Patterns of Isozyme Variation in the *Leucaena shannonii* Alliance (Leguminosae: Mimosoideae)

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Abstract

Genetic variation within, and the relationships between, 4 taxa that comprise the *Leucaena shannonii* alliance were analysed with isozymes. Six enzyme systems were used to analyse 2 natural populations of *L. salvadorensis* STANDLEY ex. B. and R., 2 of *L. shannonii* DONN. SMITH subsp. *magnifica* C. E. HUGHES, 4 of *L. shannonii* DONN. SMITH subsp. *shannonii* and 2 populations of an undescribed taxon, *L. sp. nov.*. Cluster analysis of NEI's genetic distances (NEI, 1978) and Population Aggregation Analysis (PAA) (DAVIS and NIXON, 1992) were used to delimit species. *L. sp. nov.* was found to be most closely related to *L. salvadorensis,* however, isozyme variation did not support a close relationship between the 2 subspecies of *L. shannonii.* The level and structure of genetic variation within species of the alliance was also assessed. *L. shannonii* subsp. *shannonii* was found to be the most diverse taxon, and subsp. *magnifica* the least diverse. It is postulated that this may contribute to the apparent dissimilarity of the 2 taxa in the cluster analysis. The use of PAA to delimit species in the *L. shannonii* alliance is also discussed.

Key words: Leguminosae, Mimosoideae, *Leucaena, Leucaena shannonii, L. salvadorensis,* isozymes, species delimitation, genetic variation, population structure.

FDC: 165.3; 165.53; 176.1 *Leucaena shannonii.*

Introduction

The most widely used molecular technique for assessing genetic diversity within and among plant populations has been the analysis of isozyme variation (LOVELESS and HAMRICK, 1984; SOLTIS and SOLTIS, 1989). Isozyme electrophoresis is also useful for examining variation between species (GOTTLIEB, 1977; CRAWFORD, 1983), as it allows individual character loci to be identified, and many individuals can be assayed in a cost-effective manner. The emergence of the phylogenetic species concept (NIXON and WHEELER, 1990), and a renewed interest in species delimitation, has seen the increased use of isozyme data in this area of systematics (e.g. DAVIS and NIXON, 1992; DAVIS and GOLDMAN, 1993; ELISENS and NELSON, 1993).

Leucaena BENTH. is a small genus of approximately 23 species and is placed in the tribe *Mimoseae* of the subfamily Mimosoideae of the Leguminosae. All the species are native to the New World, where the greatest diversity is found in seasonally dry, mainly tropical habitats of southern Mexico and northern Central America. One species, *L. leucocephala* (LAM.) DE WIT subsp. *glabrata* (ROSE) ZÁRATE, is cultivated pantropically as a forage and multi-purpose tree. Over the last decade, however, a wider range of *Leucaena* species has been incorporated, either into direct use, or breeding and interspecific hybridisation programmes (BREWBAKER and SORENSSON, 1993; HUGHES, 1993). One species, *L. salvadorensis* STANDLEY ex. BRITTON and ROSE, is of particular interest because of its high wood biomass production (STEWART *et al.,* 1991) and preferred use by farmers (HELLIN and HUGHES, 1993). A sound understanding of species relationships and patterns of interspecific variation in *Leucaena* are therefore vital to genetic improvement and conservation programmes which depend on the manipulation of available variation for genetic gain, or gene conservation.

Delimitation of species within the genus *Leucaena* remains, however, the greatest source of taxonomic confusion, and the number of species recognised by different authors has varied from 12 to 39, with little consistency in the use of species and subspecies ranks. While some species are narrowly distributed, morphologically uniform and distinct, others are highly variable, widely distributed and present considerable difficulties in their delimitation. One such unresolved group of taxa is the *Leucaena shannonii* DONN. SMITH alliance, which is taken to comprise *L. shannonii* DONN. SMITH subsp. *shannonii, L. shannonii* DONN. SMITH subsp. *magnifica* C. E. HUGHES, *L. salvadorensis* and *L. sp. nov.,* an undescribed taxon discovered in 1991 in Honduras. These 4 taxa have been variously treated as valid species, subspecies, or grouped under synonymy by different authors.

Although the validity of *L. shannonii* as a distinct species has never been doubted, the identity, rank and relationships of the closely related taxa remains unresolved. ZÁRATE (1984) perceived *L. shannonii* as a variable species that included *L. salvadorensis* as a synonym. BREWBAKER (1987) also discarded *L. salvadorensis,* but treated it instead, as a synonym of *L. leucocephala* subsp. *glabrata.* Over the last decade, field exploration and collection of *Leucaena* in Central America has intensified and new botanical and seed collections have been assembled (HUGHES, 1993). This has led to a number of important new findings associated with the *L. shannonii* alliance: (i) an arborescent, large leaflet variant of *L. shannonii* was discovered in 1984 in south-east Guatemala (HUGHES, 1986), and named as a distinct subspecies, *magnifica* (HUGHES, 1991); (ii) the identity, status and distribution of *L. salvadorensis* has been thoroughly investigated (HUGHES, 1988; HELLIN and HUGHES, 1993), and based on this new work, ZÁRATE (1987), resurrected *L. salvadorensis* from synonymy and placed it as a subspecies of *L. shannonii.* HUGHES (1988) and HELLIN and HUGHES (1993), however, maintain that *L. salvadorensis* is a distinct species in its own right; (iii) a new taxon with clear affinities to both *L. shannonii* and *L. salvadorensis,* designated here as *L. sp. nov.,* was discovered in northern Honduras in 1991, and has now been thoroughly collected and its distribution mapped (HUGHES, in prep.).

