Genetic Variation in Leucaena leucocephala (Lam.) de Wit. (Leguminosae: Mimosoideae)

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Abstract

Leucaena leucocephala (Lam.) de Wit. is a multi-purpose neotropical tree, with a long history of human use, which is now distributed pantropically. The level of genetic variability within and among 24 populations of *L. leucocephala* has been assessed using isozyme analysis (324 plants; 12 populations) and restriction analyses (23 populations) of the chloroplast DNA (cpDNA) and nuclear ribosomal DNA (rDNA). In a survey of three isozyme systems (AAT; PER; PGI) multi-enzyme phenotypes were encountered such that it was possible to identify the two subspecies of *L. leucocephala* and inter-population differentiation. Partitioning of phenotypic diversity within and among populations revealed that 76% of the variation resided among *L. leucocephala* populations. Evidence of either multiple introductions or hybridisation/introgression was found in a Haitian collection. Seven restriction fragments showed cpDNA variation and evidence for between 1 and 5 rDNA repeats per population was encountered. These results are discussed in relation to the utilization of *L. leucocephala* as a multi-purpose tree crop.

Key words: *Leucaena leucocephala*, Leguminosae, multi-purpose tree, genetic variation, chloroplast DNA, ribosomal DNA, isozymes.

FDC: 165.3; 165.5; 176.1 *Leucaena leucocephala*.

Introduction

The potential of multi-purpose tree species to enhance the diversity, sustainability and productivity of marginal ecosystems has recently received increased attention. However, despite their ecological and agricultural importance little is known about the basic biology of multi-purpose tropical trees (Simons, 1991) and rarely have assessments of their genetic variability been attempted. Such knowledge is vital as the genetic improvement of any organism depends on the existence, type and amount of the genetic variability available for manipulation.

Leucaena leucocephala (Lam.) de Wit. is native to the neotropics and widely planted throughout the tropics. The value of *L. leucocephala* as a multi-purpose tropical tree lies in its combination of fast growth and high quality products (fodder, fuel, green manure); qualities which have led to its wide promotion by national and international developmental agencies (Brewbaker, 1987; Hughes, 1993; Pound and Martinez, 1983; National Academy of Sciences, 1984). However, with an increase in its use a number of important limitations of *L. leucocephala* have become apparent (Hughes, 1993). These include: lack of cold and drought tolerance, poor growth on acid soils, heavy pod production, poor wood durability and susceptibility to defoliation by the psyllid bug, *Heteropsylla cubana* (Hughes, 1993). Within the neotropics *Leucaena* has been used for at least 2000 years as a minor food crop and source of timber products (Casas, 1992; Harris et al., in press) and as a result the partitioning and distribution of genetic variation within *L. leucocephala* may be complex. *Leucaena leucocephala* (along with *L. esculenta s.l.*) is the most widely cultivated, harvested and marketed species in the genus (Hughes, 1993). It has, clearly, been widely transported. Indeed, there are few settlements in the tropical and subtropical zones of Mexico where *L. leucocephala* is not found. Added to its widespread use by Man, is the high degree of artificial crossing between taxa within the genus (Sorensson and Brewbaker, in press) and the resulting possibility of hybridisation/introgression. The occurrence of hybrids in the wild has been confirmed with the discovery of sporadic, but common, *L. leucocephala* ssp. *glabrata* x *L. esculenta* ssp. *esculenta* hybrids in south-central Mexico (Hughes and Harris, in press).

Leucaena leucocephala is known to be a self-compatible tetraploid (2n=4x=104; de Freitas et al., 1991; Frahm-Leliveld, 1957 and 1960; Gonzalez et al., 1976; Hutton, 1981; Shibata, 1962; Tijo, 1948; Turner and Fearing, 1960). Two subspecies have been recognised, ssp. *leucocephala* and ssp. *glabrata* (Zarate, 1987). Subspecies *leucocephala*, a shrubby taxon, that occurs in the Yucatan Peninsula and the Isthmus of Tehuantepec (Mexico; Hughes, 1993), was introduced to the Far East in the 17th Century and has now spread pantropically, becoming a weed in certain areas (Hughes and Styles, 1989; Zarate, 1987). Subspecies *glabrata*, a more arborescent taxon, was introduced to many tropical countries in the last 3 decades and has been the focus of most of the agronomic and utilisation work in the genus. The level of genetic variation within the species is largely unknown and, although the narrow genetic base of the commonly planted varieties from El Salvador and Mexico has been appreciated (Brewbaker, 1980 and 1985), one or a few self-pollinated progenies have apparently become dominant over the tropics in the last 3 decades (Hughes, 1993). This makes *L. leucocephala* an extreme example of a tropical tree crop with a very narrow genetic base. Assessment of the levels of genetic variation between *L. leucocephala* populations, within and outside the neotropics, is important for understanding the origin of the species and for its genetic improvement.

The traditional approach to the assessment of forest genetic resources has been to examine a combination of morphological and agronomic traits which, in the main, exhibit continuous variation. However, the effectiveness of this approach has been questioned by several authors (Brown, 1979; Gottlieb, 1977). The application of biochemical and molecular techniques has provided a powerful set of tools for the study of genetic diversity within and among plant populations. The most widespread, and commonly used, approach is the analysis of protein isozymes (Soltis and Soltis, 1990). More recently restriction fragment length polymorphisms (RFLPs; Clegg, 1989a and b), DNA "fingerprinting" (Besse et al., 1993) and randomly amplified polymorphic DNA sequences (RAPDs; Welsh et

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al., 1991; WELSH, 1990) have been used to study genetic diversity. The majority of such studies have been concerned with temperate herbs or shrubs, relatively few studies have been concerned with angiosperm trees, (e.g. TORRES, 1990), and fewer still with tropical angiosperm trees (e.g. CHALMERS, 1992; HAMRICK and LOVELESS, 1989; SCHNABEL and HAMRICK, 1900; SYTSMA and SCHAAL, 1985).

