L. leucocephala*

From the isozymic survey of 77 accessions of the "common" and 36 accessions of the "giant" *L. leucocephala*, one isozymic, band Rf 80 in ACO, was found in association with the "giant" types. There were several so-called "common" accessions from Taiwan Agricultural Research Institute, which also showed Rf 80 ACO band in some of their seedlings. On the other hand, the "giant" accession K6 from New Guinea showed the "common" type phenotype in ACO.

ACO band Rf 80 was used as a reference to review accuracy of K numbers in our *Leucaena* germplasm collections. K21 (PI 188810) from the Philippines proved to be a "common" type that was mislabeled as a "giant" tree. K595 from Mexico was designed a "giant" type tree, but our collection only contained the "common" type seed, because of mislabeling.

4.4.2 Ancestors of *L. diversifolia* (2n=104) and *L. pallida* (2n=104)

Is *L. diversifolia* ssp. *diversifolia* (2n =104) an autotetraploid of *L. diversifolia* ssp. *trichandra* (2n=52), and is *L. pallida* (2n=104) an amphidiploid of *L.*

diversifolia ssp. *trichandra* and *L. esculenta* (2n=52) ? The isozyme similarity among 12 tested *Leucaena* species was examined, using the data in Table 9 to compute average taxonomic distance coefficients employing the NTSYS program (Rohlf, 1987). The distance coefficients from 12 species were presented in Table 25. The distance coefficients varied from 0.27 to 0.54 among the species. The distance coefficient between the two subspecies of *L. leucocephala* was 0.13, as expected, smaller than the coefficients between any two species. However, the distance coefficient between two subspecies of *L. diversifolia* was 0.4, in the range of coefficients between any two species.

The electromorphic distance matrix among all 14 pairwise comparisons (*L. leucocephala* and *L. diversifolia* were divided into two sub species) was subjected to a cluster analysis with UPGMA algorithm. The cluster analysis for these species resulted in several groups (Figure 22). A pronounced branching suggests a complex phylogenetic differentiation within the genus. Two subspecies of *L. leucocephala* showed a close relationship, as expected. Interestingly, *L. collinsii* (2n=52) and *L. lanceolata* (2n=52) also showed quite close relationship. *L. collinsii, L. lanceolata, L. macrophylla, L. pulverurenta* and *L. leucocephala* all showed close relationship too compared with the other species. No close relationship between two *L. diversifolia* subspecies, and between *L. pallida* and its

Table 25. Distance coefficients (dissimilarity) among twelve *Leucaena* species from NTSYS program by using electromorphic data

Species	COL	DIV2	DIV4	ESC	GRE	LAN	LEUc	LEUg	MAC	PAL	PUL	RET	SHAN
COL	0												
DIV2	0.45	0											
DIV4	0.35	0.4	0										
ESC	0.42	0.46	0.39	0									
GRE	0.44	0.53	0.49	0.46	0								
LAN	0.27	0.4	0.3	0.39	0.43	0							
LEUc	0.32	0.41	0.41	0.44	0.44	0.3	0						
LEUg	0.33	0.43	0.4	0.44	0.44	0.3	0.13	0					
MAC	0.32	0.47	0.35	0.38	0.45	0.28	0.34	0.33	0				
PAL	0.41	0.52	0.46	0.41	0.45	0.42	0.42	0.41	0.42	0			
PUL	0.37	0.44	0.41	0.39	0.37	0.33	0.31	0.29	0.34	0.41	0		
RET	0.44	0.55	0.46	0.45	0.41	0.4	0.49	0.48	0.42	0.47	0.4	0	
SHAN	0.35	0.46	0.36	0.42	0.42	0.28	0.36	0.36	0.32	0.41	0.36	0.41	0
TRI	0.43	0.52	0.45	0.46	0.54	0.37	0.46	0.45	0.43	0.5	0.45	0.51	0.32

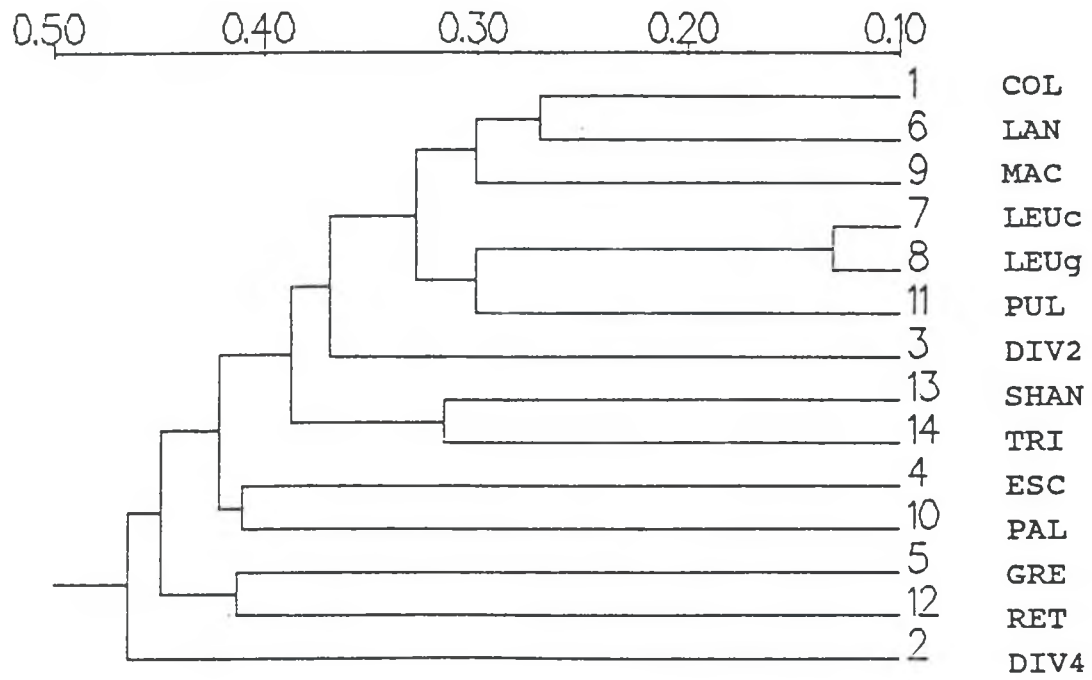


Figure 22. Cluster analysis dendrogram of six enzyme phenotypes in twelve *Leucaena* species

esculenta) was observed. From the limited isozyme markers, the results did not support conclusions that *L. diversifolia* ssp. *diversifolia* is an autotetraploid from *L. diversifolia* ssp. *trichandra*, and *L. pallida* is an amphidiploid from *L. diversifolia* ssp. *trichandra* and *L. esculenta*.

Chapter 5

DISCUSSIONS

5.1 The gene loci of the genus *Leucaena*

The genus *Leucaena* includes 14 widely recognized species, several of which have become pantropical as fodder, fuelwood, shade and ornamental trees (Brewbaker et al. 1987). Because of relatively short maturity from seed to seed, *Leucaena* could be selected as a model for genetic improvement of other tree crops (Sorensson, pers. comm.). There are several reports on the inheritance of traits in *Leucaena* species, but relatively few have been carried through to genetic analysis. Gonzales (1966) revealed the quantitative character of mimosine, a non-essential amino acid found in all *Leucaena* species. Gray (1967) reported that branching habit was controlled by two pairs of disomically inherited genes in *L. leucocephala* (2n=104). Research conducted by Pan (1985) revealed that several morphological characters (i.e. leaf size) of *Leucaena* were quantitatively inherited and that F1 hybrids were intermediate to the parents. Single gene loci were ascribed to pubescent branches and leaves, strong flower odor, pendulous inflorescence and straight stem, each of which were dominant over glabrous, no odor, upright inflorescence and branching or shrubby habit, respectively. Progeny crosses revealed that 4 S alleles controlled self-

incompatibility in diploid *L. diversifolia* and isozyme studies identified 4 peroxidase gene loci (Pan, 1985). Sorensson (1989) reported that the gene Luteus was a lethal recessive. Schiffino-Wittmann et al. (1990) reported that esterase could be used as a genetic marker for further breeding study, as noted earlier by Brewbaker (pers. comm.). The few genetic markers available limit interpretation of relationships among species and the analysis of genotypes within species.