Levels of genetic diversity within species of the *L. shannonii* alliance, or within other species in the genus, have not been investigated in detail. PAN (1985) used isozymes to investigate the systematics of the *L. diversifolia* (SCHLECHT.) BENTH. group, but found polymorphism for only 1 enzyme system, peroxidase. SCHIFINO-WITTMANN and SCHLEGEL (1990) studied isozyme variation in *L. diversifolia, L. leucocephala* and their hybrids that had been selected for acid soil tolerance, and HARRIS *et al.* (1994a) examined patterns of phenotypic variation within *L. leucocephala* using a combination of isozymes, and nuclear

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and chloroplast DNA. The purpose of this paper is to assess isozyme variation within and between populations of the species of the *L. shannonii* alliance to assist in both species delimitation and the estimation of levels of genetic diversity.

Materials and Methods

Seed samples

Ten populations representing the 4 taxa of the *L. shannonii* alliance were sampled across the range of the taxa in Mexico, Guatemala, Honduras and Nicaragua (*Table 1*). Within each population, seed from a minimum of 12, and normally 20, families was analysed. Each family comprised seed progeny from a single maternal parent, and a minimum distance of 50 m separated each parent tree to prevent bias due to co-ancestry. Distances between sampled trees, and hence the area of each population sampled, varied substantially due to significant human disturbance of the populations sampled. In all cases, trees were sampled from disturbed secondary vegetation, or highly degraded and fragmented remnant populations.

Table 1. – Collection site data for the 10 populations of *Leucaena* studied. The number of seeds per family sampled are shown in parentheses. Botanical vouchers are deposited at FHO, MEXU and K; Hughes (CEH).

OFI No.	Voucher CEH	Population	Country	Lat. (N)	Long. (W)	Altitude (m)	No. of families
Leucaena salvadorensis							
34/88	746	Calaire, Choluteca	Honduras	13º 15'	87º 06'	350-500	18 (10)
7/91	1407	San Juan de Limay, Esteli	Nicaragua	13º 12'	86º 29'	500-900	20 (10)
Leucaena shannonii subsp. *magnifica*							
19/84	412	El Rincón, Chiquimula	Guatemala	14º 40'	89º 42'	900-950	20 (10)
58/88	720	Quetzaltepeque, Chiquimula	Guatemala	14º 37'	89º 27'	600-650	20 (10)
Leucaena shannonii subsp. *shannonii*							
53/87	507	Champoton, Campeche	Mexico	19º 20'	90º 43'	0-20	20 (10)
22/83	239	Comayagua, Comayagua	Honduras	14º 22'	87º 39'	600-700	20 (10)
1/91	1417	Asunción Mita, Jutiapa	Guatemala	14º 25'	89º 41'	700	20 (10)
2/91	1401	La Puerta, Chontales	Nicaragua	12º 11'	85º 18'	340	12 (10)
Leucaena sp. nov.							
5/91	1447	Valle de Aguan, Yoro	Honduras	15º 25'	86º 50'	200-300	20 (10)
6/91	1411	Cuyamapa, Yoro	Honduras	15º 17'	87º 40'	200	20 (10)

Sample preparation

Leaf and root tissue were harvested from seedlings grown in a glasshouse maintained at 27 °C. 250 mg of each tissue was ground separately in 0.3 ml of extraction buffer comprising 50 ml gel buffer, 40 mg KCl, 100 mg $MgCl_2$, 18 mg EDTA (disodium salt), 2.0 g PVP-40, 0.5 ml Triton-X-100 and 2 ml 10% DTT. Extracts were centrifuged at 13000 rpm for 2 min, and the supernatant transferred to a clean Eppendorf tube and frozen under liquid nitrogen. Samples were then stored at – 70 °C for up to a month without any detectable loss of enzyme activity.

Preparation of polyacrylamide gels

Vertical polyacrylamide gel electrophoresis was carried out in a dual-cooled slab unit (SE 600 series, Hoeffer Scientific) on 7.5% acrylamide. One of 2 gel/electrode buffer systems were used: (i) electrode buffer which comprised 1.2 g lithium hydroxide and 11.9 g boric acid per litre distilled water (pH 8.3), and gel buffer which comprised 5.4 g Tris and 1.28 g citric acid per litre distilled water; (ii) electrode buffer which comprised 15.1 g Tris and 7.3 g citric acid per litre distilled water (pH 7.5) and gel buffer which comprised 8.36 g histidine HCl and 0.03 g EDTA (disodium salt) per litre distilled water. 10 µl of sample was loaded per sample well, and a constant current of 20 mA per gel applied for 3.5 h to 4.0 h.

