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Detection of genetic variation between and within populations of *Gliricidia sepium* and *G. maculata* using RAPD markers

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Gliricidia sepium and *G. maculata* are multi-purpose leguminous trees native to Central America and Mexico. Research programmes have been initiated to define the native distribution of *Gliricidia* and sample the spectrum of genetic variation. To date, there has been little systematic assessment of genetic variability in multi-purpose tree species. Accurate estimates of diversity between- and within-populations are considered a prerequisite for the optimization of sampling and breeding strategies. We have used a PCR-based polymorphic assay procedure (RAPDs) to monitor genetic variability in *Gliricidia*. Extensive genetic variability was detected between species and the variability was partitioned into between- and within-population components. On average, most (60 per cent) of the variation occurs between *G. sepium* populations but oligonucleotide primers differed in their capacity to detect variability between and within populations. Population-specific genetic markers were identified. RAPDs provide a cost-effective method for the precise and routine evaluation of variability and may be used to identify areas of maximum diversity. The approaches outlined have general applicability to a range of organisms and are discussed in relation to the exploitation of multi-purpose tree species of the tropics.

Keywords: genetics, *Gliricidia*, population, RAPDs, woody perennials.

Introduction

Multi-purpose tree species have recently received increased attention because they could enhance the productivity, diversity and sustainability of marginal ecosystems. Despite their ecological and agricultural importance, however, little is known of the basic biology of non-industrial trees (Simons, 1991). The genetic improvement of any organism depends on the existence, nature and extent of the genetic variability available for manipulation. The partitioning of variability between and within populations will influence the breeding strategy to be adopted. There has been little systematic assessment of genetic variability or evaluation of the spatial distribution of diversity within the gene pools of multi-purpose tree species.

Traditionally, genetic resources have been characterized by a combination of morphological and agronomic traits. The effectiveness of this approach to estimate genetic diversity, however, has been questioned by several authors (Gottlieb, 1977; Brown, 1979). As an alternative, isozymes have been used extensively to monitor diversity (Soltis & Soltis, 1990) and optimize breeding strategies for tree species (Yeh, 1989). Numerous studies have revealed the non-random distribution of genetic variation and emphasized the importance of understanding the genetic structure of a population (Loveless & Hamrick, 1984). Recent reviews of the levels of variation detected in a range of plant species, based on isozyme data, reveal that tropical tree species maintain most of their variation within populations (Hamrick, 1990). This suggests that the classical forestry approach, which considers provenance or geographical variation as an accurate predictor of the diversity spectrum within a species, may be inappropriate. The extreme disturbance of the natural gene pools of many multi-purpose tropical tree species, through human intervention, also indicates that the partitioning and distribution of variability may be complex. These factors emphasize that there is an urgent need to examine the population genetic structure of multi-purpose tree species.

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Molecular and biochemical techniques provide a powerful set of tools for the study of plant population genetics. Numerous studies have used restriction-site diversity to infer population genetic structure (Clegg, 1989a,b). Restriction fragment length polymorphisms (RFLP) are the most frequently used type of DNA marker. The RFLP assay requires large quantities of relatively pure DNA, species-specific DNA probes, and generally uses short-lived radio-isotopes in the detection system. Furthermore, RFLP analysis is laborious, making it impractical for many population-based studies.

The analysis of nucleotide sequence variability has been revolutionized by the development of the polymerase chain reaction (PCR) (Saiki *et al.*, 1988). A major limitation of the procedure, however, has been the requirement for DNA-sequence information (Innis *et al.*, 1990). Recently, Williams *et al.* (1990) and Welsh & McClelland (1990) described a novel procedure for the identification of polymorphism in plants based on PCR which is not dependent on having prior knowledge of a DNA sequence. Randomly amplified polymorphic DNA sequences or RAPD markers are based on the amplificatoin of unknown DNA sequences using single, short, random oligonucleotide primers. The RAPD method overcomes many of the technical limitations of RFLPs and has been used for genetic fingerprinting (Wilde *et al.*, 1992), creating linkage maps (Rafalski *et al.*, 1991), locating disease resistance genes (Martin *et al.*, 1991; Michelmore *et al.*, 1992), identifying chromosome-specific markers (Quiros *et al.*, 1991) and characterization of somatic hybrids (Baird *et al.*, 1992).