Attempts have been made to identify intraspecific variation within *Leucaena leucocephala* using traditional analyses of morphological and agronomic traits (BREWBAKER, 1987). Whilst significant differences in performance were detected between subspecies very little variation was detected within ssp. *glabrata* (e.g. ARORA, 1981; BREWBAKER, 1972; WHEELER et al., 1987). An initial report of isozyme variation in 12 individuals of *L. leucocephala* concluded that very little genetic variation existed in this species (SCHIFINO-WITTMANN and SCHLEGEL, 1990). The purpose of this paper is to assess the levels of genetic diversity found between *L. leucocephala* accessions collected from widely dispersed sites in Mexico and Central America, using isozymes and RFLPs derived from the chloroplast and nuclear genomes.

Material and Methods

Plant material

Three hundred and twenty four plants of *Leucaena leucocephala* from 12 populations were assessed for isozyme diversity, of which 55 individuals were from 2 populations of ssp. *leucocephala* and 268 individuals were from 10 populations of ssp. *glabrata* (*Table 1*). Ten populations came from Mexico and Central America [Mexico (6); Gua-

Table 1. — Locations of the populations analysed and the number of individuals sampled using chloroplast DNA, nuclear ribosomal DNA and isozyme markers. For isozyme markers the figures in pharentheses are the number of families sampled; 'bulk' indicates that seed sample contained material from a number of trees. All vouchers are 'Hughes' unless otherwise stated and are deposited at K, MEXU and FHO. Code refers to the number used by the OFI *Leucaena* germplasm collection.

Code	Voucher	Locality	Lat.	Long.	No's analysed cpDNA	rDNA	isozyme
		Leucaenaleucocephala (Lam.) de Wit. ssp. glabrata (Rose) Zarate					
		Guatemala					
139/92	1698	Barillas, Huehuetenango	15° 48' N	91° 48' W	1	1	–
44/88	-	Gualan, Zacapa	15° 06' N	89° 22' W	1	1	25 (bulk)
117/92	1689	Ixtahuacan, Huehuetenango	15° 23' N	91° 50' W	1	1	20 (7)
45/88	-	Masagua, Escuintla	-	-	1	1	25 (bulk)
		Haiti					
32/88	-	Operation Double Harvest	-	-	1	1	25 (bulk)*
		Honduras					
19/81	Stead & Styles705	Duyure, Choluteca	13° 38' N	86° 55' W	1	1	36 (18)
		Madagascar					
mad	–	Montagne des Français	12° 19' S	49° 20' E	–	–	25 (bulk)
		Mexico					
91/92	1547	El Pescadero, Baja California	23° 22' N	110° 09' W	1	1	–
95/92	1557	Loreto, Baja California Sur	26° 00' N	111° 21' W	1	1	–
136/92	1679	Cintalapa de Figueroa, Chiapas	16° 46' N	93° 26' W	1	1	–
93/92	1578	Ixmiquilapan, Hidalgo	20° 30' N	99° 12' W	1	1	8 (3)
84/92	1638	Teotitlan del Camino, Oaxaca	18° 10' N	97° 05' W	1	1	51 (20)
123/92	1625	Santiago Acatepec, Puebla	18° 14' N	97° 33' W	1	1	–
121/92	1618	Zapotitlan Salinas, Puebla	18° 19' N	97° 28' W	1	1	–
86/92	1596	Lagunita, Queretaro	21° 14' N	99° 15' W	1	1	27 (10)
85/92	1591	Tamazunchale, San Luis Potosi	21° 17' N	98° 48' W	1	1	27 (10)
145/91	1519	Caitime, Sinaloa	25° 13' N	107° 59' W	1	1	–
94/92	1568	Empalme, Sonora	27° 57' N	110° 48' W	1	1	–
92/92	1574	Tecupa, Sonora	28° 31' N	109° 10' W	1	1	–
		U.S.A.					
74/91	-	Waimanalo, Hawaii	-	-	1	1	–
		Leucaenaleucocephala (Lam.) de Wit. ssp. leucocephala					
		Mexico					
80/92	1734	Francisco Escarcega, Campeche	18° 35' N	90° 46' W	1	1	30 (10)
133/92	1671	Matias Romero, Oaxaca	16° 48' N	95° 03' W	1	1	25 (bulk)
147/92	1735	Chetumal, Quintana Roo	18° 31' N	88° 24' W	1	1	–
		U.S.A.					
73/91	-	Waimanalo, Hawaii	-	-	1	1	–

*) Seed was collected from 18 individuals and bulked. Other bulked seed collections were made from an unspecified number of individuals.

temala (3); Honduras (1)], 1 population came from Haiti and 1 population from Madagascar. The Haitian and Madagascan populations have been introduced, the Duyure (Mexico) population was derived from cultivated material introduced from elsewhere, whilst the Gualan (Guatemala) and Masagua (Guatemala) populations were derived from recently established plantations. Populations were sampled in 2 ways: (i) seeds from individuals within a population were bulked to form a single sample (32/88; 44/88; 45/88; 133/92; Madagascar) and then 25 randomly sampled seeds were examined from the bulk collection; (ii) seeds from between 3 and 20 families per population were collected in the field (117/92; 19/81; 84/92; 85/92; 86/92; 93/92; 80/92) and then between 1 and 3 individuals per family were randomly sampled for examination.

Twelve additional populations were examined for DNA variation (*Table 1*). Ten populations were identified as ssp. *glabrata* and 2 populations were identified as ssp. *leucocephala*. Ten of the populations were from Mexico and Central America [Mexico (9); Guatemala (1)] and 2 were introductions to Hawaii. Single individuals were sampled for DNA analyses.