Enzyme stains

When electrophoresis was complete, standard staining protocols (HAMES and RICKWOOD, 1981; SOLTIS *et al.,* 1983) were used to resolve the isozymes. The enzyme systems used in the analysis were: aspartate aminotransferase (AAT; E.C. 2.6.1.1), isocitrate dehydrogenase (IDH; E.C. 1.1.1.42), leucine aminopeptidase (LAP; E.C. 3.4.-.-), peroxidase (PER; E.C. 1.11.1.7), phosphoglucose mutase (PGM; E.C. 5.4.2.2), and shikimic dehydrogenase (SDH; E.C. 1.1.1.25).

Analysis of isozyme polymorphisms

As full-sib progeny tests were not possible, and as the sampled trees were assumed to be diploid and open-pollinated, genetic interpretations of the isozyme gel, or zymogram, banding patterns were based on the evaluation of isozyme polymorphisms in other well-documented investigations (WENDEL and WEEDEN, 1989). These interpretations have been corroborated by genetic analysis, and the sub-unit structure of many isozymes is known (SHIELDS *et al.,* 1983). In most cases, the banding patterns implied a simple diploid genetic model and could be interpreted in terms of loci and alleles. A genotype was assigned to each seed on this basis, and the allele frequencies for each enzyme system in each family were calculated (summarised in *Table 2*) and entered into BIOSYS-1 (SWOFFORD, 1989). Chi-square test for deviation from HARDY-WEINBERG equilibrium (summarised in *Table 3*) and measures of heterozygosity within and between populations were computed. NEI's genetic distances (NEI, 1978) between all pairs of populations were also computed and used to cluster the populations by the unweighted pair group method (UPGMA). In addition, species delimitation was attempted using Population Aggregation Analysis (PAA), a method described by DAVIS and NIXON (1992) enabling the identification of phylogenetic species *sensu* NIXON and WHEELER (1990). Briefly, this procedure begins by scoring each allele (x) in each population as either absent (0), present and fixed (1), or present and not fixed (*; $0 < x < 1$). Each population allele profile is then compared with all others, and populations are 'aggregated' when they are not distinct from one another, i.e. when they do not differ by the fixed occurrence of at least one allele in one population and its absence from the other (as an example, see DAVIS and GOLDMAN (1993)). In this way, multipopulation species, characterised by the occurrence of a unique fixed character combination, are identified, meeting the requirement of the phylogenetic species concept that descent relationships among such populations are hierarchic. PAA was conducted manually.

Results

Enzyme descriptions

In interpreting those zones of enzyme activity which showed variation, the alleles that were inferred to exist at each locus were identified by letters. Each zymogram band, and the genotype to which it corresponds, is then identified by a combination of letters indicating which alleles contribute to it (*Figure 1*).

Table 2. – Summary allele frequencies for 7 polymorphic loci among species and populations of the *Leucaena shannonii* alliance and the results of PAA (*) allele present, not fixed; 1 allele present, fixed; 0 allele absent).

Population	Locus/Allele																
	Aat-2		Idh-1		Idh-2		Lap-2			Per-1				Pgm-3		Sdh-1	
	A	B	A	B	A	B	A	B	C	A	B	C	D	A	B	A	B
L. salvadorensis																	
Calaire	1.00	0.00	0.43	0.57	0.61	0.39	0.41	0.59	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.25	0.75
San Juan de Limay	1.00	0.00	0.35	0.65	0.52	0.48	0.71	0.29	0.00	0.00	1.00	0.00	0.00	1.00	0.00	0.09	0.91
PAA	**1.00**	**0.00**	*	*	*	*	*	*	**0.00**	**0.00**	**1.00**	**0.00**	**0.00**	**1.00**	**0.00**	*	*
L. shannonii subsp. *magnifica*																	
El Rincón	1.00	0.00	1.00	0.00	0.00	1.00	0.41	0.59	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.91	0.09
Quetzaltepeque	1.00	0.00	1.00	0.00	0.00	1.00	0.46	0.54	0.00	1.00	0.00	0.00	0.00	1.00	0.00	0.49	0.51
PAA	**1.00**	**0.00**	**1.00**	**0.00**	**0.00**	**1.00**	*	*	**0.00**	**1.00**	**0.00**	**0.00**	**0.00**	**1.00**	**0.00**	*	*
L. shannonii subsp. *shannonii*																	
Champoton	1.00	0.00	0.77	0.23	0.54	0.46	0.36	0.27	0.36	0.00	0.71	0.29	0.00	0.72	0.27	0.00	1.00
Comayagua	0.31	0.69	0.98	0.02	0.98	0.02	0.09	0.50	0.41	0.00	0.55	0.40	0.05	0.52	0.48	0.11	0.89
Asunción Mita	0.56	0.44	0.97	0.03	0.18	0.82	0.12	0.41	0.47	0.00	0.50	0.50	0.00	0.47	0.53	0.14	0.86
La Puerta	1.00	0.00	1.00	0.00	0.37	0.63	0.23	0.38	0.39	0.000	0.52	0.48	0.00	0.55	0.45	0.18	0.82
PAA	*	*	*	*	*	*	*	*	*	**0.00**	*	*	*	*	*	*	*
L. sp. nov.																	
Valle de Aguan	1.00	0.00	0.50	0.50	1.00	0.00	0.08	0.37	0.55	0.58	0.42	0.00	0.00	0.50	0.50	0.86	0.14
Cuyamapa	0.61	0.39	0.73	0.27	1.00	0.00	0.02	0.79	0.19	0.00	1.00	0.00	0.00	0.72	0.28	0.82	0.18
PAA	*	*	*	*	**1.00**	**0.00**	*	*	*	*	*	**0.00**	**0.00**	*	*	*	*
PAA (all pops.)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	