Most studies of diversity and population structure of tree species have concentrated on commercial species, primarily temperate zone conifers. In this paper, we report the use of RAPD markers to investigate the extent and distribution of genetic diversity in *Gliricidia sepium* and *G. maculata*. These are nitrogen-fixing trees native to Central America and Mexico (Hughes, 1987), but are important components of many agroforestry systems in the topics. *G. sepium* is an aggressive colonizing species often forming dense pure stands. It is entomophilous and an obligate out-breeder (A. J. Simons, unpublished observations). A systematic evaluation and quantification of the variability available in the *Gliricidia* gene pool will make it easier to exploit this species as a multi-purpose tree for tropical agroforestry.

Materials and methods

Plant material

The species and populations studied together with their geographical origin are given in Table 1.

DNA extractions

DNA was isolated from fresh leaf material using a modification of the method described by Edwards *et al.* (1991). Samples were collected using the lid of a sterile Eppendorf tube to pinch out a disc of leaf material. This ensured a uniform sample size and reduced the possibility of contamination arising from handling the tissue. The tissue was macerated in the original tube at room temperature using disposable Eppendorf grinders, with the addition of 10–20 mg of Polyclar AT (polyvinylpyrrolidone, insoluble). Four-hundred microlitres of extraction buffer (200 mM Tris HCl, pH 7.0, 250 mM NaCl, 25 mM EDTA, 0.5 per cent SDS, 10 mM mercaptoethanol) were added and the sample vortexed for 5 s. The extracts were centrifuged at 13,000 r.p.m. for 1 min and the supernatant transferred to a clean tube. The supernatant was extracted with phenol/chloroform, then chloroform

Table 1 *Gliricidia sepium* and *G. maculata* samples studied

Sample number	Species	Indent number	Origin	
1–5	*G. maculata*	42/85	Mexico	Quintana
6–10	*G. maculata*	43/87	Mexico	Xeb
11–15	*G. sepium*	72/87	Thailand	Chumphon
16–20	*G. sepium*	40/85	Mexico	Arriaga
21–25	*G. sepium*	59/87	Guatemala	Vado Hondo
26–30	*G. sepium*	1/86	Venezuela	Mariara
31–35	*G. sepium*	13/86	Panama	Pedasi
36–40	*G. sepium*	11/86	Costa Rica	El Roblar
41–45	*G. sepium*	12/86	Costa Rica	Playa Tamarindo
46–50	*G. sepium*	31/84	Nicaragua	Managua

and the resulting aqueous fraction were mixed with 300 μl isopropanol and left at room temperature for 2 min to precipitate the DNA. Following centrifugation at 13,000 r.p.m. for 5 min, the DNA pellet was vacuum dried and dissolved in 100 μl TE buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA). The DNA was stable at 4°C for at least 12 weeks. Four microlitres of this sample was sufficient for a standard 100-μl PCR reaction.

Polymerase chain reaction (PCR)

PCR reaction mixtures (100 μl final volume) contained approximately 100 ng genomic DNA, dATP, dCTP, dGTP and dTTP each at 100 μM final concentration, 200 nM primer, 1 × Taq polymerase buffer and 1.3 units of Taq polymerase (Northumbria Biologicals Ltd). Each reaction was overlaid with 100 μl of mineral oil to prevent evaporation. The random sequence 10-mer primers used in this study (Table 2) were synthesized on an applied Bio-systems 391 PCR-mate oligonucleotide synthesizer. Samples for enzymatic amplification were subjected to 45 repeats of the following thermal cycle: 1 min at 92°C, 3 min at 35°C and 2 min at 72°C. After the final cycle, samples were incubated for a further 3 min at 72°C then held at 4°C prior to analysis. Fragments generated by amplification were separated according to size on 2 per cent agarose gels run in 1 × TBE (89 mM Tris HCl, pH 8.3, 89 mM Boric acid, 5 mM EDTA), stained with ethidium bromide and visualized by illumination with ultraviolet light (312 mm).

Primer and product indexing

The following conversion was adhered to throughout. Consider, for example, the amplification product designated SC10-60. SC10-60-G900 was generated by primer SC10-60 and was synthesized at the Scottish Crop Research Institute, it is 10 nt in length and it is number 60 in our primer collection. The product of interest, G900, generated with this primer, is from *Gliricidia* and is 900 nt in length.