5.2 A standard description of six isozyme systems in *L. leucocephala* (K997) and modified techniques

A standard description of phenotypes is essential for the comparison of the results from different gels and different accessions. A local Hawaiian population of *L. leucocephala* ssp. *leucocephala* (K997) was chosen as a check in this study to make the results comparable because it showed genetic uniformity in these isozyme systems.

Six isozyme systems (ACO, ADH, IDH, MDH, PGI and PGM) were used to elicit consistent band patterns in a histidine-citric acid buffer system. Research revealed about a dozen suspected genetic loci. Procedures for gel preparation were modified and reduced use of chemicals were tried during the initial experiments. A simple modification of normal procedure was to prepare the gel 12 hours previous to electrophoresis. This technique provided improved band resolution, and made daily isozyme analysis easier.

Chemical ingredients for staining solution of these isozyme systems were reduced by one-half concentration and still produced reliable results. Extensive literature exists on modifications of sample preparation, ingredients of buffer, extraction and staining solutions, and even concentrations of starch gel (Soltis et al. 1980). Many staining solution recipes recommend larger quantities of ingredients than required for normal procedures (Aradhya, 1992). This not only wastes the chemicals, but also may produce bands too dark to be recorded, e.g., PGM in this study.

5.3 Ontogenetic variations

Mowrey et al. (1990) reported nine isozyme activities were developmentally regulated in peach (*Prunus persica*). Pan's (1985) work on peroxidase revealed the same result in *Leucaena*. However, ontogenetic experiments of this study did not show isozymic phenotype variation in different plant tissues and life-cycle stages in these isozyme systems except for the ADH system. Results of ADH ontogenic variation in *Leucaena* agree with ADH activity in several angiosperm species and conifers which were developmentally regulated (Harry et al. 1987, Mowrey et al. 1990). ADH activity was highest the first 20 days after sowing in two *L. diversifolias* and *L. pulverurenta*, then, slowly decreased thereafter. Other isozyme systems only showed variation in intensity of their bands in developmental stages and

tissues. Perhaps, they are involved with the strong biochemical activities in these tissues at the certain developmental stages. Variations observed in this study indicated that isozymic phenotypes of ADH associated with ontogenic. Shoot and cotyledon tissues of the other tested *Leucaena* species consistently displayed complete and invariable isozymic phenotypes for ACO, IDH, MDH, PGI and PGM. Consequently, these isozyme systems could be used in genetic studies without further concern for the tissue and life-stage difference.

5.4 Isozymic polymorphism in *Leucaena* species

All six isozymes tested in this study, were found to be variable. The least variable isozyme, MDH, was polymorphic in 66 percent of the tested species, while the most variable isozymes, ACO and PGM, were polymorphic in all species. The array of multiple isozyme phenotypes found in each species were distinctly different from other species; In other words, each species had its own unique phenotypes. Furthermore, in most outcrossing species, isozymic phenotypes found in each accession within species were distinctly different from those of other accessions. Two kinds of isozymic phenotype variability within species were noticed: 1) mosaic pattern, e.g., PGM phenotype distribution in eight *L. diversifolia* ssp. *diversifolia* accessions (Table 18), and 2) distinct pattern, e.g., ACO and PGI phenotype

distribution in the same diploid *diversifolia* accessions (Table 18). These kinds of isozymic variability patterns were also observed in *Avena barbata*. It was suggested that mosaic patterns of isozymic variation were correlated to mosaic pattern habitat and distinct patterns were correlated to specific habitat (Kahler et al., 1980).

Leucaena species were quite diverse isozymically. Although genetic interpretation of these diverse patterns was not attempted, the phenotypic polymorphism of these isozymically analyzed accessions in twelve species were assessed. The average weighted P_w in the tested species varied from 0.01 to 0.28. The results clearly indicated that outcrossing species showed higher levels of electrophoretic variability than self-pollinated species. This study suggests that the levels of electrophoretic variability observed appear to be more strongly correlated with breeding systems than to ploidy levels. Among the species, *L. diversifolia* ssp. *trichandra* accessions had the highest average P_w (0.28), this was visually comfirmed as the species showed great variability morphologically. *L. leucocephala* ssp. *leucocephala* accessions had the lowest average P_w (0.01), too as the species showed uniformity in morphology. Among the three tetraploid species, outcrossing *L. pallida* (2n=104) showed higher P_w (0.13) than those of the self-pollinated species of *L. diversifolia* ssp. *diversifolia* (0.08) and *L. leucocephala* ssp. *glabrata*

(0.07) . This phenomenon was also reported in *Clarkia* species (Smith-Huerta, 1986). The effects of ploidy level on isozyme variability were reported in several other plant groups (Weeden, 1989), such as *Cucurbita* and *Malus*. However, polyploidy *Leucaena* species did not show more variability in these isozyme expression. Average P_w across species was 0.15, similar to that of *Beta vulgaris L.* (Nagamine et al., 1990).

Isozymic polymorphism not only existed among species, but also within species. The limited number of accessions analyzed in this study representing each *Leucaena* species does not allow for a comprehensive estimation of population diversity within each species. However, within the constraints of phenotype polymorphism analysis, it does provide information on diversity within accessions and provides a basis for further evaluation and collection. The results clearly indicated that there was considerable variability within species. Average P_w values varied from accession to accession. For example, the P_ws varied from 0.14 to 0.52 in diploid *diversifolia* accessions. This higher variability within the species could be attributed to the species breeding systems and its wide range of ecological habitats.

Correlation between isozymic and morphological variations were seldom studied especially in forest tree crops (NRC, 1991). Parallel isozyme and morphological

analysis was not conducted in this study, but agreement between variation in isozymic phenotypes in this study and variation in tree growth performance rate and tree form conducted by Dr. Brewbaker in early 1960s was noticed in some of *L. leucocephala ssp. leucocephala* accessions.

5.5 Genetics of ACO and IDH, characterization of species hybrids

Genetic markers of isozyme polymorphism could be confirmed by three methods: 1) examining progeny of controlled crosses, 2) comparing haploid and diploid tissues of an individual, and 3) applying Hardy-Weinberg's law in theoretical models (Rothe, 1990). Contolled progeny crosses were employed in this study. Five loci of these isozymes were confirmed in *L. lanceolata*, three loci in ACO and two loci in IDH. Both ACO and IDH are monomers. Segregation of these loci did not show any linkage among them. ACO also showed three loci in the other diploid *Leucaena* species. However, IDH did not show the same pattern of phenotypes and supposed to be dimmers. ACO was found useful for genetic mapping in soybean, and linkage group of ACO3-Sp1-y12-t was constructed (Kiang, 1991). Linkage studies should be carried out between these loci and loci governing morphological traits in *Leucaena*.

Identification of interspecific hybrids in early growth stages in *Leucaena* may be performed by observing the morphological traits such as number of leaflets per pinna

(Sorensson et al., 1990). However, confusion may result if the two species under study have relative similarities for morphological characters, e.g., diploid and tetraploid *L. diversifolia*. It would be almost impossible to identify intraspecific hybrids morphologically because of similarities in traits. Isozyme characterization was found to be useful in identifying inter and intra-specific hybrids in many studies (Chapparro et al., 1987; Parfitt et al., 1985). This technique was useful to identify the *Leucaena* hybrids.