Two zones of activity were observed on AAT gels. The first zone (Aat-1) exhibited up to 6 bands and suggested 2 dimer isozymes with overlapping mobilities. A number of inconsistencies in the data, however, made this region difficult to interpret and hence was ignored for the purpose of the analysis. The second zone (Aat-2) exhibited up to 3 bands, 2 homodimers and a band of intermediate mobility, the heterodimer. This locus was polymorphic in the Comayagua and Asunción Mita populations of *L. shannonii* subsp. *shannonii* and the Cuyamapa population of *L. sp. nov.*. One zone of banding was exhibited on IDH gels. The complex pattern of bands exhibited in this region was typical of 2 dimer isozymes with overlapping mobilities. A maximum of 7 bands could be observed when both heterozygous genotypes were expressed. Both populations of *L. shannonii* subsp. *magnifica* were monomorphic at both loci. Two regions of activity were observed on LAP gels. Poor resolution of the first banding zone made it impossible to score, but the faster moving putative locus (Lap-2), exhibited up to 3 bands, a slow (A), an intermediate (B) and a fast (C). Only 2 of these bands occurred in any one sample; hence the isozyme was presumed to be a monomer encoded by a tri-allelic locus. All 3 allozymes were expressed by the populations of *L. shannonii* subsp. *shannonii* and *L. sp. nov.,* but only 2 were exhibited by the populations of *L. salvadorensis* and *L. shannonii* subsp. *magnifica.*

Table 3. – Observed (O) and expected (E) numbers of genotypes, and chi-square test for departure from random mating proportions for up to 7 loci in the progeny generation of each of the 10 populations of the *Leucaena shannonii* alliance.

Population	Chi squared test/ Locus						
	Aat-2	Idh-1	Idh-2	Lap-2	Per-1	Pgm-3	Sdh-1
L. salvadorensis							
Calaire	–	4.260	115.4*	0.246	–	–	44.34*
San Juan de Limay	–	2.544	46.5*	1.710	–	–	2.078
L. shannonii subsp. *magnificia*							
El Rincon	–	–	–	0.419	–	–	14.30*
Quetzaltepeque	–	–	–	3.336	–	–	20.40*
L. shannonii subsp. *shannonii*							
Champoton	–	3.060	0.281	25.38*	3.410	0.069	–
Comayagua	33.33*	0.083	0.131	5.779	0.710	2.040	0.024
Asuncion Mita	6.276	0.191	1.591	0.785	32.00*	31.35*	4.820
La Puerta	–	–	5.813	0.851	0.143	20.58*	19.69*
L. sp. nov.							
Valle de Aguan	–	147.9*	–	88.80*	64.74*	141.1*	40.07*
Cuyamapa	27.72*	56.90*	–	28.39*	–	8.516	1.457

*) significant at $P < 0.05$.
df = 1 for all chi-square values quoted

One zone of banding was observed on PER gels. Bands always occurred in pairs for all populations of the alliance. A maximum of 4 allozymes (8 bands) were exhibited (A, B, C and D, slow to fast respectively). Only 2 allozymes (4 bands) were exhibited in any one sample; hence this isozyme was presumed to be a monomer encoded by a bi-allelic locus. The *L. salvadorensis* and *L. shannonii* subsp. *magnifica* populations were both monomorphic for this locus at different allozymes. The *L. sp. nov.* shared allozymes in common with *L. salvadorensis* and *L. shannonii* subsp. *magnifica,* whereas *L. shannonii* subsp. *shannonii* expressed the B allozyme only, in combination with the 2 faster allozymes, C and D. Three zones of activity were distinguishable on PGM gels.

Poor resolution of the first 2 banding zones made them impossible to score, but the third putative locus (Pgm-3) exhibited 2 bands, a slow (A) and a fast (B); hence this isozyme was presumed to be a monomer encoded by a bi-allelic locus. Pgm-3 was polymorphic within populations of *L. shannonii* subsp. *shannonii* and *L. sp. nov.,* and monomorphic within subsp. *magnifica* and *L. salvadorensis.* One zone of activity was observed on SDH gels, with 2 bands being apparent, a slow (A) and a fast (B); hence, the isozyme was presumed to be a monomer encoded by a bi-allelic locus. This isozyme was polymorphic within all the taxa of the *L. shannonii* alliance.