Seeds were grown in a mixture of 8 parts Levingtons Professional (M2) compost and 2 parts fine gravel [powdered calcium carbonate was added at the rate of 5 g/l] under a 16 hour day length. Five days after germination seedlings were inoculated with *Rhizobium* strains TAL1145 and TAL1187.

Isozyme analyses

Electrophoresis was used to separate isozymes by standard techniques (Wendel and Weeden, 1990). Briefly electrophoresis was carried out on 12 % starch using a gel buffer, at pH 8.3, which comprised 5.4 g Tris-base, 1.28 g anhydrous citric acid and 100 ml electrode buffer per litre of distilled, deionised water. The electrode buffer, at pH 8.3, comprised 1.2 g lithium hydroxide and 11.9 g boric acid per litre of distilled, deionised water. The terminal pair of eophyll leaflets from 20 to 30 day old seedlings were ground in 1 drop of extraction buffer. For AAT and PGI, the extraction buffer comprised 50 ml gel buffer, 37 mg KCl, 10 mg $MgCl_2$, 18 mg tetrasodium EDTA, 25 mg PVPP, 0.5 ml Triton-X-100 and 2 ml β-mercaptoethanol (β-ME). For peroxidase the PVPP, Triton-X-100 and β-ME were replaced by 811 mg ascorbic acid. Enzyme extracts were absorbed onto filter-paper wicks which were loaded onto the gels and run at 4 °C and 70 mA until the 1% bromophenol blue tracker dye had migrated 8 cm from the origin. Gels were stained for the following isozymes: aspartate aminotransferase (AAT; E.C. 2.6.1.1); peroxidase (PER; E.C. 1.11.1.7); phosphoglucose isomerase (PGI; E.C. 5.3.1.9).

DNA analyses

Intact, total DNA was extracted from fresh leaf material of individual plants (*Table 1*) according to the methods of Doyle and Doyle (1987), but with the following modifications: 1% PVP-40T was added to the 2 x CTAB extraction buffer and the mixture incubated for 30 minutes at 65 °C. The extraction buffer: fresh weight ratio was greater than 25 ml/g FW. Samples were purified on caesium chloride density gradients containing 0.75 g/ml caesium chloride and 100 μg/ml ethidium bromide and centrifuged overnight at 20 °C at 40,000 rpm (Sorvall OTD65B centrifuge with T865.1 rotor; Maniatis et al., 1982). Extracted DNA was digested with 14 different 6bp-cutting restriction enzymes [*Bam*HI; *Bcl*I; *Bgl*II; *Bsc*I; *Eco*RI; *Eco*RV; *Hin*DIII; *Nru*I; *Nsi*I; *Pst*I; *Pvu*II; *Sac*I; *Stu*I; *Xho*I]. Restriction fragments were separated on 1 % agarose gels at 60 mA overnight. DNA fragments were blotted on to Amersham Hybond N or BDH nylon membrane and baked for 2 hours at 80 °C. Filters were then sequentially probed with the following *Vigna* chloroplast DNA clones (Palmer and Thompson, 1981): MB1; MB2; MB3; MB5+MB7; MB9; MB11+MB12 and the wheat ribosomal DNA probe, pTA71 (Gerlach and Bedbrook, 1979). Probes (60 ng) were labelled by the random primer method (Feinberg and Vogelstein, 1983) using ^{32}P-dCTP (3000 Ci/mM, 10 μCi). Hybridisation took place overnight at 65 °C in Buffer III [0.6 M sodium chloride, 10 mM PIPES (pH 6.8), 1 mM disodium-EDTA (pH 8.5), 10 x Denhardt's solution]. Filters were washed using the conditions recommended by Palmer (1986) and then exposed to X-ray film for 6 hours to 6 days.

Results

Isozyme variation

The 3 enzyme systems analysed, AAT, PER and PGI, were polymorphic. In addition, superoxide dismutase (SOD; E.C. 1.15.1.1.) was scored from PGI gels as achromatic 'ghost' bands; this system was monomorphic.