This could be used to facilitate our *Leucaena* breeding programs. For example, the understanding of breeding systems in the genus by selecting isozyme markers could be sought. Sorensson (1987) found there were some selfed progeny within many interspecific crosses. A seven percent self pollinating rate was found in intraspecific crosses of *L. lanceolata* (K10) by ACO system in this study. This result indicated that there was a certain degree of selfing in self-incompatible species.

5.6 Classification of *Leucaena* species and evolutionary tests

Taxonomic classification of *Leucaena* species is very complex. At one time, there were more then 50 species reported in the genus. Brewbaker (1985) regrouped them to 13 species using morphological characters of living

specimen. New species and subspecies are still being added to the genus. Variation in isozyme phenotypes has long been used to distinguish difference tree species, seedlots and clones (Rothe, 1990). Results from isozyme analysis of 169 accessions from 12 species supports the current classification of the genus. The specific and subspecific bands in ACO, ADH, IDH, MDH, and PGM systems were individually identified. *L. leucocephala* ssp. *glabrata* lines were easily distinguished from *L. leucocephala* ssp. *leucocephala* by simply analyzing ACO phenotypes. Further, for identifying new species and subspecies in the genus, criteria of these specific bands should be considered.

L. leucocephala ssp. *leucocephala*, a tropical legume tree is originated in Central America. It spread to the Philippines in the early 1600s by Spanish galleons, from whence it was pantropically distributed in the 19th century (Brewbaker et al. 1989). If it was true, then, it is hypothesized that all *L. leucocephala* ssp. *leucocephala* outside of its native habitat may be offsprings of one pure line or possibly a single tree, and therefore should be isozymically identical. The results from the six isozyme survey confirmed this hypotheses. Seventy-five percent of the "common" types of *L. leucocephala ssp. leucocephala* showed identical phenotypes for all six isozyme systems. The tested common *L. leucocephala* accessions compared with the other species showed the lowest isozyme phenotypic

polymorphism values (P_j and P_w) in the genus. However, there were a few accessions from the study that showed higher P_j and P_w values. The result from this study is in agreement with field trials at Waimanalo Research Station from the early 1960s to 1970s. Most accessions showed little variation in growth performance and morphology with the exception of a few accessions. Accessions showing higher variation in growth performance and morphlogy in the early study which also showed isozymic variation in this study. These few accessions were mostly from Taiwan and New Guinea, perhaps where early "giant" *L. leucocephala* type introductions were involved.

An analysis of morphological traits in the genus and geographical distributions in Central America suggest that *L. diversifolia* ssp. *diversifolia* (2n=104) is an autotetraploid derived from diploid *L. diversifolia* (2n=52), and that *L. pallida* (2n=104) is an amphidiploid from diploid *L. diversifolia* and *L. esculenta* (2n=52) (Pan, 1985). Electrophoresis techniques were employed to verify the ancestors of these plants. Most bands in the six isozyme systems observed in *L. pallida* were detected in *L. diversifolia* ssp. *trichandra* and *L. esculenta* (Table 9). Pan (1985) also reported that all peroxidase isozyme in *L. pallida* were also present in *L. diversifolia* ssp. *trichandra*. However, the distance dissimilarity coefficient values (Table 33) did not show any close relationship

between *L. pallida* and its postulated ancestors. The distance dissimilarity coefficient value between two sub species of L. diversifolia was higher than as expected. The results were inconclusive in confirming that *L. diversifolia* ssp. *diversifolia* is an autotetraploid from *L. diversifolia* ssp. *trichandra*, and that *L. pallida* is an amphidiploid of *L. esculenta* and *L. diersifolia* ssp. *trichandra*

Interestingly, cluster analysis revealed that *L. collinsii* and *L. lanceolata* show a very close relationship between them. However, the two species exhibit quite different morphological traits. Interspecific crosses between all possible pairs suggest that these two species were closely related producing abundant pods and nearly 100 percent fertile seeds (Sorensson, 1992). This could lead us to suggest that isozyme similarity could also provide clues as to the ease with which species can be crossed in addition to closeness of taxonomic relationship and geographic distribution. *L. macrophylla*, *L. pulverulenta*, and in addition to *L. collinsii* and *L. lanceolata* also showed close relationship with *L. leucocephala*. This interesting result could suggest that tetraploid *L. leucocephala* could be derived from these species.

Chapter 6

SUMMARY AND CONCLUSIONS

There is considerable information in the literature on the electrophoretic technique and its usefulness as a tool for population geneticists and forestry breeders in understanding the population structure and evolution of tree species. However, few reports have been published on electrophoresis for specific isozyme systems in *Leucaena* species. Furthermore, the reported isozyme markers were highly environmentally dependent, like peroxidase and esterase, which were reluctantly selected by research workers for the population study or as a selection marker.

One goal of this study was to define electrophoretic techniques for identifying useful isozyme markers by surveying different buffer and isozyme systems in different plant tissues. A method utilizing a histidine-citrate buffer system was selected, whereby the cotyledon protein could be assayed for isozyme polymorphism by starch gel electrophoresis. Using this technique, several isozyme systems provided useful markers for identification of *Leucaena*, cultivars and hybrids, for the evaluation of genetic variation in *Leucaena* species. Consistent and well resolved variable band patterns were observed for ACO, ADH, IDH, MDH, PGI and PGM. Phenotypes of ADH systems were found to be developmentally regulated and the other isozyme

systems were not. This finding was helpful in conducting genetic studies of ACO, IDH, MDH, PGI and PGM systems without further concern for tissue difference. Gdh, G6pdh, 6-Pgdh and Skdh did not reveal clear and recordable band patterns in any of the three selected buffer systems. Improved procedures, as well as modifications of existing buffer systems, might be required for the successful analysis of these isozyme systems. More isozyme systems should be explored for the enrichment of genetic markers in *Leucaena*.

For convenience of comparison between the different gel results, a local Hawaiian population of *L. leucocephala ssp. leucocephala* (K997) was selected as a check because of its monomorphic expression of these isozyme systems. A standard description of phenotypes for K997 was established.

Genetic analysis revealed that isozyme systems of ACO and IDH were under the control of three loci and two loci, respectively in sibcrosses of *L. lanceolata* (K10). These two systems were inherited independently and may be used as genetic markers in *Leucaena*. Three loci of ACO were also found in the other diploid *Leucaena* species. In the furture, linkage studies could be carried out between these loci and loci governing morphological traits, especially those of economic importance.

Understanding genetic structure in tree species is a

prerequisite for genetic management and conservation. Extensive collection of *Leucaena* species has been conducted by University of Hawaii under Dr. Brewbaker and Oxford Forestry Institute. Little information about genetic structure of these collections has been available. Another purpose of this study was to characterize the genetic structures of species in the genus Leucaena by surveying available *Leucaena* accessions with electrophoresis technique. Extensive ioszyme polymorphism was observed among twelve *Leucaena* species. The P_ws of weighted polymorphism were high among the outcrossing species and low among self-pollinated species. The level of electrophoretic variability was more highly correlated with breeding system than to ploidy levels. Isozyme polymorphism was analyzed to clarify phylogeny of *Leucaena* species and hybrids.

The genus Leucaena has received a variety of taxonomic treatments and confusion still exists as to which species and subspecies should be recognized. Sometimes, accessions are difficult to classify within the present species classification, as they show characteristics intermediate between two species. Electrophoretic characterization of species in the genus Leucaena was found reliable and could be used as an aid to their classification. Twelve species, comprising 169 accessions were surveyed for six isozyme

systems. Five systems have been found useful in identifying species and subspecies by presence or absence of specific bands. These isozyme systems were also found effective in identifying inter and intra-specific hybrids.