Nei's genetic distances and cluster analysis

A matrix of Nei's unbiased genetic distances (*D*; Nei, 1978) for pairwise comparisons across the 10 populations of the *L. shannonii* alliance is presented in *table 4.* The information in the matrix is represented graphically in a phenogram produced using UPGMA (*Figure 2*). The matrix and the phenogram both show that all populations have greater affinities with others of their own taxon than with populations of any

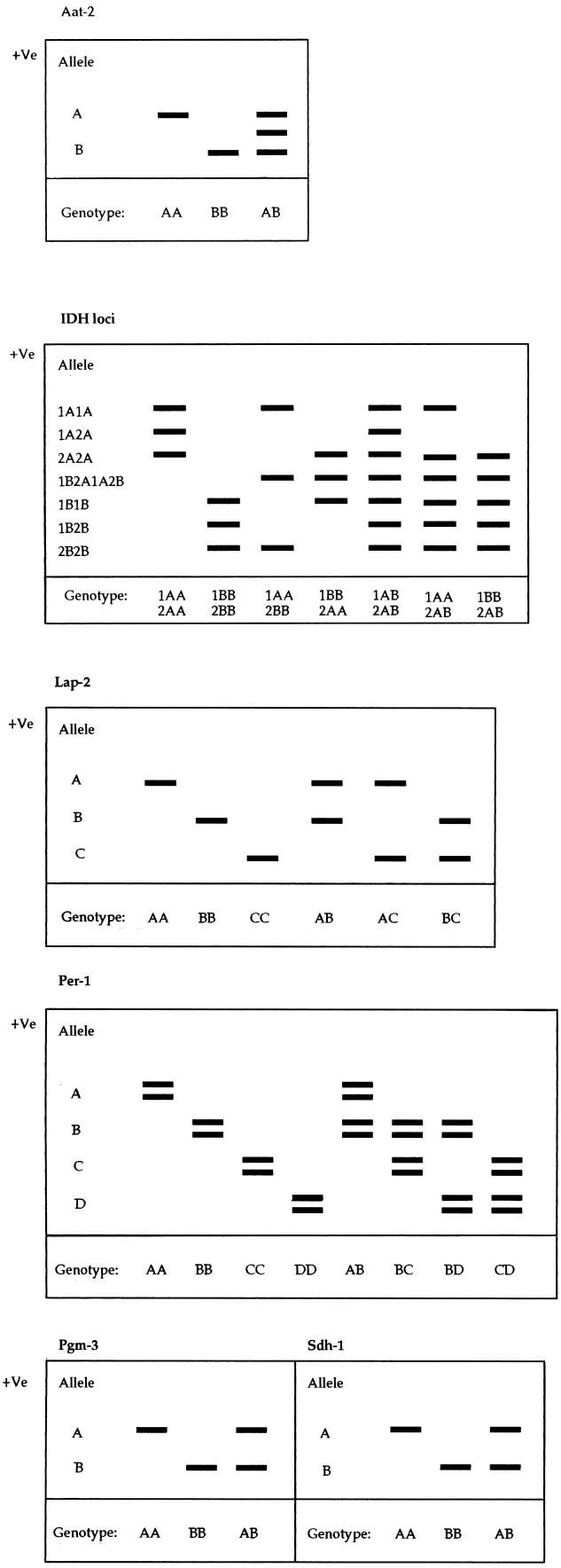

Figure 1. – Schematic illustration of the zymatogram banding patterns for the putative polymorphic enzyme loci Aat-2, Idh-1, Idh-2, Lap-2, Per-1, Pgm-3, and Sdh-1 found in the *Leucaena shannonii* complex. The letters refer to the genotype designation used in the text.

other taxon. There is greatest differentiation between *L. shannonii* subsp. *magnifica* and all other taxa of the alliance. This taxon was highly differentiated from subsp. *shannonii,* with a mean genetic distance between the 2 subspecies of 0.289 (D values between populations range from 0.184 to 0.454). The populations of *L. sp. nov.* showed greatest affinity with the populations of *L. salvadorensis* (mean $D = 0.198$). The similarity of *L. sp. nov.* to *L. shannonii* subsp. *shannonii* is indicated by a mean genetic distance of 0.206, and between *L. salvadorensis* and *L. shannonii* subsp. *shannonii* there is a mean genetic distance of 0.163.

Population Aggregation Analysis

If the PAA procedure, described by DAVIS and NIXON (1992), is strictly adhered to, all sampled populations of the *L. shannonii* alliance aggregate into one multipopulational isozyme species, as no fixed differences between all populations were observed (*Table 2*). If, however, populations corresponding to 2 of the 4 taxa are compared and aggregated, some fixed differences between taxa are apparent. *L. shannonii* subsp. *magnifica* is distinct from both subsp. *shannonii* and *L. salvadorensis* on the basis of the fixed occurrence of allele A of Per-1, with *L. salvadorensis* also exhibiting the fixed occurrence of allele B at this locus. Subsp. *magnifica* is also distinct from *L. sp. nov.* on the basis of the fixed occurrence of allele B of Idh-2, with allele A of this locus being fixed in *L. sp. nov.*. There are, however, no fixed differences between the populations of *L. salvadorensis, L. shannonii* subsp. *shannonii* and *L. sp. nov.*.

Heterozygosity and population structure

Levels of variation at the 10 polymorphic putative loci identified in the *L. shannonii* alliance were assessed on the basis of the average number of alleles per locus (A), the observed mean heterozygosity (H_o), the expected mean heterozygosity (H_e) and the percentage of polymorphic loci (P) (*Table 5*). The most variable taxon by these criteria was *L. shannonii* subsp. *shannonii,* the most variable population being Asunción Mita. *L. sp. nov.* showed comparable mean values for P and A of 71.4% and 1.9 respectively, but displayed low levels of heterozygosity ($H_o = 0.183$ and 0.102 for Cuyamapa and Valle de Aguan respectively). The least variable taxon for all criteria was *L. shannonii* subsp. *magnifica,* the population from El Rincón having the lowest mean observed heterozygosity. *L. salvadorensis* showed intermediate variability for all criteria.