Table 2 Random oligonucleotide primer sequences

Primer	Sequence
SC10-30	5′CCGAACGGGT
SC10-34	5′GGTGGGTGCT
SC10-35	5′GTGCGGACAG
SC10-36	5′TCACCGAACG
SC10-37	5′GCCAATCCTG
SC10-39	5′GGACGGGTGC
SC10-45	5′GGACCACCAT
SC10-49	5′CCACGACGAT
SC10-58	5′CGGGAGACCC
SC10-59	5′GCATGGAGCT
SC10-60	5′GGCCTTGAGT

Hybridization analysis

Amplified DNA fragments separated according to their length by polyacrylamide gel electrophoresis were transferred electrophoretically to Biodyne Nylon membrane (PALL) in a Biorad electroblotting apparatus in 0.4 M TBE at 1.5 A constant current for 1 h. Fragments to be used as probes were excised and the DNA recovered from the gel by electroelution in dialysis tubing as described by Maniatis *et al.* (1982). Isolated fragments were labelled by random priming (Feinberg & Vogelstein, 1984) with ^{32}P dCTP (3,000 Ci mmol^{-1}, ICN Biomedicals), and used to probe the prepared blots of standard procedures (Maniatis *et al.*, 1982). After hybridization, the blots were washed in several changes of 0.1 × SSC, 0.1 per cent SDS at 65°C and the hybridizing fragments revealed by autoradiography.

Results

The DNA mini-preparation extraction procedure, using a single-leaf disc, produced DNA of sufficient quality for 25 PCR reactions using the 10-mer oligonucleotides listed in Table 2. Ten of the 11 primers evaluated generated amplification products of varying sizes which detected polymorphism between *Gliricidia* genotypes. An example of the polymorphism detected with SC10-60 is given in Fig. 1, which clearly discriminates between *G. sepium* and *G. maculata*. In addition, molecular variability is detected between and within the *G. sepium* populations. Samples 21–25, originating from Guatemala, and samples 16–20, from Mexico, are clearly polymorphic for a major 0.9 kb amplification product generated by SC10-60 (designated SC10-60-G900). Amplification products, which are unique to the *G. sepium* samples from Thailand, were also identified with SC10-38 (Fig. 2). The Panamanian and Venezuelan populations exhibited relatively low levels of variability, by contrast with the polymorphism detected in the remaining populations (Fig. 3).

Partitioning of genetic variability between and within G. sepium *populations*

The number of polymorphic loci detected with the nine primers for the eight *G. sepium* populations is given in Table 3. The total number of polymorphic loci

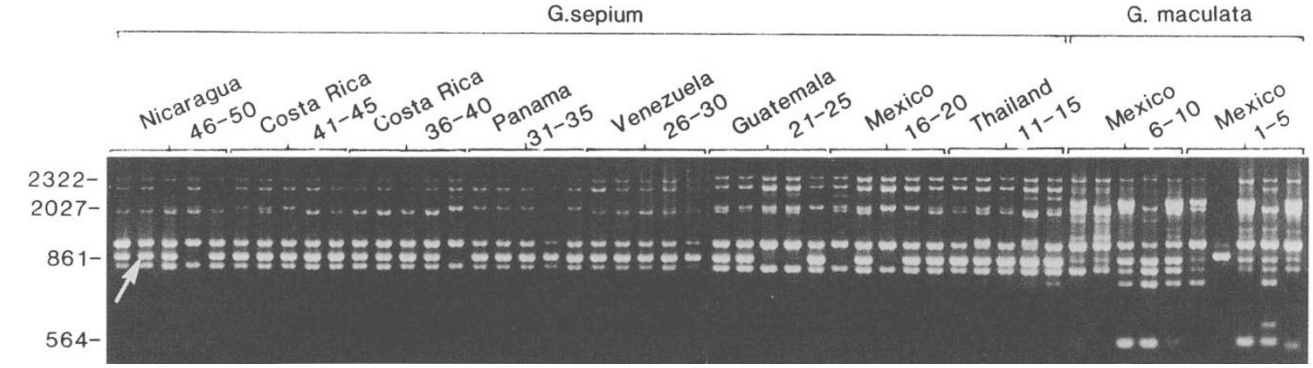

Fig. 1 Amplification products generated from five individuals of 10 populations of *Gliricidia* spp. using primer SC10-60. The products were generated and resolved as described in the Materials and Methods. The polymorphic 900 bp fragment (SC10-60-G900) is highlighted (arrow).