Regarding *Leucaena* species evolution, it was suggested that the "common" *L. leucocephala ssp. leucocephala* first reached the Philippines in the early 1600s via Spanish sailors, from whence it was distributed throughout tropics by the 19th century. From the electrophoretic survey of 77 pantropical accessions, it was confirmed that all "common" types of *L. leucocephala ssp. leucocephala* in the world with the exception of those from Central America, were offspring from a pure line variety or from a single tree.

The determination of the ancestral parentage of present polyploids is made through a number of traditional evaluations including morphological similarities, geographic distributions, and the chromosome paring in meiosis. Electrophoresis was employed in this study to test some of the evolutionary hypotheses regarding the origin of polyploidy in *Leucaena*. It has been suggested that *L. pallida* (2n=104) is an amphiploid derived from *L. diversifolia* ssp. *trichandra* (2n=52) and *L. esculenta* (2n=52), and that *L. diversifolia* ssp. *diversifolia* (2n=104) is an autotetraploid from diploid *L. diversifolia ssp. trichandra*. Isozyme data were inconclusive and did not

support these hypotheses, but did indicate that tetraploid *L. leucocephala* may be derived from two of these species, *L. collinsii, L lanceolata, L. macrophylla and L. pulverulenta*.

Appendix A

Listed K number, taxon, origin, latutude, longitude, PI number, ID number, and elevation of tested *Leucaena* accessions

L. collinsii ssp. *collinsii* (2n=52)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
905	COL	Chiapas	16.36 N	93.00 W		OFI 45/85	500

L. collinsii ssp. *zacapana* (2n=52)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
911	COL	Guatemala	14.47 N	90.47 W		J88-5	800
912	COL	Guatemala	14.47 N	90.04 W		J88-6	650
913	COL	Guatemala	14.50 N	90.04 W		J88-7	600
914	COL	Guatemala	14.56 N	89.31 W		J88-8	225
917	COL	Guatemala	14.36 N	89.40 W		J88-11	750

L. diversifolia ssp. *trichandra* (2n=52 diploids)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
408	DIV2	Guatemala	14.55 N	89.57 W	443491	J78-3	900
483	DIV2	Honduras	14.01 N	86.21 W	443501	J78-80	1200
821	DIV2	Oaxaca	17.05 N	96.55 W		J85-54	2475 e
823	DIV2	Oaxaca	17.20 N	96.26 W		J85-56	2600 e
907	DIV2	Guatemala	14.43 N	91.32 W		J88-1b	1600
919	DIV2	Guatemala	14.38 N	89.48 W		J88-13	1500
927	DIV2	Guatemala	14.24 N	90.26 W		J88-22	1250
936	DIV2	Honduras	14.15 N	87.28 W		J88-32	1000

L. diversifolia ssp. *diversifolia* (2n=104 tetraploids)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
776	DIV4	Veracruz	18.54 N	97.00 W		J85-6c	1130 e
782	DIV4	Veracruz	18.52 N	97.03 W		J85-13	1175 e
790	DIV4	Veracruz	18.52 N	96.55 W		J85-22	775 e
796	DIV4	Veracruz	19.33 N	96.56 W		J85-28	1525 e
802	DIV4	Veracruz	19.23 N	96.57 W		J85-34	1200 e
946	DIV4	Mexico	19.26 N	96.45 W		OFI 45/87	800
947	DIV4	Veracruz	19.32 N	96.55 W		OFI 46/87	1275

L. esculenta ssp. *esculenta* (2n=52)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
689	ESC	Morelos	18.45 N	99.15 W e			1150 e
948	ESC	Guerrero	18.18 N	99.45 W		OFI 47/87	1550 e
949	ESC	Guerrero	18.38 N	100.48 W		OFI 48/87	600

(Appendix A. cont.)

L. esculenta ssp. *matudae* (2n=52)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
950	ESC	Guerrero	17.51 N	99.40 W		OFI 49/87	650 e

L. greggii (2n=56)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
854	GRE	Nuevo Leon	24.40 N	99.55 W		VS85-8	1830 e
855	GRE	Nuevo Leon	24.50 N	100.05 W		VS85-9c	1830 e
858	GRE	Coahuila	25.25 N	101.00 W		VS85-12	1880
862	GRE	Nuevo Leon	25.45 N	100.50 W		VS85-16c	1830 e
956	GRE	Nuevo Leon	24.53 N	100.01 W		OFI 82/87	1550 e

L. lanceolata ssp. *lanceolata* (2n=52)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
10	LAN	Nayarit	21.50 N	105.07 W	286248		100 e
381	LAN	Oaxaca	16.21 N	95.09 W		J77-39c	90 e
401	LAN	Guerrero	17.58 N	101.49 W			150 e
470	LAN	Chiapas	16.13 N	93.52 W		J78-67	225 e

L. lanceolata ssp. sousae (2n=52, tentative subspecific epithet)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
952	LAN	Oaxaca	15.40 N	96.30 W		OFI 51/87	50

L. leucocephala ssp. *leucocephala* (2n=104)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
2	LEUc	Senegal	No original locale		281784	MX35	
12	LEUc	Honduras	14.05 N	86.46 W e	282405		500e
13	LEUc	Taiwan	No original locale		282474	CSR31182	
21	LEUc	Philippines	No original locale		188810		
22	LEUc	Philippines	No original locale		241167		
23	LEUc	Zaire	No original locale		247682		
24	LEUc	S.Africa	No original locale		274470		
25	LEUc	India	No original locale		279180		
26	LEUc	Virgin Isl.	No original locale		281605		
31	LEUc	Australia	No original locale		281627		
32	LEUc	Tanzania	No original locale		281636		
35	LEUc	New Britain	No original locale		281775		
36	LEUc	New Caledonia	No original locale		281777		
37	LEUc	New Caledonia	No original locale		281778		
38	LEUc	New Caledonia	No original locale		281779		
40	LEUc	Sri Lanka	No original locale		281781		
41	LEUc	Ghana	No original locale		281782		
42	LEUc	Sierra Leone	No original locale		281783		
43	LEUc	Philippines	No original locale		282396		
46	LEUc	Australia	No original locale		282461	Q544	
47	LEUc	Fiji	No original locale		282462	CPI19580	
48	LEUc	New Caledonia	No original locale		282463	CPI19852	
49	LEUc	Brazil	No original locale		282464	CPI2	