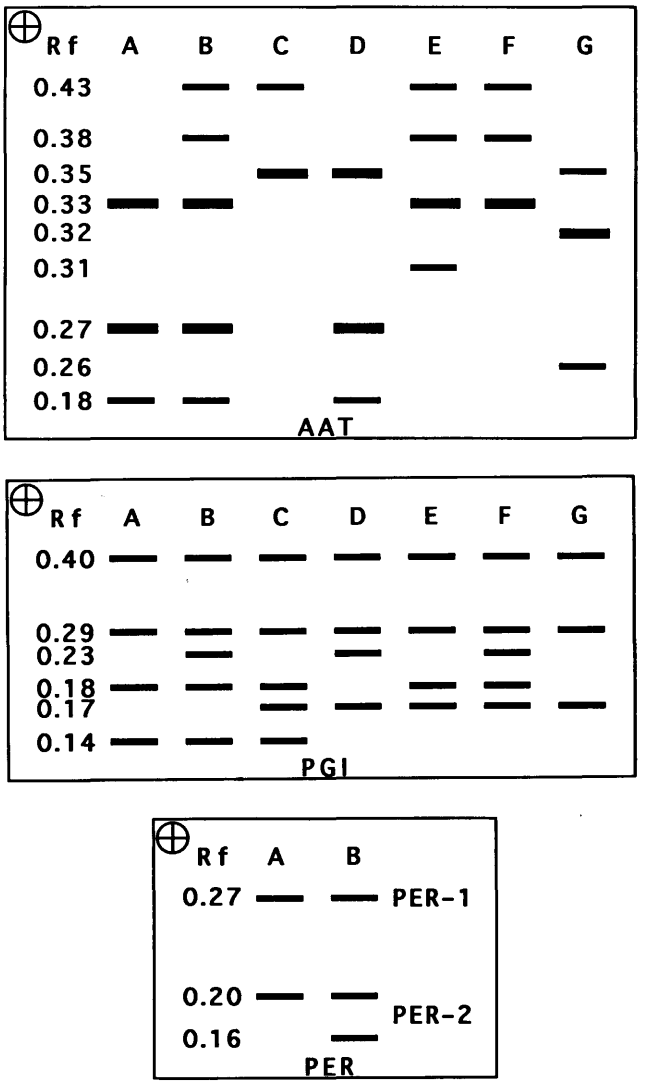

Figure 1. — Zymogram for the polymorphic enzymes found in *Leucaena leucocephala*. The letters refer to the phenotype designations used in the text. For PER, putative loci are identified.

Table 2. — Frequency of single-enzyme phenotypes for the three polymorphic enzymes across the 12 populations of *Leucanea leucocephala*. Phenotype designations are given in *figure 1*. Gua — Guatemala; Hai — Haiti; Hon — Honduras; Mad — Madagascar; Mex — Mexico.

Enzyme	Phenotype	ssp. glabrata										ssp. leucocephala	
		Gua 44/88	Gua 45/88	Gua 117/92	Hai 32/88	Hon 19/81	Mad mad	Mex 84/92	Mex 85/92	Mex 86/92	Mex 93/92	Mex 80/92	Mex 133/92
AAT	A	1.00	1.00	–	0.92	–	–	1.00	0.89	–	0.25	–	–
	B	–	–	–	0.08	1.00	1.00	–	0.11	1.00	–	–	–
	C	–	–	–	–	–	–	–	–	–	–	1.00	–
	D	–	–	–	–	–	–	–	–	–	–	–	1.00
	E	–	–	0.60	–	–	–	–	–	–	0.38	–	–
	F	–	–	0.25	–	–	–	–	–	–	–	–	–
	G	–	–	0.15	–	–	–	–	–	–	0.38	–	–
PER	A	1.00	1.00	0.85	1.00	1.00	–	1.00	1.00	–	0.75	–	1.00
	B	–	–	0.15	–	–	1.00	–	–	1.00	0.25	1.00	–
PGI	A	–	–	–	0.28	–	–	0.06	1.00	–	1.00	1.00	1.00
	B	1.00	1.00	1.00	0.24	–	1.00	–	–	1.00	–	–	–
	C	–	–	–	0.40	1.00	–	0.02	–	–	–	–	–
	D	–	–	–	0.04	–	–	0.84	–	–	–	–	–
	E	–	–	–	–	–	–	0.02	–	–	–	–	–
	F	–	–	–	–	–	–	0.06	–	–	–	–	–
	G	–	–	–	0.04	–	–	–	–	–	–	–	–

(i) Aspartate aminotransferase

Several AAT bands occurred between Rf 0.18 and Rf 0.43 producing a total of 7 distinct phenotypes (*Fig. 1*). Of these, between 1 (e.g. Duyure, Honduras) and 3 (Ixtahuacan, Guatemala) were found in a given population (*Table 2*). However, the banding patterns were such that it was not possible to provide a simple model of genetic control for the variation exhibited.

(ii) Peroxidase

Two regions of peroxidase activity were identified which were interpreted as loci (*Per*-1 and *Per*-2). *Per*-1 was monomorphic (Rf 0.27). *Per-2* was polymorphic for apparently 2 alleles which behaved as monomeric units (Rf 0.16 and 0.20; *Fig. 1*). Given the assumption is correct that the isozyme variation of *Per*-2 is caused by allelic variation at a single locus, then the phenotypes of *Per-2* can be considered genotypes. None of the populations contained the homozygous slow genotype, 58 % had the homozygous fast genotype only, 25 % had the heterozygous genotype only and 17 % of the populations had a mixture of homozygous fast and heterozygous genotypes (*Table 2*).

(iii) Phosphoglucose isomerase

Two regions of PGI activity were resolved. The most anodally migrating region (Rf 0.40) was monomorphic and probably corresponded to the region of plastid PGI activity (Weeden and Wendel, 1990). Bands within the

Table 3. — Frequency of multi-enzyme phenotypes (AAT:PER:PGI) within populations of *Leucaena leucocephala* subspecies.

AAT: PER: PGI	ssp. glabrata										ssp. leucocephala	
	Gua 44/88	Gua 45/88	Gua 117/92	Hai 32/88	Hon 19/81	Mad Mad	Mex 84/92	Mex 85/92	Mex 86/92	Mex 93/92	Mex 80/92	Mex 133/92
A:A:A	-	-	-	0.24	-	-	0.06	0.89	-	-	-	-
A:A:B	1.00	1.00	-	0.24	-	-	-	-	-	-	-	-
A:A:C	-	-	-	0.36	-	-	0.02	-	-	-	-	-
A:A:D	-	-	-	0.04	-	-	0.84	-	-	-	-	-
A:A:E	-	-	-	-	-	-	0.02	-	-	-	-	-
A:A:F	-	-	-	-	-	-	0.06	-	-	-	-	-
A:A:G	-	-	-	0.04	-	-	-	-	-	-	-	-
A:B:A	-	-	-	-	-	-	-	-	-	0.24	-	-
B:A:A	-	-	-	0.04	-	-	-	0.11	-	-	-	-
B:A:C	-	-	-	0.04	1.00	-	-	-	-	-	-	-
B:B:B	-	-	-	-	-	1.00	-	-	1.00	-	-	-
C:B:A	-	-	-	-	-	-	-	-	-	-	1.00	-
D:A:A	-	-	-	-	-	-	-	-	-	-	-	1.00
E:A:A	-	-	-	-	-	-	-	-	-	0.38	-	-
E:A:B	-	-	0.60	-	-	-	-	-	-	-	-	-
F:A:B	-	-	0.25	-	-	-	-	-	-	-	-	-
G:A:A	-	-	-	-	-	-	-	-	-	0.38	-	-
G:B:B	-	-	0.15	-	-	-	-	-	-	-	-	-

Gua — Guatemala; Hai — Haiti; Hon — Honduras; Mad — Madagascar; Mex — Mexico.