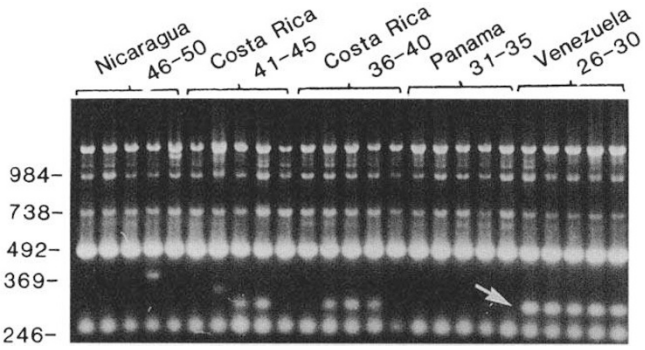

Fig. 2 Monomorphism in the Panamanian (tracks 31–35) and Venezuelan (tracks 26–30) populations for the presence or absence of SC10-38-G300 (arrow) are contrasted with the polymorphism within the Costa Rican (tracks 36–40 and 41–45) and Nicaraguan (tracks 46–50) populations.

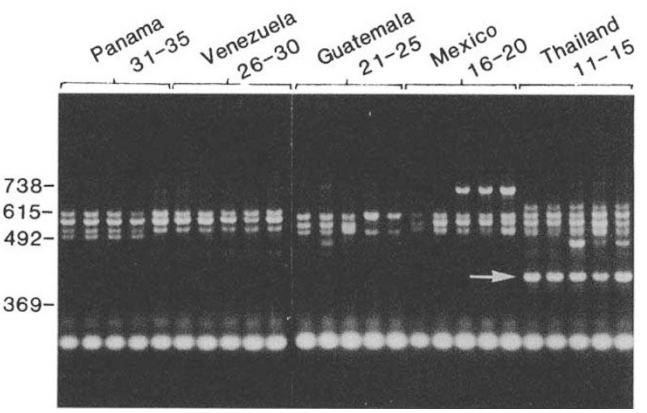

Fig. 3 Products of PCR amplification from five populations with SC10-58. The presence of SC10-58-G420 (arrow) clearly distinguishes the Thailand population from the other populations.

detected varied between primers and populations. For example, primer SC10-36 and SC10-59 detected a total of 12 and 11 loci respectively. The Venezuelan and Panamanian populations are characterized by being monomorphic for seven of the nine primers evaluated. The phenotypic frequencies detected with the nine primers evaluated for the eight *G. sepium* populations were calculated and estimates of diversity (H_0) within populations were obtained using Shannon's information measure $H_0 = -\Sigma p_i \log_2 p_i$, where p_i is the phenotypic frequency (King & Schaal, 1989). Estimates of genetic diversity within populations are given for each primer in Table 4. Overall the Venezuelan and Panama populations exhibit relatively low levels of within-population variability. The five individual genotypes assayed were found to be monomorphic for seven primers in the case of the Venezuelan populations and for six of the nine primers in the case of the Panamanian population. The Nicaraguan and Guatemalan populations exhibited the highest levels of within-population variability. Information on those primers which identify unique phenotypes with a particular *G. sepium* population is also presented in Table 4. For example, SC10-39 identified unique phenotypes with three populations, two of which originate from Venezuela and Panama.

Shannon's index of phenotypic diversity was used to partition the diversity into within- and between-population components (Table 5). H_{pop} provides a measure of the average diversity within populations. SC10-59 detects the most and SC10-39 the least within-population variability. An examination of the proportion of diversity present within populations (H_{pop}/H_{sp}) and between populations ($H_{sp} - H_{pop}/H_{sp}$) indicates that, on average, most of the diversity (60 per cent) occurs between *G. sepium* populations. The distribution of

Table 3 Polymorphic loci detected with nine primers for eight populations of *G. sepium* (proportion of polymorphic loci)