(Appendix A. cont.)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
50	LEUc	Philippines	No original locale		282465	CPI29215	
51	LEUc	Thailand	No original locale		282466	CPI29633	
52	LEUc	Vietnam	No original locale		282467	CPI30479	
54	LEUc	Australia	No original locale		282469		
55	LEUc	Australia	No original locale		282470		
56	LEUc	Australia	No original locale		282471		
57	LEUc	Australia	No original locale		282472		
53	LEUc	Vietnam	No original locale		282468	CPI30481	
58	LEUc	Australia	No original locale		282473		
60	LEUc	Taiwan	No original locale		282817		
61	LEUc	New Caledonia	No original locale		283697		
62	LEUc	Ivory Coast	No original locale		286295		
63	LEUc	Oahu	No original locale		288000		
64	LEUc	Uganda	No original locale		288001		
68	LEUc	Philippines	No original locale		288006		
70	LEUc	Singapore	No original locale		288008		
71	LEUc	Indonesia	No original locale		288009		
72	LEUc	Maui	No original locale		288011		900
73	LEUc	Philippines	No original locale		290753		
76	LEUc	Taiwan	No original locale		279577	C32	
77	LEUc	Taiwan	No original locale		295360	C60	100
78	LEUc	Taiwan	No original locale		295361	C61	
79	LEUc	Taiwan	No original locale		295362	C62	
80	LEUc	Taiwan	No original locale		295363	C63	
81	LEUc	Taiwan	No original locale		295364	C64	
82	LEUc	Taiwan	No original locale		295364	C65	
83	LEUc	Indonesia	No original locale		317908		
84	LEUc	Fiji	No original locale		317909		
85	LEUc	Am. Samoa	No original locale		317910		
86	LEUc	Tahiti	No original locale		317911		200
87	LEUc	S. Africa	No original locale		300010		
88	LEUc	S. Africa	No original locale		300011		
89	LEUc	Sierra Leone	No original locale		305453		
115	LEUc	Tanzania	No original locale		319842		
278	LEUc	Kauai	No original locale				10
279	LEUc	Kauai	No original locale				10
281	LEUc	Oahu	No original locale				10
296	LEUc	Yucatan	20.22 N	89.46 W	324914		50e
304	LEUc	Benin	No original locale		330481		
313	LEUc	Thailand	No original locale				60
314	LEUc	Thailand	No original locale				
315	LEUc	Thailand	No original locale				
316	LEUc	Thailand	No original locale				10
317	LEUc	Thailand	No original locale				15
318	LEUc	Thailand	No original locale			<=K8?>	
319	LEUc	Thailand	No original locale				
320	LEUc	Thailand	No original locale				
321	LEUc	Thailand	No original locale				
322	LEUc	Thailand	No original locale				
323	LEUc	Thailand	No original locale				
325	LEUc	Thailand	No original locale				
326	LEUc	Thailand	No original locale				
341	LEUc	Hawaii	20.14 N	155.5 W			
676	LEUc	Malaysia	No original locale				
677	LEUc	Philippines	No original locale				
678	LEUc	Thailand	No original locale			J80-3c	
997	LEUc	Hawaii	No original locale			UH2	

(Appendix A. cont.)

L. leucocephala ssp. *glabrata* (2n=104)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
1	LEUg	N.Guinea	No original locale		281770	CPI18623?	
6	LEUg	N.Guinea	No original locale		281772	N.G.4164	
8	LEUg	Zacatecas	21.16 N	103.10 W	263695		1100e
28	LEUg	El Salvador	13.41 N	89.17 W	281607		750e
29	LEUg	Honduras	14.24 N	89.13 W	281608		750e
132	LEUg	Morelos	18.39 N	99.13 W	324391	J67-9	925e
140	LEUg	Puebla	18.22 N	97.15 W	324393	J67-17	1200e
217	LEUg	El Salvador	13.36 N	88.29 W	324310	J67-98	750e
218	LEUg	El Salvador	13.36 N	88.29 W	324300	J67-98	750e
358	LEUg	Campeche	19.51 N	90.32 W		J77-16	5e
378	LEUg	Oaxaca	16.40 N	96.18 W		J77-35	775e
395	LEUg	Oaxaca	16.22 N	95.22 W		J77-53	240e
397	LEUg	Oaxaca	16.29 N	95.55 W		J77-55	750e
417	LEUg	El Salvador	13.26 N	88.42 W	443480	J78-12c	120e
418	LEUg	El Salvador	13.21 N	88.29 W	443481	J78-13c	275e
419	LEUg	El Salvador	13.21 N	88.28 W	443482	J78-14c	275 e
452	LEUg	Oaxaca	16.20 N	95.14 W	443575	J78-47c	100 e
499	LEUg	Quintana Roo	18.30 N	88.18 W			60 e
517	LEUg	Merida	20.45 N	89.00 W	443591	Oak3193	30 e
538	LEUg	Morelos	18.44 N	99.16 W	443610	Oak3216	1100 e
562	LEUg	Colima	19.12 N	103.48 W	443629	Oak3253	450 e
565	LEUg	Colima	19.13 N	103.42 W	443631	Oak3256	485 e
584	LEUg	Veracruz	19.46 N	96.25 W	443643	Oak3280	25 e
598	LEUg	Tamaulipas	22.24 N	97.55 W	443655	Oak3296	10 e
607	LEUg	Tamaulipas			443663	Oak3305	
608	LEUg	Tamaulipas	24.51 N	98.10 W	443664	Oak3306	60 e
614	LEUg	Tamaulipas	25.55 N	97.35 W	443670	Oak3314	15 e
617	LEUg	Tamaulipas	25.58 N	98.00 W	443673	Oak3317	25 e
619	LEUg	Tamaulipas	26.32 N	99.00 W e	443675	Oak3319	100 e
622	LEUg	Tamaulipas	26.09 N	98.25 W	443538	Oak3322	50 e
633	LEUg	Coahuila	25.33 N	100.58 W	443688	Oak3333	1400 e
636	LEUg	Coahuila	25.25 N	101.00 W	443740	Oak3336	1575 e
638	LEUg	Nuevo Leon	25.40 N	100.15 W	443692	Oak3338	500 e
664	LEUg	Tamaulipas			443710	Oak3366	
665	LEUg	Nuevo Leon	25.12 N	99.49 W	443711	Oak3368	275 e
678	LEUg	Thailand	No original locale			J80-3c	

L. macrophylla ssp. *nelsonii* (2n=52,tentative subspecific epithet)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
902	MAC	Oaxaca	15.59 N	97.16 W		CEH47/85	10

L. pallida (2n=104)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
376	PAL	Oaxaca	17.08 N	96.46 W		J77-33c	1675 e
806	PAL	Puebla	18.37 N	97.24 W		J85-38	2000 e
819	PAL	Oaxaca	17.21 N	96.50 W		J85-52	1925 e
953	PAL	Puebla	18.38 N	97.24 W		OFI 52/87	2100

(Appendix A. cont.)

L. pulverulenta (2n=56)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
870	PUL	Texas	26.15 N	98.15 W		V85-24	60
957	PUL	Tamaulipas	23.36 N	99.14 W		OFI 83/87	1250 e
958	PUL	Texas				OFI 84/87	

L. retusa (2n=56)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
502	RET	Texas	29.33 N	103.05 W	435920	J79-2	915
503	RET	Texas	29.33 N	103.05 W	435921	J79-3	1005
506	RET	Texas	29.51 N	102.48 W	435924	J79-6	800
899	RET	Texas	29.44 N	102.43 W		Kirmse	
900	RET	Texas	30.37 N	104.03 W		Kirmse	

L. salvadorensis (2n=52)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
746	SAL	Honduras	13.52 N	87.18 W		OFI(H&S129	660

L. shannonii ssp. *magnifica* (2n=52)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
916	SHAN	Guatemala	14.36 N	89.38 W		J88-10	770
769	SHAN	Guatemala	14.36 N	89.34 W			950

L. shannonii ssp. *shannonii* (2n=52)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
741	SHAN	Honduras	14.22 N	87.39 W		OFI 22/83	650
924	SHAN	Guatemala	14.22 N	89.43 W		J88-18	500
925	SHAN	Guatemala	14.16 N	89.56 W		J88-19	900
954	SHAN	Campeche	19.20 N	90.43 W		OFI 53/87	5

L. trichodes (2n=52)

K No.	TAXON	ORIGIN	LAT	LONG	PI No	ID No	ELEV
903	TRI	Venezuela	9.38 N	70.18 W		OFI 2/86	700

Appendix B

Distribution and frequencies of six isozymic phenotypes in ten *Leucaena* species through table B.1 to table B.10