Table 4. — Number of single-enzyme phenotypes (S.E.) for the analysed polymorphic isozymes AAT, PER and PGI. For multi-enzyme phenotypes, the number of phenotypes (M.E.), the number of individuals (n) and families (f) analysed, SIMPSON'S diversity index (D) for the 12 populations and SHANNON'S information measure (H_{pop}) for those populations with family information are shown.

Population	S.E.	M.E.	n	f	D	H_{pop}*
117/92	6	3	20	7	0.407	1.379
19/81	3	1	36	18	0.000	0.000
32/88	8	7	25	–	0.681	–
44/88	3	1	25	–	0.000	–
45/88	3	1	25	–	0.000	–
84/92	7	5	51	20	0.274	0.592
85/92	4	2	27	10	0.152	0.503
86/92	3	1	27	10	0.000	0.000
93/92	6	3	8	3	0.470	1.585
mad	3	1	25	–	0.000	–
133/92	3	1	25	–	0.000	–
80/92	3	1	30	10	0.000	0.000

*) for ssp. *glabrata*: $H_{pop} = 0.677$.
$H_{sp} = 2.761$.
$H_{pop}/H_{sp} = 0.245$.
$(H_{sp} - H_{pop})/H_{sp} = 0.755$.

other region could not be easily interpreted as the products of particular loci or alleles, however seven distinct phenotypes were resolved (Rf 0.14—0.29; *Fig. 1*). Of these, between 1 (e.g. Duyure, Honduras) and 5 (Ixtahuacan, Guatemala; Operation Double Harvest, Haiti) were found in a given population (*Table 2*).

Treating the data set as multi-enzyme phenotypes for AAT, PER and PGI, 18 individual phenotypes were distributed across the 12 populations and 324 individuals analysed (*Table 3*). Two multi-enzyme phenotypes (12%) were represented by a single individual. Eleven of the multi-enzyme phenotypes (61%) were limited to a single population, i.e. few of the populations shared phenotypes. In contrast, the most widespread multi-enzyme phenotypes were present in 56 (3 populations) and 33 (3 populations) individuals respectively. The number of multienzyme phenotypes per population ranged from one (Duyure, Honduras; Gualan, Guatemala; Masagua, Guatemala; Matias Romero, Mexico; Francisco Escarcega, Mexico; Montagne des Français; Madagascar) to 7 (Operation Double Harvest, Haiti), with a mean of 3.1. Individuals from each of the families examined were identical, except in 2 cases. Two individuals from one family of the Teotitlan del Camino (Mexico) population gave multi-enzyme phenotypes A:A: C and A:A:E respectively. Three individuals from one family of the Tamazunchale (Mexico) population gave multi-enzyme phenotypes A:A:A (2 individuals) and B:A:A (1 individual). These 2 families were excluded from the Shannon information analysis of the data set.

To obtain a measure of multi-enzyme phenotype diversity in each population, PIELOU'S correction of the SIMPSON index (D: PEET, 1974; PIELOU, 1969) was calculated (based on all of the individuals analysed). This index of heterogeneity takes into account the number of phenotypes present and the evenness which they are distributed in a finite sample. D may range from zero (a uniform sample) to one (a completely heterogeneous sample). In this study the values of D ranged from 0.000 to 0.681 (*Table 4*). Those populations for which family material was available were used to calculate estimates of within population diversity (H_{pop}) using SHANNON'S information measure, $H_{pop} = -\Sigma p_i \cdot \log_2 p_i$, where p_i is the multi-enzyme phenotype frequency (KING and SCHAAL, 1989; LEWONTIN, 1972; *Table 4*). The relative ranking of SHANNON'S information measure among the populations was the same as for SIMPSON'S measure (*Table 4*). For *Leucaena leucocephala* ssp. *glabrata* populations, SHANNON'S measure of phenotypic diversity was used to partition the diversity into within- and among-population components (*Table 4*). An examination of the proportion of diversity present within populations (H_{pop}/H_{sp}) and among populations $[H_{sp} - H_{pop})/H_{sp}]$ indicated that, on average, most of the diversity (76%) occurred among populations of ssp. *glabrata* (*Table 4*).

The number of shared multi-enzyme phenotypes for the 12 populations was low. Only 8 (12 %) of the 66 possible pair-wise comparisons between populations shared any multi-enzyme phenotypes. Four populations (117/92; 93/92; 133/92; 80/92) did not share any multi-enzyme phenotypes with any other *Leucaena leucocephala* populations (*Table 3*).

Chloroplast DNA variation

The *Vigna* cpDNA probes that cross-hybridised successfully to the *Leucaena leucocephala* chloroplast genome covered approximately 85% of the *Vigna* chloroplast genome. The whole of the invert repeat and small single copy region were covered; approximately 15% of the large single copy region was not covered (see PALMER, 1981). The 14 6bp-cutting restriction enzymes used in this study sampled 3636 bp, representing 2.4 % of the *Leucaena* chloroplast genome (assuming that the genome size is approximately 150 kb; PALMER et al., 1988). Each accession was screened for 84 probe enzyme combinations (PECs), i.e. 14 restriction enzymes with 6 cpDNA probes. Four PECs showed intraspecific variation for 7 restriction fragments among the 23 populations analysed (*Table 5*).