Primer	Location sample number								Total number polymorphic loci
	Thailand 11–15	Mexico 16–20	Guatemala 21–25	Venezuela 26–30	Panama 31–35	Costa Rica 36–40	Costa Rica 41–45	Nicaragua 46–50	
SC10-30	1 (0.059)	2 (0.118)	1 (0.059)	1 (0.059)	0 (0.000)	1 (0.059)	3 (0.176)	2 (0.118)	6 (0.353)
SC10-34	0 (0.000)	1 (0.250)	1 (0.250)	0 (0.000)	0 (0.000)	3 (0.750)	2 (0.250)	2 (0.250)	7 (0.875)
SC10-36	7 (0.583)	5 (0.417)	4 (0.333)	0 (0.000)	0 (0.000)	0 (0.000)	3 (0.250)	6 (0.500)	12 (1.000)
SC10-39	3 (0.428)	1 (0.125)	2 (0.250)	0 (0.000)	0 (0.000)	0 (0.000)	1 (0.125)	1 (0.125)	7 (0.636)
SC10-45	9 (0.000)	2 (0.500)	2 (0.500)	0.(0.000)	0 (0.000)	1 (0.250)	1 (0.250)	1 (0.250)	4 (0.219)
SC10-49	1 (0.250)	1 (0.250)	1 (0.250)	0 (0.000)	0 (0.000)	1 (0.250)	1 (0.250)	2 (0.500)	2 (0.500)
SC10-58	1 (0.111)	1 (0.111)	2 (0.222)	0 (0.000)	1 (0.111)	2 (0.222)	3 (0.333)	4 (0.444)	8 (0.889)
SC10-59	4 (0.267)	4 (0.267)	5 (0.333)	2 (0.133)	1 (0.067)	3 (0.200)	7 (0.467)	3 (0.200)	11 (0.733)
SC10-60	2 (0.222)	3 (0.333)	3 (0.333)	0 (0.000)	0 (0.000)	4 (0.444)	3 (0.333)	3 (0.333)	6 (0.667)

Table 4 Estimates of genetic diversity (H_0) within populations for *G. sepium* sampled from eight locations

Primer	Location sample number							
	Thailand 11–15	Mexico 16–20	Guatemala 21–25	Venezuela 26–30	Panama 31–35	Costa Rica 36–40	Costa Rica 41–45	Nicaragua 46–50
SC10-30	0.722	1.585	0.722	0.722	0.000	0.722	1.522	1.371
SC10-34	0.000*	0.918	0.722	0.000	0.000	2.386	1.371	2.386
SC10-36	2.386*	1.585*	2.322	0.000	1.500	1.371	1.522	2.322
SC10-39	0.971*	0.971	1.522	0.000*	0.000*	0.000	0.722	0.971
SC10-45	0.000*	0.722*	1.371	0.000	0.000	0.971	0.971	0.722
SC10-49	0.811	1.000	0.722	0.000	0.000	0.971	0.971	0.722
SC10-58	0.971*	0.971	1.522	0.000	0.722	1.371	1.522	0.918
SC10-59	2.322	2.386	2.322	0.722	1.522*	2.386	2.322	2.322
SC10-60	2.386	2.322	2.386	0.000	0.000	1.371	1.522	2.386
$\bar{x}$	1.174	1.384	1.512	0.160	0.416	1.277	1.382	1.754

*Indicates populations consisting entirely of unique RAPD phenotypes.

Table 5 Partitioning of the genetic diversity between- and within-populations of *G. sepium* for nine random oligonucleotide primers

Primer	H_{pop}	H_{sp}	H_{pop}/H_{sp}	$(H_{sp} - H_{pop})/H_{sp}$
SC10-30	0.921	2.344	0.393	0.607
SC10-34	0.973	2.292	0.424	0.576
SC10-36	1.626	4.229	0.384	0.616
SC10-39	0.645	3.146	0.205	0.795
SC10-45	0.595	2.336	0.255	0.745
SC10-49	0.851	1.807	0.471	0.529
SC10-58	1.547	2.804	0.552	0.448
SC10-59	2.038	4.682	0.435	0.565
SC10-60	1.547	3.146	0.491	0.509
$\bar{x}$	1.194	2.976	0.401	0.599

variability between and within populations, however, does vary between primers. For example, SC10-39 detects most variability between populations (80 per cent) whereas primer 58 detects most variation within populations (55 per cent).