Table B.1. Frequencies of six isozyme phenotypes in *L. diversifolia* ssp. *diversifolia* (2n=104)

Phenotypes	Accessions							Total	Frequency %
	K776	K782	K790	K796	K802	K946	K947		
ACO									
1	24	25	9	25	25	22	19	149	85.14
2	1		14					15	8.57
3							6	6	3.43
4			2					2	1.14
5						2		2	1.14
6						1		1	0.57
ADH									
1			25	25	25	13	14	102	58.29
2	25	25				12	11	73	41.71
MDH									
1	25	25	25	25	25	25	25	175	100.00
IDH									
1	21	25	25					71	40.57
2				19		24	18	61	34.86
3					25			25	14.29
4				6		1	7	14	8.00
5	4							4	2.29
PGI									
1	14	25	25	25	25	25	17	156	89.14
2	11							11	6.29
3							5	5	2.86
4							3	3	1.71
PGM									
1	21		25			22	25	93	53.14
2				25	25			50	28.57
3		21						21	12.00
4	4							4	2.29
5		4						4	2.29
6						3		3	1.71
Total Samples	25	25	25	25	25	25	25	175	

Table B.2. Frequencies of six isozyme phenotypes in *L. esculenta*

Phenotypes	Accessions				Total	Frequency %
	K689	K948	K949	K950		
ACO						
1	3				3	3.80
2	6				6	7.59
3	2				2	2.53
4	1				1	1.27
5				14	14	17.72
6				3	3	3.80
7		2	1		3	3.80
8		8	22		30	37.97
9		2			2	2.53
10		2			2	2.53
11		6			6	7.59
12		2			2	2.53
13		1			1	1.27
14		1			1	1.27
15		1			1	1.27
16			2		2	2.53
ADH						
1	12	3			15	19.23
2				5	5	6.41
3				5	5	6.41
4				2	2	2.56
5				2	2	2.56
6				3	3	3.85
7		6	16		22	28.21
8		6	4		10	12.82
9		5			5	6.41
10		4	2		6	7.69
11		1			1	1.28
12			3		3	3.85
MDH						
1	12	25	25	17	79	100.00
IDH						
1	3				3	3.90
2	2				2	2.60
3	7				7	9.09
4				16	16	20.78
5				1	1	1.30
6		5			5	6.49
7		5			5	6.49
8		8	12		20	25.97
9		3			3	3.90
10		1			1	1.30
11		1			1	1.30
12		1			1	1.30
13		1	9		10	12.99
14			2		2	2.60
15			1		1	1.30

(Table B.2. cont.)

Phenotypes	Accessions				Total	Frequency %
	K689	K948	K949	K950		
PGI						
1	12				12	15.19
2		1	9	7	17	21.52
3		2	5	6	13	16.46
4		15	8	4	27	34.18
5		3	2		5	6.33
6			1		1	1.27
7		2			2	2.53
8		2			2	2.53
PGM						
1	12				12	15.19
2				3	3	3.80
3				4	4	5.06
4				3	3	3.80
5				2	2	2.53
6				2	2	2.53
7				1	1	1.27
8				1	1	1.27
9				1	1	1.27
10		5			5	6.33
11		13			13	16.46
12		3	8		11	13.92
13		1	11		12	15.19
14		1			1	1.27
15		1			1	1.27
16		1			1	1.27
17			3		3	3.80
18			1		1	1.27
19			1		1	1.27
20			1		1	1.27
Total samples	12	25	25	17	79	

Table B.3. Frequencies of six isozyme phenotypes in *L. greggii*

Phenotypes	Accessions					Total	Frequency %
	K854	K855	K858	K862	K956		
ACO							
1	23	9	25	25	15	97	94.17
2	2					2	1.94
3					2	2	1.94
4					2	2	1.94
ADH							
1	17	9	13	25	19	83	80.58
2	8		12			20	19.42
MDH							
1	15	9	16	15	19	74	71.84
2	10		9	10		29	28.16
IDH							
1	25	9	25	25	19	103	100.00
PGI							
1	1	5			8	14	13.59
2		2			6	8	7.77
3		1			2	3	2.91
4					1	1	0.97
5					1	1	0.97
6		1			1	2	1.94
7	3		6	9		18	17.48
8	21		19			40	38.83
9				16		16	15.53
PGM							
1					1	1	0.97
2	13				6	19	18.45
3		3	1		3	7	6.80
4					2	2	1.94
5			1		2	3	2.91
6	6	5	4		5	20	19.42
7		1				1	0.97
8	1		7			8	7.77
9	4					4	3.88
10	1					1	0.97
11			4			4	3.88
12			1			1	0.97
13			1	5		6	5.83
14			1			1	0.97
15			1			1	0.97
16			1	1		2	1.94
17			1			1	0.97
18			1	5		6	5.83
19			1			1	0.97
20				5		5	4.85
21				1		1	0.97
22				2		2	1.94
23				5		5	4.85
24				1		1	0.97
Total samples	25	9	25	25	19	103	

Table B.4. Frequencies of six isozyme phenotypes in *L. lanceolata*

Phenotypes	Accessions					Total	Frequency %
	K10	K381	K401	K470	K952		
ACO							
1	4					4	3.74
2	1					1	0.93
3	3		22			25	23.36
4	2		3			5	4.67
5	1					1	0.93
6	3					3	2.80
7	1					1	0.93
8	2					2	1.87
9	2					2	1.87
10	1					1	0.93
11	1					1	0.93
12	1					1	0.93
13	1					1	0.93
14	1					1	0.93
15	1					1	0.93
16		7		5	3	15	14.02
17				4	1	5	4.67
18				1	1	2	1.87
19				3	1	4	3.74
20				1	3	4	3.74
21				2	6	8	7.48
22		2				2	1.87
23		5				5	4.67
24		2				2	1.87
25					1	1	0.93
26					2	2	1.87
27					4	4	3.74
28					2	2	1.87
29					1	1	0.93
ADH							
1	25	16	17	16	25	99	92.52
2			8			8	7.48
MDH							
1	25					25	23.36
2		16		16	25	57	53.27
3			25			25	23.36
IDH							
1	11			4	25	40	37.38
2	10					10	9.35
3	1		22			23	21.50
4	3					3	2.80
5		9		12		21	19.63
6		7				7	6.54
7			3			3	2.80
PGI							
1	3					3	2.80
2	10	6		4		20	18.69
3	2					2	1.87

(Table B.4. cont.)

Phenotypes	Accessions					Total	Frequency %
	K10	K381	K401	K470	K952		
PGI							
4	1		1			2	1.87
5	7		13			20	18.69
6	1					1	0.93
7	1					1	0.93
8		2		7		9	8.41
9		8		5		13	12.10
10			7			7	6.54
11			3			3	2.80
12			1		18	19	17.76
13					7	7	6.54
PGM							
1	10					10	9.35
2	15		5			20	18.69
3		1	8	16	10	35	32.71
4		3	7			10	9.35
5		2				2	1.87
6		2				2	1.87
7		3			5	8	7.48
8		4				4	3.74
9		1				1	0.93
10			2			2	1.87
11			3			3	2.80
12					3	3	2.80
13					2	2	1.87
14					2	2	1.87
15					1	1	0.93
16					2	2	1.87
Total samples	25	16	25	16	25	107	

Table B.5. Frequencies of six isozyme phenotypes in *L. macrophylla*

Phenotypes	Accessions				Total	Frequency %
	K836	K880	K902	K955		
ACO						
1	25				25	25.00
2		6			6	6.00
3		2			2	2.00
4		6			6	6.00
5		11			11	11.00
6			7		7	7.00
7			2	1	3	3.00
8			2		2	2.00
9			14		14	14.00
10				12	12	12.00
11				3	3	3.00
12				4	4	4.00
13				2	2	2.00
14				3	3	3.00
IDH						
1	7				7	7.00
2	13		5		18	18.00
3	5	25	18	25	73	73.00
4			2		2	2.00
ADH						
1	25		25	22	72	72.00
2		25			25	25.00
3				3	3	3.00
MDH						
1	12		25	25	62	62.00
2		20			20	20.00
3		5			5	5.00
4	13				13	13.00
PGI						
1	25	25	7	25	82	82.00
2			18		18	18.00
PGM						
1	15		9	2	26	26.00
2	3		1		4	4.00
3	7		15		22	22.00
4		2			2	2.00
5		3			3	3.00
6		4			4	4.00
7		6			6	6.00
8		3			3	3.00
9		1			1	1.00
10		2			2	2.00
11		2			2	2.00
12		2			2	2.00
13				5	5	5.00
14				6	6	6.00

(Table B.5. cont.)