There was generally good agreement between the observed and expected heterozygosities, except in the populations at Cuyamapa and Valle de Aguan. This suggests that these populations were in genetic disequilibrium.

A comparison of WRIGHT's F-statistics (WRIGHT, 1951) for all 4 taxa is also given in *table 5.* The mean value of F_{ST} for *L. salvadorensis* is very low (0.036) and indicates very little population differentiation between Calaire and San Juan de Limay. The mean F_{ST} value for *L. shannonii* subsp. *magnifica* (0.100) suggests a moderate degree of population differentiation, as does that for *L. sp nov.* (0.149), but population differentiation is slightly greater in *L. shannonii* subsp. *shannonii* (0.152).

Discussion

Species delimitation

The cluster analysis of NEI's genetic distances in the 10 populations studied provides evidence for the existence of 4 groups in the *L. shannonii* alliance. In all cases, populations grouped according to their original taxonomic identities, suggesting that there may be four distinct taxa and that gross

Table 4. – Matrix of NEI's unbiased genetic distances (1978) for pairwise comparisons of 10 populations of the *Leucaena shannonii* alliance over 7 polymorphic isozyme loci.

Species	Population	1	2	3	4	5	6	7	8	9
L. salvadorensis	1. Calaire									
	2. San Juan de Limay	0.019								
L. shannonii	3. El Rincón	0.304	0.350							
subsp. *magnifica*	4. Quetzaltepeque	0.250	0.272	0.026						
L. shannonii	5. Champoton	0.066	0.068	0.310	0.223					
subsp. *shannonii*	6. Comayagua	0.211	0.256	0.454	0.384	0.120				
	7. Asunción Mita	0.197	0.216	0.288	0.221	0.075	0.101			
	8. La Puerta	0.133	0.154	0.247	0.184	0.027	0.124	0.034		
L. sp. nov.	9. Valle de Aguan	0.191	0.254	0.273	0.293	0.196	0.221	0.269	0.203	
	10.Cuyamapa	0.129	0.217	0.347	0.366	0.190	0.136	0.231	0.203	0.106

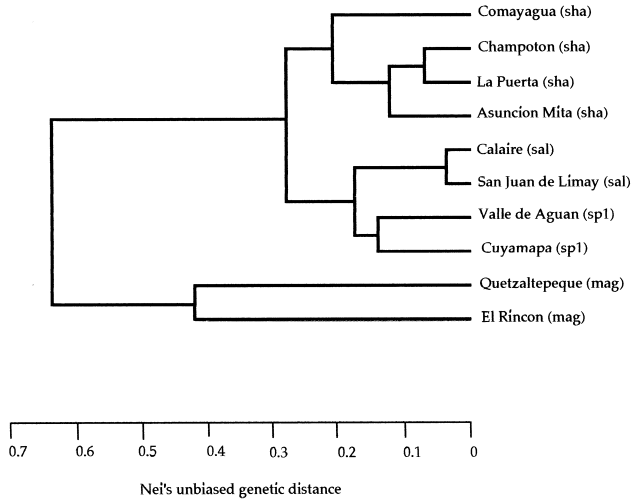

Figure 2. – Phenogram showing the isozyme similarities between 10 populations of the *Leucaena shannonii* alliance. Key: sal – *L. salvadorensis,* mag – *L. shannonii* subsp. *magnifica,* sha – *L. shannonii* subsp. *shannonii* and sp1 – *L. sp. nov.*.

Table 5. – Genetic variability at 7 isozyme loci for 10 populations of *Leucaena shannonii* alliance (standard errors in parentheses).

Species	Population	Mean no of alleles per locus	% polymorphic loci	Mean heterozygosity (H)		F_{ST}
				Observed	Expected	
L. salvadorensis	Calaire	1.6 (0.2)	57.1	0.193 (0.092)	0.261 (0.093)	0.036
	San Juan de Limay	1.6 (0.2)	57.1	0.236 (0.105)	0.220 (0.087)	
L. shannonii subsp. *magnifica*	El Rincón	1.3 (0.2)	28.6	0.089 (0.071)	0.093 (0.069)	0.100
	Quetzaltepeque	1.3 (0.2)	28.6	0.129 (0.086)	0.143 (0.092)	
L. shannonii subsp. *shannonii*	Champoton	1.9 (0.3)	71.4	0.312 (0.090)	0.334 (0.094)	0.152
	Comayagua	2.3 (0.2)	100.0	0.290 (0.088)	0.332 (0.088)	
	Asunción Mita	2.1 (0.1)	100.0	0.373 (0.115)	0.385 (0.072)	
	La Puerta	1.9 (0.3)	71.4	0.367 (0.072)	0.345 (0.097)	
L.sp. nov.	Valle de Aguan	1.9 (0.3)	71.4	0.102* (0.034)	0.327 (0.092)	0.149
	Cuyamapa	1.9 (0.3)	71.4	0.183* (0.051)	0.273 (0.074)	

*) significant at deviation from HARDY-WEINBERG expectations at $P < 0.05$.

morphology as used in the original identifications is correlated with isozyme variation. Although isozyme data, analysed using phenetic methods, may not taken alone, infer species relationships or taxonomic rank, it is interesting to examine the results of the isozyme analysis in relation to studies of seed protein (CHAMBERLAIN *et al.,* in prep.) and chloroplast DNA (cpDNA) variation (Harris *et al.,* 1994b).