To complement the analysis based on phenotypic frequencies Nei's estimate of similarity (Nei, 1972), based on the number of shared amplification products (Nei & Li, 1979), was used to generate a similarity matrix (Table 6). The populations from Venezuela and Panama exhibit the least variability and the Mexican population exhibits the most. The proportion of shared fragments ranges from 0.50 to 0.81 and the population introduced into Thailand is most similar to the Mexican and Guatemalan populations. The similarity matrix

Table 6 Similarity matrix generated from Nei & Li's (1976) estimate of similarity based on the number of shared fragments. The variability within populations is represented by the leading diagonal. The remaining components of the matrix are based on the means of all five individuals for each population

	Thailand	Mexico	Guatemala	Panama	Venezuela	Costa Rica (11/86)	Costa Rica (12/86)	Nicaragua
Thailand	0.8098							
Mexico	0.6523	0.7222						
Guatemala	0.6481	0.7407	0.8245					
Panama	0.5467	0.6518	0.7091	0.9417				
Venezuela	0.5160	0.6314	0.6703	0.7365	0.9298			
Costa Rica (11/86)	0.5291	0.6340	0.7013	0.8077	0.7753	0.8499		
Costa Rica (12/86)	0.5245	0.6005	0.6705	0.7572	0.7267	0.7814	0.7798	
Nicaragua	0.4983	0.5955	0.6794	0.7442	0.6767	0.7555	0.7483	0.7822

highlights the distinction between these populations and the others analysed. The populations sampled from Panama and Venezuela exhibit greatest similarity with the Costa Rican (11/86) population and may therefore have originated from this region of Costa Rica. A dendrogram displaying hierarchial associations is given in Fig. 4. The dendrogram is generated by group-average clustering where the similarity between two groups is defined as the average similarity of all points of unit involving a member of each group. The Thailand, Guatemalan and Mexico populations appear to form a distinct group.

The molecular profiles generated for the Guatemalan (59/87) population with SC10-39 combine the amplification products generated from the Thailand populations of *G. sepium* (SC10-39-G-210 and SC10-39-G-180). The three phenotypes identified in the Guatemalan sample (Fig. 5a) could be due to allelic amplification products at a single genetic locus. Williams *et al.* (1990) have demonstrated the RAPD markers tend to be inherited in a dominant/recessive manner. Thus allelism at a single locus would appear to be unlikely. To resolve this issue, the amplification pro-

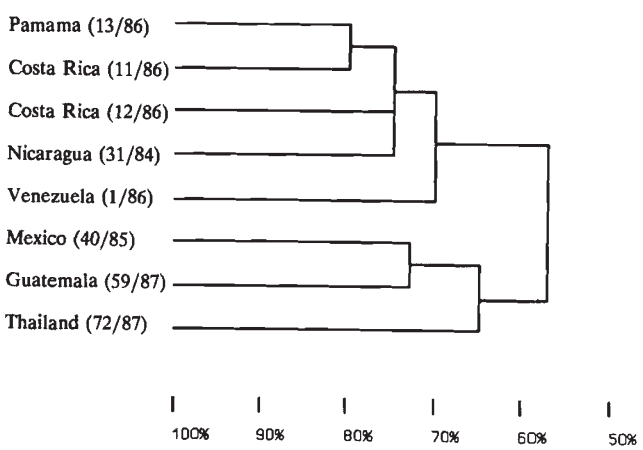

Fig. 4 Dendrogram of *G. sepium* populations generated by group average clustering analysis.

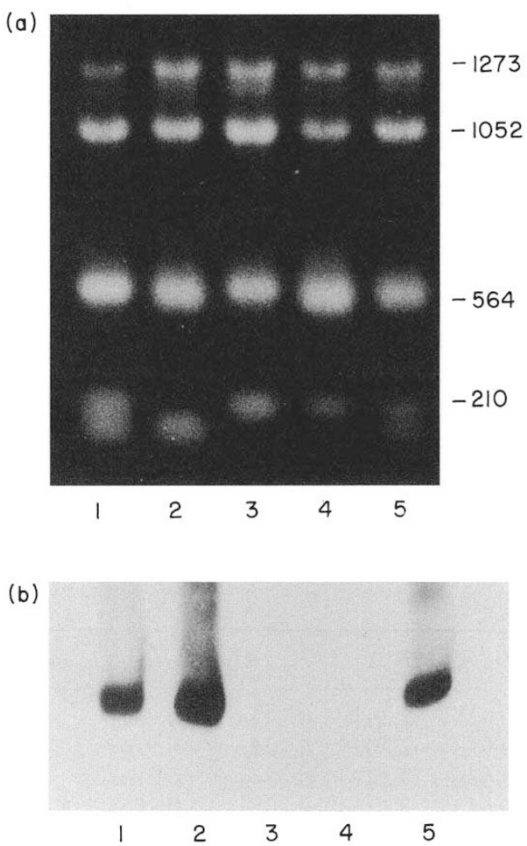

Fig. 5 (a) Amplification products of *Gliricidia* population from Guatemala with SC10-39. Polymorphic bands SC10-39-G-210 and SC10-39-G-180 are highlighted. (b) SC10-39-G-180 was excised from lane 2 in (a) and used to probe a Southern blot of the same amplification products.

duct SC10-39-G-180 was excised labelled with [^{32}P] dCTP and used to probe Southern blots of the amplified products. If the amplification products are allelic then one would expect the labelled fragment to hybridize to products from all five genotypes. Figure 5b clearly demonstrates that the labelled product only hybridizes to the product with the corresponding size (SC10-39-G-180) and that the products are non-allelic. The amplification products must therefore correspond to two loci.