Nuclear ribosomal DNA variation

Fourteen 6bp-cutting restriction enzymes were used to assess the nuclear 18S—5.8S—25S ribosomal DNA (rDNA) for intraspecific variation. Five of the enzymes did not provide information on rDNA diversity, either as a result of not cutting (*Bsc*I; *Nru*I; *Pst*I; *Pvu*II) or cutting only very sporadically over the 23 populations studied (*Xho*I). No intraspecific rDNA variation was detected with 4 en-

Table 5. — Distribution of chloroplast DNA variants among accessions of *Leucaena leucocephala*. Type A — MB5+MB7/*Xho*I (3.9 kb); Type B — MB9/*Bsc*I (4.5 kb); Type C — MB11+MB12/*Eco*RV (7.0 kb); Type D — MB11+MB12/*Eco*RV (6.6 kb); Type E — MB11+MB12/*Eco*RV (6.4 kb); Type F — MB11+MB12/*Eco*RV (3.6 kb); Type G — MB11+MB12/*Hin*DIII (3.3 kb).

Population	Fragment presence (1) or absence (0)						
	A	B	C	D	E	F	G
Leucaena leucocephala ssp. glabrata							
Guatemala							
139/92	1	?	?	?	0	0	1
44/88	1	1	1	1	0	1	0
117/92	0	1	0	0	1	0	1
45/88	1	1	0	0	1	0	0
Haiti							
32/88	1	1	0	0	1	0	0
Honduras							
19/81	1	0	1	1	0	1	0
Mexico							
91/92	1	?	?	?	?	?	0
95/92	1	?	?	?	?	?	0
93/92	1	?	?	?	?	?	0
136/92	0	?	0	0	0	0	0
84/92	1	?	?	?	?	?	0
123/92	1	?	0	0	0	0	1
121/92	0	?	?	?	?	?	0
86/92	1	?	?	?	?	?	0
85/92	1	?	?	?	?	?	0
145/91	1	1	0	0	1	0	0
94/92	0	?	?	?	?	?	0
92/92	0	?	?	?	?	?	?
USA							
74/91	0	?	?	?	?	?	0
Leucaena leucocephala ssp. leucocephala							
Mexico							
80/92	1	?	0	0	0	0	1
133/92	0	?	0	0	0	0	1
147/92	0	?	0	0	0	0	1
USA							
73/91	1	?	0	0	0	0	1

zymes (*Bgl*II; *Eco*RV; *Sac*I; *Stu*I). Five enzymes showed intraspecific rDNA variation (*Bam*HI; *Bcl*I; *Eco*RI; *Hin*DIII; *Nsi*I), of which only data from *Eco*RI will be presented.

Analysis of *Eco*RI digests showed the majority of the intraspecific variation could be explained due to differences in rDNA repeat length. Mapping of the *Eco*RI sites relative to a conserved *Eco*RV site (Baldwin, 1993; Rafalski, 1983) showed that the length variation occurred

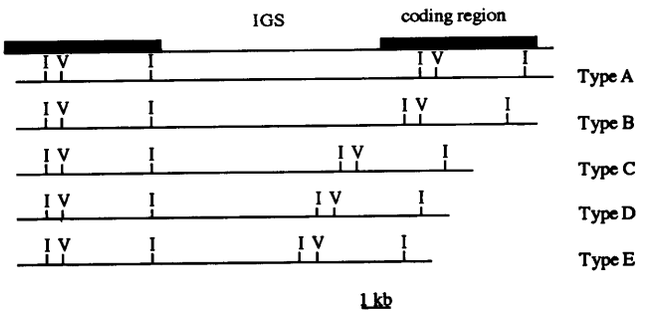

Figure 2. — Ribosomal DNA repeat types found in *Leucaena leucocephala*. Type A — 13.4 kb; Type B — 12.8 kb; Type C — 10.5 kb; Type D — 9.7 kb; Type E — 9 kb. Black boxes above the repeat types indicates the approximate position of the coding region. 'I' — *Eco*RI site and 'V' — *Eco*RV site.

Table 6. — Distribution of ribosomal DNA repeat length variants among accessions of *Leucaena leucocephala*.

Population	Repeat type and size (kb)					Rpt
	A	B	C	D	E	No.
	15.4	14.8	12.5	11.7	11.0	
Leucaena leucocephala ssp. glabrata						
Guatemala						
139/92	*	*		*		3
44/88	*			*		2
117/92	*		*	*		3
45/88	*		*	*		3
Haiti						
32/88	*			*		2
Honduras						
19/81	*			*		2
Mexico						
91/92		*	*		*	3
95/92	*	*			*	3
93/92		*	*		*	3
84/92	*	*	*			3
123/92	*	*	*	*	*	5
121/92	*			*		2
86/92		*	*		*	3
85/92		*			*	2
145/91	*			*		2
94/92	*		*			2
92/92	*					1
Leucaena leucocephala ssp. leucocephala						
Mexico						
80/92	*			*	*	3
133/92	*	*		*		3
147/92	*		*	*		3
USA						
73/91		*				1

within the intergenic spacer (IGS) region (*Fig. 2*). Five rDNA repeat lengths (15.4 kb. 14.8 kb, 12.5 kb, 11.7 kb and 11 kb) were detected due to variation in the number and size of DNA fragments in the 9.7 kb to 5.3 kb region. The fragments in this region covered the IGS of the rDNA repeat, whilst the majority of the coding region was covered by a single, invariable 3.7 kb fragment. Two broad groups of rDNA repeat lengths were found in the *L. leucocephala* accessions (*Table 6*): (i) 13.4 kb (Type A) and 12.8 kb (Type B) (ii) 10.5 kb (Type C); 9.7 kb (Type D) and 9kb (Type E). All of the accessions analysed possessed at least 1 repeat from each group, except an accession of *L. leucocephala* ssp. *glabrata* from Tecupa (Mexico) and an accession of L. *leucocephala* ssp. *leucocephala* introduced to Hawaii (USA). No information was obtained about the occurrence of repeat length variation within populations of *L. leucocephala*.