Phenotypes	Accessions				Total	Frequency %
	K836	K880	K902	K955		
PGM						
15				3	3	3.00
16				3	3	3.00
17				1	1	1.00
18				1	1	1.00
19				1	1	1.00
20				2	2	2.00
21				1	1	1.00
Total samples	25	25	25	25	100	

Table B.6. Frequencies of six isozyme phenotypes in *L. pallida*

Phenotypes	Accessions			Total	Frequency %
	K376	K819	K953		
ACO					
1	9	10	24	43	57.33
2	12	7	1	20	26.67
3	2			2	2.67
4	2	3		5	6.67
5		2		2	2.67
6		3		3	4.00
IDH					
1	9			9	12.00
2	16	25		41	54.67
3			12	12	16.00
4			13	13	17.33
ADH					
1	25	25	18	68	90.67
2			7	7	9.33
MDH					
1	25	25	25	75	100.00
PGI					
1	25	25	2	52	69.33
2			23	23	30.67
PGM					
1	9	13		22	29.33
2	1	6		7	9.33
3	8	5		13	17.33
4	3		23	26	34.67
5	1			1	1.33
6	3			3	4.00
7		1		1	1.33
8			2	2	2.67
Total samples	25	25	25	75	

Table B.7. Frequencies of six isozyme phenotypes in *L. pulverulenta*

Phenotypes	Accessions			Total	Frequency %
	K870	K957	K958		
ACO					
1	4			4	5.33
2	2			2	2.67
3	3			3	4.00
4	14			14	18.67
5	1			1	1.33
6	1			1	1.33
7		7		7	9.33
8		3		3	4.00
9		3		3	4.00
10		1		1	1.33
11		1		1	1.33
12		1		1	1.33
13		5		5	6.67
14		3		3	4.00
15		1		1	1.33
16			10	10	13.33
17			3	3	4.00
18			9	9	12.00
19			2	2	2.67
20			1	1	1.33
ADH					
1	25	15	25	65	86.67
2		10		10	13.33
MDH					
1	25	25	25	75	100.00
IDH					
1	8	22		30	40.00
2	16			16	21.33
3	1			1	1.33
4		3		3	4.00
5			18	18	24.00
6			3	3	4.00
7			3	3	4.00
8			1	1	1.33
PGI					
1	2		21	23	30.67
2			4	4	5.33
3		25		25	33.33
4	23			23	30.67
PGM					
1	21			21	28.00
2	4		8	12	16.00
3		25	17	42	56.00
Total samples	25	25	25	75	

Table B.8. Frequencies of six isozyme phenotypes in *L. retusa*

Phenotypes	Accessions				Total	Frequency %
	K503	K506	K899	K900		
ACO						
1	1				1	1.15
2	3				3	3.45
3	1				1	1.15
4	7				7	8.05
5	1				1	1.15
6	2				2	2.30
7	1				1	1.15
8	2				2	2.30
9	1				1	1.15
10	5				5	5.75
11	1				1	1.15
12			1		1	1.15
13			1		1	1.15
14			1		1	1.15
15			1		1	1.15
16			3		3	3.45
17			5		5	5.75
18			8		8	9.20
19			2		2	2.30
20			1		1	1.15
21			2		2	2.30
22				6	6	6.90
23				5	5	5.75
24				14	14	16.09
25		1			1	1.15
26		1			1	1.15
27		3			3	3.45
28		1			1	1.15
29		4			4	4.60
30		1			1	1.15
31		1			1	1.15
ADH						
1	25		20	25	70	80.46
2			4		4	4.60
3			1		1	1.15
4		12			12	13.79
MDH						
1	25	12	25	25	87	100.00
IDH						
1	10	10	25	11	56	64.37
2	8	1		10	19	21.84
3	7				7	8.05
4		1			1	1.15
5				3	3	3.45
6				1	1	1.15
PGI						
1	12	12	2		26	29.89
2	2		12		14	16.09
3	4				4	4.60

(Table B.8. cont.)

Phenotypes	Accessions				Total	Frequency %
	K503	K506	K899	K900		
PGI						
4	7				7	8.05
5			8		8	9.20
6			3		3	3.45
7				19	19	21.84
8				2	2	2.30
9				4	4	4.60
PGM						
1	19	2		18	39	44.83
2	2	2	3	7	14	16.09
3	4				4	4.60
4		1			1	1.15
5		1			1	1.15
6		2	4		6	6.90
7		2			2	2.30
8		1			1	1.15
9		1			1	1.15
10			4		4	4.60
11			6		6	6.90
12			1		1	1.15
13			4		4	4.60
14			3		3	3.45
Total samples	25	12	25	25	87	

Table B.9. Frequencies of six isozyme phenotypes in *L. shannonii*

Phenotypes	Accessions					Total	Frequency %
	K741	K916	K924	K925	K954		
ACO							
1	1					1	0.82
2	1					1	0.82
3	7					7	5.74
4	3					3	2.46
5	1					1	0.82
6	3					3	2.46
7	1					1	0.82
8	1					1	0.82
9	2					2	1.64
10	1					1	0.82
11	1					1	0.82
12	2					2	1.64
13	1					1	0.82
14		21				21	17.21
15		4				4	3.28
16			13			13	10.66
17			2			2	1.64
18			1			1	0.82
19			6			6	4.92
20			2			2	1.64
21			1			1	0.82
22				19		19	15.57
23				2		2	1.64
24				1		1	0.82
25					10	10	8.20
26					6	6	4.92
27					4	4	3.28
28					1	1	0.82
29					1	1	0.82
30					2	2	1.64
31					1	1	0.82
ADH							
1	25			5		30	24.59
2		25				25	20.49
3			11		25	36	29.51
4			12			12	9.84
5			2			2	1.64
6				9		9	7.38
7				1		1	0.82
8				5		5	4.10
9				2		2	1.64
MDH							
1	25		25	22	11	83	68.03
2		25				25	20.49
3					14	14	11.48
IDH							
1	25		24	19		68	55.74
2		25				25	20.49
3			1	3		4	3.28
4					12	12	9.84
5					13	13	10.66

(Table B.9. cont.)