Discussion and conclusions

Methods have been established to detect and monitor genetic variability routinely and on a large scale for *Gliricidia*. The value of RAPD for detecting intra-population variation is endorsed by the fact that the provenance ranks of within-provenance variation, given in Table 4, correspond closely with estimates of variation for many quantitative characters assessed in field trials (A. J. Simons, unpublished observations). The polymorphism revealed by amplification of arbitrary primers is extensive and, to our knowledge, this is the first report of the partitioning of genetic variability within and between populations using RAPD markers. Accurate estimates of diversity are a prerequisite for optimizing sampling strategies and for conserving tree genetic resources. The high diversity revealed by RAPD is in agreement with the conclusion that outbreeding woody plants retain considerable variability (Hamrick, 1990). The polymorphic assay procedures used in the present study discriminate well between *G. sepium* and *G. maculata* and provide molecular support for the conclusion that *G. maculata* should be considered a distinct taxon.

Most of the diversity was detected between populations. This is contrary to the results accumulated by Hamrick (1990), who indicated that for outbreeding, woody perennial plants, most variation is exhibited within populations. This conclusion, however, is based on isozyme analyses and because these represent only coding regions of the genome, such results may not be directly comparable with the RAPD data for *Gliricidia*. Primers did differ, however, in their capacity to detect within- and between-population variability (e.g. SC10-58 detects most variation within populations). Overall, there appears to be population differentiation, and population-specific amplification products (loci) were identified. This finding lends support to the concept of keeping provenances separate. Therefore, where *Gliricidia* is to be used as an exotic, it is essential to carry out provenance testing to identify the most appropriate provenance and discourage the multiple introduction of untested seed sources by agencies releasing germplasm of *Gliricidia*.

Ecological and geographical differentiation are important factors which influence the breeding and sampling strategies of tree crops. It is also essential to consider the relationship between population structure in natural and domesticated populations. Namkoong (1986) has stressed the importance of high levels of genetic variation as a safeguard against co-evolving biotic factors such as pests and diseases. Although *Gliricidia* is native to Central America, introductions have been made to other tropical regions (Hughes, 1987). In some cases (e.g. in Sri Lanka), introductions were based on single tree progenies and may therefore suffer from inbreeding depression. Multi-purpose trees are generally grown on poor sites with variable climates and differing management practices. It is therefore essential to retain a genetic buffering capacity and identify diverse stocks for distribution. The relatively low levels of polymorphism detected within the Panamanian and Venezuelan populations concur with their putative history of domestication from a restricted genetic base.

At least two of the *Gliricidia* populations studied, those from Thailand and Venezuela, lie outside the native range. Group-average cluster analysis (Fig. 4) indicates that the Venezuelan population may originate from Costa Rica or Nicaragua. The Thailand population is quite distinct from the other provenances examined but may have originated from Guatemala or Mexico.

Genetic polymorphism detected with RAPD reveals one allele per locus which corresponds to the amplification product visualized (Fig. 1). RAPD are not expected to identify heterozygous loci. Gene flow and mating system are important determinants of the genetic structure of tree populations. Methods of estimating gene flow and mating system are dependent of the ability to detect heterozygotes (Clegg, 1980). In this context, RAPD are limited. However, each primer detected on average seven polymorphic amplification products or loci (Table 3). RAPD may be used to identify heterozygous individuals when a single primer generates at least one complementary polymorphic amplification product from each parent (Baird *et al.*, 1992). Species- and population-specific loci were also identified in this study. The methodology and approach outlined may therefore have a range of applications. In particular, RAPD are a useful predictive tool to identify areas of maximum diversity and may be used to estimate levels of genetic variability in natural populations. We anticipate that RAPD will have a major impact on the management, conservation and improvement of tropical tree crops.

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