L. salvadorensis and *L. sp. nov.* are shown to be more similar to each other than to any other taxon on the basis of the cluster analysis of isozyme data. A close association between these 2 taxa is also supported by evidence from seed protein markers and cpDNA. CHAMBERLAIN *et al.,* (in prep.), in their phenetic analysis of seed protein markers across the genus, showed that *L. salvadorensis* and *L. sp. nov.* were distinct, but that they clustered together. These 2 taxa were also distinct, but closely grouped as sister taxa in a phylogenetic analysis of cpDNA (HARRIS *et al.,* 1994b)[3]), the relationship being strongly supported by its appearance in 96% of replicates in a bootstrap analysis. It is interesting to note that the original collectors of *L. sp. nov.* (ALVARADO and HELLIN, unpublished) pointed out its morphological similarity to *L. salvadorensis,* although they also noted its smaller leaflets and greater number of pinnae pairs per leaf. All 3 analyses thus support a close relationship between *L. salvadorensis* and *L. sp. nov.*.

L. shannonii subsp. *magnifica* was originally described by HUGHES (1991) who distinguished it morphologically from the typical subspecies, most notably by its larger leaves with more pinnae per leaf, more and larger leaflets per pinna and its larger pods. Exploration in Guatemala has revealed that subsp. *magnifica* has a restricted distribution of no more than 400 km^2 in the Department of Chiquimula. In contrast, subsp. *shannonii* is widely distributed and abundant in seasonally dry forest from southern Mexico to central Nicaragua (HUGHES, 1991). Evidence from cpDNA (HARRIS *et al.,* 1994b), supports the recognition of subsp. *magnifica* as a distinct taxon. It was separated from subsp. *shannonii* by 4 autapomorphic fragment changes, 2 of which were unique, and indicates a close relationship between the 2 taxa, a relationship supported by its appearance in 85% of bootstrap replicates. The analysis of seed proteins (CHAMBERLAIN *et al.,* in prep.) also showed the 2 taxa to be distinct, but closely associated. In the cluster analysis, the populations of subsp. *magnifica* formed a unique group separated from all other members of the *L. shannonii* alliance. This is in clear contrast to the cpDNA and seed protein evidence and the original description of *magnifica* as a subspecies within *L. shannonii.*

The separation of subsp. *magnifica* from typical subsp. *shannonii* in the cluster analysis is partly attributable to differences in genetic variability between the 2 taxa. Subsp. *magnifica* was the least variable taxon in the *L. shannonii* alliance on the basis of the percentage of polymorphic loci, the mean number of alleles per locus, and the mean observed and expected heterozygosities. The 2 populations from Quetzaltepeque and El Rincón were each monomorphic at 5 enzyme loci. In contrast, its subspecies, *shannonii,* was the most variable taxon on all the above criteria. The *shannonii* population at Asunción Mita, located only 40 km from El Rincón, was polymorphic at all 7 enzyme loci used in the analysis. It is likely that this difference in allele frequencies, or genetic variability, in addition to the fixed difference at allele A of Per-1, has contributed to the separation of the 2 subspecies seen in the cluster analysis.

[3]) *L. sp. nov*. is referred to as *L. sp. nov.* 1. in HARRIS *et al.* (1994b).

Isozyme data allows quantification of the similarity, or difference, between populations, groups of populations and species (GOTTLIEB, 1977 and 1981). The populations and species can be characterised on the basis of differences in allele frequencies, fixed or otherwise. What has been recognised for some years now, and has perhaps meant that enzyme electrophoresis has not been routinely used by plant taxonomists, is that divergence of genes specifying soluble enzymes is often uncorrelated with plant speciation (GOTTLIEB, 1973 and 1974; GOTTLIEB and PILZ, 1976). PAA, however, specifically sets out to utilise isozyme data for the delimitation of phylogenetic species (DAVIS and NIXON, 1992). In the *L. shannonii* alliance, the strict standards imposed by PAA cause all populations to aggregate into a single species (although fixed differences can be observed between *L. shannonii* subsp. *magnifica* and subsp. *shannonii, L. salvadorensis* and *L. sp. nov.*). A single polymorphic population may cause otherwise distinct populations to become aggregated into a common species, as seen for the Valle de Aguan population of *L. sp. nov.* at the Per-1 locus. The most likely explanation for this is that too few loci were examined, thereby reducing the ability of PAA to resolve differences between actual phylogenetic species. Every unsampled attribute, i.e. every allele at a locus, is a potential species-delimiting character, and undersampling of attributes will consistently bias the results towards recognition of fewer species than actually exist (DAVIS and NIXON, 1992). In this study, undersampling of attributes may have been exacerbated by the selection of enzyme systems that exhibit polymorphism, given our additional interest in species heterozygosity and population structure. For PAA to be effective, a large number of attributes must be sampled, including some that may be monomorphic within species.