Discussion

The analysis of 3 isozyme systems (AAT, PER, PGI) of *Leucaena leucocephala* has revealed high levels of intraspecific variability such that it was possible to distinguish between the 2 subspecies of *L. leucocephala* and identify inter-populational variation in ssp. *glabrata*. This was in contrast to the study of Schifino-Wittmann and Schlegel (1990) who failed to report any intraspecific variation in *L. leucocephala* using 7 enzyme systems, 2 of which (AAT and PGI) showed polymorphism in this study. The 2 subspecies of *L. leucocephala*, ssp. *leucocephala* and ssp. *gla-*

brata, were differentiated from each other by the occurrence of the AAT phenotypes C and D in ssp. *leucocephala*. In addition, restriction analyses of cpDNA and rDNA revealed intraspecific variation, although in these cases it was not possible to distinguish the 2 subspecies. Intraspecific variation in *L. leucocephala* has not been previously detected, except between the 2 subspecies (BREWBAKER, 1987; HUGHES, 1993). The isozyme data were analysed as multi-enzyme phenotypes since: (i) *L. leucocephala* is a high polyploid, of potentially ancient origin (GOLDBLATT, 1981.), (ii) artificial crosses between *Leucaena* species are technically difficult and result in low numbers of crossed seed (SORENSSON and BREWBAKER, in press; SORENSSON et al., 1984); (iii) variation was only detected in 2 of the half-sib families examined, however little seed was available therefore segregation ratios could not be determined. These factors therefore made any characterisation of the AAT or PGI phenotypes in terms of loci and alleles difficult and, furthermore, any hypotheses potentially difficult to test.

The history of migration and introduction of *Leucaena leucocephala* around the tropics has been relatively poorly documented. Many introductions are thought to have been based on very few genotypes and a concomitant reduction in the levels of genetic variation. Accurate estimates of genetic diversity are an important prerequisite for the optimisation of sampling strategies and the conservation of genetic resources (SCHNABEL and HAMRICK, 1990; SURLES et al., 1990). High levels of genetic variation are essential as a safe-guard against co-evolving pests and diseases (NAMKOONG, 1986) in tree crops. *Leucaena leucocephala* is susceptible to the psyllid bug, *Heteropsylla cubana,* which is a serious pest that leads to defoliation. This pest is now well established outside the neotropics and is continuing to expand its range (BREWBAKER, 1987). It is therefore important to maintain a high level of genetic variation in a diverse array of seed material for distribution to field trials, by using either other *L. leucocephala* genotypes or other *Leucaena* species. Such requirements have lead to the need for effective breeding and sampling strategies for *L. leucocephala* and have emphasised the importance of identifying the parentage of this polyploid taxon (HARRIS et al., in press). In addition, given its long history of use it is of interest to consider the relationships between the structures of 'natural' and introduced populations of *L. leucocephala* (HARRIS et al., in press).

Migration and population establishment in cultivated species often involve a small number of phenotypes, which can have significant effects on the levels of variation due to the occurrence of genetic bottlenecks (NEI et al., 1975). In a self-fertilising plant the most significant factors in determining the levels of genetic variability are the number of different populations which have been introduced and the likelihood of cross-fertilisation between individuals in the immigrant population (BARRETT and SHORE, 1990). One of the most widely planted accessions of *Leucaena leucocephala* ssp. *glabrata* is the Hawaiian accession "K8" (BREWBAKER, 1987). "K8" was derived, as seed, from one or a few cultivated trees collected in Zacetecas (Mexico) in 1959. This is the so-called "giant" type of *L. leucocephala.* Four seed accessions used in this study were collected from material planted as "K8" (19/81; 32/88; 44/88; 45/88). Two of the accessions (44/88; 45/88; Guatemala) had identical multi-enzyme isozyme phenotypes. One of the other "K8" accessions (19/81; Honduras) contained a single multi-enzyme phenotype; but different to that found in the 2 Guatemalan "K8" populations. The final "K8" accession (32/88; Haiti) was the single most variable population encountered (7 multi-enzyme phenotypes). The high level of variation in this accession may be due to either multiple introductions of *L. leucocephala* to Haiti or hybridisation/ introgression of "K8" with other introduced *Leucaena* taxa on Haiti.