Phenotypes	Accessions					Total	Frequency %
	K741	K916	K924	K925	K954		
PGI							
1	12					12	9.84
2	2					2	1.64
3	1					1	0.82
4	3					3	2.46
5	7					7	5.74
6		12				12	9.84
7		9				9	7.38
8		4				4	3.28
9			11		3	14	11.48
10			4	9	5	18	14.75
11			1		17	18	14.75
12			6			6	4.92
13			1			1	0.82
14			2			2	1.64
15				13		13	10.66
PGM							
1	1					1	0.82
2	1				3	4	3.28
3	1	5				6	4.92
4	3					3	2.46
5	5					5	4.10
6	1					1	0.82
7	1					1	0.82
8	3				9	12	9.84
9	1					1	0.82
10	3					3	2.46
11	1					1	0.82
12	1					1	0.82
13	1				1	2	1.64
14	1				3	4	3.28
15	1					1	0.82
16		12				12	9.84
17		8				8	6.56
18			8			8	6.56
19			3			3	2.46
20			3			3	2.46
21			1			1	0.82
22			2			2	1.64
23			1			1	0.82
24			6			6	4.92
25			1			1	0.82
26				5		5	4.10
27				3		3	2.46
28				4		4	3.28
29				1		1	0.82
30				2		2	1.64
31				1		1	0.82
32				4		4	3.28
33				1		1	0.82
34				1		1	0.82
35					8	8	6.56
36					1	1	0.82
Total samples	25	25	25	22	25	122	

Table B.10.. Frequencies of six isozyme phenotypes in *L. trichodes* (K903)

Phenotypes	Total	Frequency %
ACO		
1	5	20.00
2	1	4.00
3	8	32.00
4	7	28.00
5	1	4.00
6	1	4.00
7	1	4.00
8	1	4.00
ADH		
1	22	88.00
2	3	12.00
IDH		
1	22	88.00
2	3	12.00
MDH		
1	13	52.00
2	10	40.00
3	2	8.00
PGI		
1	25	100.00
PGM		
1	9	36.00
2	5	20.00
3	11	44.00

Appendix C

Frequencies (%) of six isozyme phenotypes in *L. leucocephala* ssp. *leucocephala*

K No.	Isozymes and its phenotypes													
	ACO			ADH		IDH			MDH	PGI		PGM		
	1	2	3	1	2	1	2	3	1	1	2	1	2	3
K2	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K12	12	87.5	0	43.8	56	37.5	50	13	100	50	50	50	18.8	31
K13	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K21	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K22	100	0	0	100	0	100	0	0	100	18.8	81	37.5	25	38
K23	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K24	100	0	0	100	0	100	0	0	100	75	25	87.5	0	13
K25	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K26	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K31	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K32	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K35	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K36	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K37	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K38	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K40	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K41	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K42	100	0	0	100	0	100	0	0	100	50	50	100	0	0
K43	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K46	100	0	0	100	0	100	0	0	100	58.3	42	100	0	0
K47	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K48	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K49	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K50	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K51	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K52	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K53	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K54	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K55	100	0	0	100	0	0	63	38	100	87.5	13	93.8	6.25	0
K56	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K57	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K58	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K60	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K61	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K63	60	0	40	100	0	90	10	0	100	20	80	40	20	40
K64	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K68	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K70	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K71	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K72	100	0	0	100	0	90	10	0	100	100	0	100	0	0
K73	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K76	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K77	40	0	60	100	0	90	10	0	100	100	0	100	0	0
K78	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K79	40	0	60	100	0	10	90	0	100	100	0	100	0	0
K80	70	0	30	100	0	40	60	0	100	90	10	100	0	0
K81	80	0	20	100	0	70	30	0	100	100	0	100	0	0

(Appendix C. cont.)

K No.	Isozymes and its phenotypes													
	ACO			ADH		IDH			MDH	PGI		PGM		
	1	2	3	1	2	1	2	3	1	1	2	1	2	3
K82	50	0	50	100	0	20	80	0	100	90	10	90	10	0
K83	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K84	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K85	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K86	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K87	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K88	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K89	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K109	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K115	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K269	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K278	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K279	100	0	0	100	0	100	0	0	100	100	0	90	10	0
K281	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K304	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K313	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K314	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K315	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K316	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K317	100	0	0	100	0	70	30	0	100	100	0	100	0	0
K318	0	0	100	100	0	100	0	0	100	70	30	10	0	90
K319	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K320	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K321	100	0	0	100	0	90	10	0	100	100	0	100	0	0
K322	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K323	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K325	100	0	0	100	0	100	0	0	100	50	50	100	0	0
K326	100	0	0	100	0	100	0	0	100	50	50	100	0	0
K341	100	0	0	100	0	100	0	0	100	100	0	100	0	0
K676	100	0	0	100	0	100	0	0	100	40	60	100	0	0
K677	100	0	0	100	0	100	0	0	100	100	0	100	0	0

Appendix D

Frequencies (%) of six isozyme phenotypes in *L. leucocephala* ssp. *glabrata*

K No.	Isozymes and its phenotypes														
	ACO			ADH	IDH				MDH	PGI		PGM			
	1	2	3	1	1	2	3	4	1	1	2	1	2	3	4
K1	0	100	0	100	100	0	0	0	100	100	0	100	0	0	0
K6	40	60	0	100	0	50	10	50	100	60	40	100	0	0	0
K8	0	100	0	100	100	0	0	0	100	0	100	20	10	70	0
K28	10	90	0	100	100	0	0	0	100	0	100	60	0	40	0
K29	0	100	0	100	100	0	0	0	100	100	0	20	0	80	0
K132	0	100	0	100	0	100	0	0	100	0	100	100	0	0	0
K140	0	100	0	100	0	100	0	0	100	40	60	0	0	100	0
K217	0	100	0	100	100	0	0	0	100	0	100	0	0	100	0
K329	0	100	0	100	0	100	0	0	100	100	0	100	0	0	0
K358	0	100	0	100	0	90	10	0	100	70	30	100	0	0	0
K378	0	100	0	100	0	20	10	70	100	50	50	100	0	0	0
K395	0	100	0	100	0	100	0	0	100	100	0	100	0	0	0
K397	0	21	79	100	0	64	7.1	29	100	42.86	57	100	0	0	0
K417	0	100	0	100	40	60	0	0	100	0	100	0	0	20	80
K418	0	100	0	100	100	0	0	0	100	0	100	0	0	80	20
K419	0	100	0	100	80	10	10	0	100	0	100	0	20	70	10
K499	0	83	17	100	33.3	67	0	0	100	0	100	33.3	17	50	0
K452	0	0	100	100	50	40	10	0	100	70	30	90	10	0	0
K517	0	90	10	100	0	100	0	0	100	20	80	10	0	90	0
K538	0	0	100	100	70	30	0	0	100	70	30	30	30	40	0
K562	0	100	0	100	50	50	0	0	100	50	50	100	0	0	0
K565	0	0	100	100	0	100	0	0	100	0	100	100	0	0	0
K584	0	0	100	100	70	20	10	0	100	0	100	80	10	10	0
K595	100	0	0	100	100	0	0	0	100	100	0	100	0	0	0
K598	0	100	0	100	0	80	10	10	100	80	20	100	0	0	0
K607	0	100	0	100	70	30	0	0	100	50	50	100	0	0	0
K608	0	100	0	100	0	100	0	0	100	20	80	50	20	30	0
K614	0	0	100	100	80	20	0	0	100	0	100	60	20	20	0
K617	0	100	0	100	20	40	30	10	100	40	60	100	0	0	0
K619	0	0	100	100	100	0	0	0	100	0	100	100	0	0	0
K633	0	100	0	100	70	30	0	0	100	40	60	10	40	50	0
K636	0	90	10	100	70	30	0	0	100	20	80	20	20	60	0
K638	0	0	100	100	100	0	0	0	100	0	100	100	0	0	0
K662	0	0	100	100	100	0	0	0	100	100	0	90	0	10	0
K664	0	90	10	100	50	50	0	0	100	0	100	50	50	0	0
K665	0	100	0	100	10	90	0	0	100	0	100	50	0	50	0
K678	0	100	0	100	0	100	0	0	100	40	60	80	20	0	0

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