On the basis of isozyme variation, species delimitation within the *L. shannonii* alliance is inconclusive. The study partly supports the recognition of 4 taxa, but does not support the current usage of subspecies and species ranks within the alliance. The distinction of *L. shannonii* subsp. *magnifica* from subsp. *shannonii* is apparent, however, suggesting that the former should be treated as a separate species. HARRIS *et al.* (1994) also failed to resolve fully the species relationships within the plastome clade containing the *L. shannonii* alliance due to the lack of synapomorphies in the cpDNA dataset. Combined analysis of morphology, isozymes and DNA evidence will be needed to resolve these conflicting species delimitations in the *L. shannonii* alliance.

Variation between and within populations

Given the importance of *Leucaena* in tropical reforestation and the considerable efforts being made to collect, evaluate and hybridise the genetic resources in the genus, it is surprising there have been very few attempts to assess levels of genetic variation within species of *Leucaena* and their populations. PAN (1985) used only one enzyme system, peroxidase, to study the *L. diversifolia* complex. SCHIFINO-WITTMANN and SCHLEGEL (1990) studied isozyme variation in *L. diversifolia, L. leucocephala* and their hybrids that had been selected for acid soil tolerance and found little diversity either within or between taxa, whilst HARRIS *et al.* (1994a) used multi-enzyme phenotypes, i.e. did not characterize enzyme banding patterns in terms of loci and alleles, to investigate variation within and between 12 populations of *L. leucocephala.* This study is, therefore, the first to assess the levels of genetic variation within and between populations of *Leucaena* taxa other than *L. diversifolia* or *L. leucocephala.* A clear understanding of the level and structure of population genetic variation is needed for genetic improvement programmes to proceed, for optimisation of germplasm sampling strategies and for genetic conservation.

The very low level of variation observed within subsp. *magnifica* (mean $H = 0.109$), compared to the other species in the *L. shannonii* alliance, corresponds to the observation by LOVELESS (1992) that lower levels of within-population variation ($H = 0.045$) are more common in narrowly restricted endemic species, such as subsp. *magnifica,* than in widely distributed species ($H = 0.181$; LOVELESS, 1992), such as subsp. *shannonii* (mean $H = 0.335$). The intermediate levels of variation found within populations of *L. salvadorensis* (mean $H = 0.215$) are comparable to those found in woody tropical species that are predominantly outcrossed and entomophilous ($H = 0.211$; HAMRICK and LOVELESS, 1989). Within *L. sp. nov.,* both populations showed high levels of variation in terms of percentage polymorphic loci and mean number of alleles per locus, but low levels in terms of the mean observed heterozygosity, due to a highly significant deficiency in the number of heterozygotes. Both populations appear to be in severe disequilibrium. *L. sp. nov.* is known, to date, from only the 2 populations included in this study. The populations are isolated from one another and confined to 2 valley systems in the northern Honduran department of Yoro. In both areas, the trees are abundant. However, at Cuyamapa the population is apparently restricted and has been subjected to severe disturbance; there are some reports that it resulted from a introduction of material from the Valle de Aguan. In the Valle de Aguan population, the trees are widely scattered forming highly fragmented sub-populations. Human interference and resulting genetic bottlenecks may, therefore, be factors in the population disequilibrium observed for *L. sp. nov.*.

Although the analysis of between-population variation is limited in this study by the small number of populations analysed for each taxon, some interesting preliminary conclusions can be drawn. The two populations of *L. salvadorensis,* from San Juan de Limay in northern Nicaragua and Calaire in southern Honduras, show little between-population variation ($F_{ST} = 0.039$) in comparison to that found in other tropical trees ($F_{ST} = 0.119$; HAMRICK *et al.,* 1992), and to that observed in the other members of the *L. shannonii* alliance. *L. salvadorensis* is highly prized by local farmers for construction and fuelwood, and given the proposed strategy for *in-situ* genetic conservation of the species through use by farmers in tree planting programmes (HELLIN and HUGHES, 1993), knowledge of population differentiation is vital to guide seed collection strategies. HELLIN and HUGHES (1993) delimited 5 broad provenance regions for *L. salvadorensis,* based solely on geography, as an interim framework for seed collection and *in-situ* conservation. Although the 2 populations studied are currently isolated due to severe fragmentation of the dry forest cover in this region, the limited level of population differentiation suggests that *L. salvadorensis* formerly occupied a more or less continuous distribution that presented few barriers to gene flow in the past. Strict adherence to the zones identified for seed collection by HELLIN and HUGHES (1993) may not, therefore, be warranted, although further testing of material from the extreme western end of the species distribution in Honduras and in eastern El Salvador, would be necessary to verify the lack of population differentiation within *L. salvadorensis.*

Much higher levels of population differentiation were detected for the other species in the alliance, with the highest level of between-population variation found in *L. shannonii* subsp. *shannonii.* This species has a wide distribution with many disjunctions. Mexican populations in the Yucatan Peninsula and the central depression of Chiapas are distantly isolated

from the remainder of the populations in Central America. Even within Honduras and Nicaragua, the species occupies the isolated inland seasonally dry valley systems, again in a series of disjunct populations with limited scope for between-population gene flow. Isozyme variation in the *L. shannonii* alliance has therefore provided a useful insight into the forces that have shaped the populations seen today, and provides valuable data that may impact on the future improvement and conservation of these taxa.

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