The most common multi-enzyme phenotypes found in the Haitian population (32/88) were not those associated with accessions identified as "K8" (A:A:B, 44/88; 45/88; B:C:A, 19/81) but A:A:C, an otherwise rare phenotype across the populations studied. The only phenotype found in 32/88, not found in other *Leucaena leucocephala* populations, was A:A:G (*Table 5*). These data would tend to support the view that the variation found in 32/88 is the result of multiple introductions of *L. leucocephala* to Haiti. The alternative hypothesis of hybridisation/introgression would require that a high level of gene exchange were occurring between *L. leucocephala* and other *Leucaena* taxa on the island. Although this explanation cannot be wholly rejected it would appear to be very unlikely, given the evidence that *L. leucocephala* is largely self-pollinating (BREWBAKER, 1987; SORENSSON and BREWBAKER, in press). However, nothing is known of the breeding system and levels of gene exchange in wild populations of *L. leucocephala.* The data reported here, indicating a high level of interpopulation variation compared to intra-population variation (*Table 4*), would tend to support the view that *L. leucocephala* is largely self-pollinated (BARRET and SHORE, 1990). Additional evidence against the hybrid/introgressive origin of the variation found in 32/88 is that one would expect to find unusual rDNA phenotypes; these were not found. All rDNA variation in 32/88 was similar to that found in other *L. leucocephala* ssp. *glabrata* accessions. Nuclear rDNA may not, however, be an ideal introgression marker in *Leucaena* since studies of known *L. leucocephala* ssp. *glabrata* x *L. esculenta* ssp. *esculenta* hybrids have shown that hybrids do not possess additive rDNA profiles for some restriction enzymes (HUGHES and HARRIS, in press). Additional nuclear markers will therefore be needed to study this particular population. Accession 32/88 has been used world-wide in *Leucaena* trials under the assumption that it is a single genotype. This results from this study indicate that this is not the case, furthermore if hybridisation/introgression are responsible for the observed high level of intrapopulation multi-enzyme phenotype variation then this could have important implications for results derived from such trials. The Duyure (Honduras) population has also been widely distributed for evaluation in field trials; in this case there appears to be only one genotype.

The 2 subspecies of *Leucaena leucocephala* may readily be distinguished on the basis of their isozyme multi-enzyme phenotypes. There is some evidence from the distribution of multi-enzyme phenotypes to indicate that accession 117/92 is different to the other accessions identified as ssp. *glabrata.* Accession 117/92 is from an isolated site in Northern Guatemale (Ixtahuacan, Huehuetenango) where it is cultivated by indigenous peoples. Morphologically the population appears to be separable from the other subspecies of *L. leucocephala.* These data would tend to support that view.

Intraspecific variation in cpDNA type is being detected more frequently in angiosperms as increasing sample sizes are used (reviewed in HARRIS and INGRAM, 1991; SOLTIS et al., 1992). Previously, levels of cpDNA variability in woody

plants and palms were thought to be very low, even between species (CLEGG et al., 1991; DOYLE et al., 1992; WILSON et al., 1990). However, extensive intraspecific cpDNA variation has been found in *Gliricidia sepium* (LAVIN, 1991) and to a lesser extent in *Leucaena leucocephala*. Indeed in *Leucaena* the occurrence of intraspecific variation appears to be rule rather than the exception (HARRIS, unpubl.).

Intra-individual rDNA length variation has been reported extensively (reviewed by SCHAAL, 1988). ROGERS (1986) found *Vicia faba* plants that had up to 20 different rDNA length variants whilst SCHAAL and LEARN (1988) report work by BAUM showing that *Lupinus texensis* has up to 11 length variants per individual. However, length variation on this scale is apparently exceptional. The mean number of length variants per *Leucaena leucocephala* individual is 2.6, which is towards the upper end of moderate intraindividual length variation (1.98 to 2.65 length variants per individual; SAGHAI-MAROOF et al., 1984; SCHAAL et al., 1987; SCHAAL and LEARN, 1988). The Santiago Acatepec (Mexico; 123/92) accession showed the greatest number of rDNA repeat length variants (5; *Table 6*). This material, collected as L. *leucocephala*, has been shown to resemble *L. leucocephala* ssp. *glabrata* x *L.* esculenta ssp. esculenta triploid hybrids on the basis of leaf morphology although it produced abundant seed. Detailed analyses of morphology and rDNA showed that this material was not typical of either *Le. leucocephala* ssp. *glabrata* or the hybrid. Furthermore, pollen grains of this accession appeared to be aborted and shrivelled prior to anthesis (HUGHES and HARRIS, in press). Additional evidence for the unusual nature of 123/92 has been provided by this study of the rDNA repeat number. Further studies will be needed to determine the exact nature of this accession.

Studies of rDNA organisation have been used to study the hybrid and polyploid origins in a range of genera, for example *Claytonia* (DOYLE et al., 1984), *Tragopogon* (SOLTIS and SOLTIS, 1991) and *Senecio* (HARRIS and INGRAM, 1992). These studies have indicated the value of rDNA markers to look at such questions. The apparent occurrence of two groups of rDNA repeats within *Leucaena leucocephala* may provide a clue regarding its parentage. Preliminary data indicates that the diploid taxa within the genus *Leucaena* fall into one of the 2 rDNA groups (HARRIS, unpubl.), which indicates that one parent of *L. leucocephala* may be derived from each of the 2 groups of taxa. Detailed analyses of the nuclear genome of *Leucaena* are in progress to address the intriguing questions of the origin of *L. leucocephala*.

Isozyme analyses of *Leucaena leucocephala* accessions promise to be a rapid and effective means of identifying interspecific and inter-population variation. Increasing the number and distribution of the populations sampled for isozyme diversity, and complementing this with RAPD analyses, may provide important evidence regarding the centre of origin of this economically important species. Furthermore, the occurrence of high levels of variation within an introduced accession (32/88) and the differences between populations in the neotropics suggests that such analyses may be an effective method of identify the origin of *L. leucocephala* accessions introduced to other parts of the tropics. The evidence of population differentiation lends support to the view that accessions (provenances) should be kept separate. Such data are likely to have considerable impact on the management, conservation and improvement of this important multi-purpose legume.

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Isozyme Variation and Mating System in Eucalyptus urophylla S. T. Blake

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Summary

The natural distribution of *Eucalyptus urophylla* is a series of disjunct population areas on a number of islands in eastern Indonesia. Thirteen loci in 8 enzyme systems were used to estimate genetic diversity and outcrossing rates, using seed collections from the full range of the species. Levels of genetic diversity were similar to other *Eucalyptus* species that have widespread distributions. Most diversity is located within populations (G_{ST} = 11.75

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