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Genetic Relatedness and Identities of Cultivated Hevea *Clones Determined by Isozymes*

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Isozyme markers were used to determine genetic relatedness between sixty cultivated Hevea *clones originating from the Wickham collection. The mean observed heterozygosity for the clones was 0.567. A dendrogram representing phenetic relationships among the clones in the study was constructed using the unweighted pair-group method with arithmetic averaging. Considerable genetic diversity was observed (mean genetic similarity = 0.754) despite the fact that the gene pool was derived from a small number of plants originally sourced from a single collection area in Brazil. Principal component analysis of the genetic diversity among the sixty clones showed that PR clones and PB clones formed distinctly different clusters. On the other hand, the RRIM clones were evenly distributed, reflecting the selection of RRIM clones from a generally wider genetic base. Isozyme polymorphism was evaluated as a tool for clonal identification and verification among source bushes (from which buds for vegetative propagation are generally obtained). A panel of seven isozymes identified fifty-two out of sixty commercially planted clones outright, while four pairs of clones shared common zymograms. A 0.2% probability of identification error was estimated for the chance occurrence that a rogue plant would have an isozyme profile identical to that of the purported clone. Isozymes were also used to validate 24 clonal progenies derived from hand-pollination. Genotypes of 23 clones were found to be consistent with those of their assigned pedigrees. The exception was RRIM 936 which had some alleles that were absent in either of the purported parents.*

Isozyme markers have been usefully employed by plant breeders to study genetic diversity of crop plants and their evolution. The availability of genetic markers are especially valuable in the breeding of tree crops in view of the length of their reproductive generations. Isozyme polymorphism has facilitated research related to *Hevea* breeding, such as in the measurement of genetic diversity and relatedness[1–3], the estimation of out-crossing rates[4], the estimation of pollen dispersal distances[5] and for clonal identification[6]. Although isozymes have been superseded by newer molecular markers in many areas, the technique remains a useful laboratory tool that is rapid and inexpensive to use.

This paper reports the inter-relatedness of sixty cultivated *Hevea brasiliensis* clones determined by the isozyme technique. Isozyme

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polymorphism was also employed for clonal identification and for pedigree verification of selected cultivars.

MATERIALS AND METHODS

Plant Materials

Sixty *Hevea brasiliensis* clones, representing a cross-section of cultivars planted in Malaysia, were selected to study their genetic relatedness. All the clones were believed to be descended from the original 1876 Henry Wickham collection[7]. The selection included the six *Class I* clones which are clones recommended for large scale planting and the twenty *Class II* clones recommended for moderate scale planting as listed in the Rubber Research Institute of Malaysia (RRIM) Clonal Planting Recommendations[8] 1992–94. (This classification scheme has since been discontinued.) The remaining 34 clones comprised some of the older cultivars (*e.g.* PB 86), several promising new clones (the later RRIM 900 series) and four experimental clones (the PC series and PM 8). Of the sixty clones, 25 originated from the RRIM. These were the RRIM and PC clones. Fifteen clones were bred by the Prang Besar Research Station in Malaysia and these bear the PB prefix. The two PM clones were from a Malaysian ortet selection of seedling plants. The eight PR clones were Javanese in origin from the Tjiomas Experimental Estate (now Indonesian Rubber Research) in Bogor. The clone GT 1 (Gondang Tapen Estate) was also of Javanese origin while the four BPM (Balai Penelitian Medan) clones were bred in Sumatra, Indonesia. There were five clones from Sri Lanka identified by the prefixes RRIC (Rubber Research Institute of Ceylon) and NAB (Nabutemme Estate).

Enzyme Extraction

Leaf samples of various clones were obtained from source bush nurseries in the Rubber Research Institute of Malaysia Experiment Station, Sungai Buloh. Preliminary experiments showed that best results were obtained with newly expanded and hardened leaves.

Leaf tissue (0.5 g, midrib discarded) were ground in 1.5 ml of 0.07M Tris-HCl, pH 7.4 containing 10% w/v polyvinylpyrrolidone (PVP-40) in a ice-cold mortar. The homogenate was poured into microcentrifuge tubes and centrifuged for 30 min at 10 000 g in a pre-chilled Sigma 201 M microcentrifuge. The clear supernatant was recovered and stored at -70°C.

Starch Gel Electrophoresis and Isozyme Staining

Wicks of Whatman 1M filter paper (3.5 × 10 mm) were saturated with leaf abstracts and loaded onto 12% starch gels. The leaf proteins were separated by horizontal starch gel electrophoresis using four different buffer systems. Electrophoretic and staining methods were adopted from Lebrun and Chevallier[9] and from Wickneswari and Norwati[10] (*Table 1*). Each leaf sample was scored for electrophoretic variation at seven loci from the seven enzyme systems, *viz.* Esterase (EST), shikimate dehydrogenase (SDH), aspartate aminotransferase (AAT), peroxidase (PER), isocitrate dehydrogenase (IDH), uridine diphosphogluconate pyrophosphatase (UDP) and phosphogluconate dehydrogenase (PGD). For each enzyme system, the most anodally negative locus was scored and designated locus 1. Within each locus, the fastest migrating band was designated allele 1, and each successively slower band was allele 2 and allele 3.

TABLE 1. BUFFERS FOR STARCH GEL ELECTROPHORETIC SYSTEMS FOR ISOZYME ANALYSIS

Enzyme	Gel buffer	Electrode buffer	Reference
Esterase (EST) E.C. 3.1.1.-	Tris-histidine, pH6	Tris-citrate, pH 6.6	Le Brun and Chevallier[9]
Shikimate dehydrogenase (SDH) E.C. 1.1.1.25	Histidine, pH 8	Citrate, pH 8	Wickneswari and Norwati[10]
Aspartate aminotransferase (AAT) E.C. 2.6.1.1	Tris-citrate, pH 7.9	Lithium-borate, pH 8.5	Wickneswari and Norwati[10]
Peroxidase (PER) E.C. 1.11.1.7			
Isocitrate dehydrogenase (IDH) E.C. 1.1.1.42	Morpholine citrate, pH 6.1	Morpholine citrate, pH 6.1	Wickneswari and Norwati[10]
Uridine diphosphogluconate pyrophosphotase (UGP)			
Phosphogluconate dehydrogenase (PGD) E.C. 1.1.1.44			

Data Analyses

Heterozygosity. Observed heterozygosity for each clone was scored against each enzyme locus. The calculated heterozygosity for the sixty clones based on allele frequency was estimated as the average of $1 - \sum p_i^2$ over the loci studied, where p_i is the frequency of the ith allele of a locus[11].

Genetic similarity. Genetic similarity (F) and genetic distance (1-F) were computed between each pair of sixty *Hevea* clones for all combinations based on allele frequencies at the selected isozyme loci as described by Nei[11]. These values were then used to generate a dendrogram using the unweighted pair-group method with arithmetic averaging as described by Sneath and Sokal[12].

A principal component analysis[13–15] was performed on the binary matrix of the isozyme data based on the presence or absence of allelic bands for the seven isozymes.

Probability of identity. The proportion of zymogram band sharing, S, for each pair of clones, x and y, was calculated using the formula:

$$S = 2m_{xy}/(m_x + m_y)$$

where m_{xy} was the number of shared bands and m_x and m_y were the number of bands for

each clone, x and y combined for all seven isozyme systems. The mean proportion of band sharing was averaged from the S values of all possible combinations of clone pairs. The probability, P, of two randomly selected individuals being identical in all the zymogram bands of the seven isozyme systems was calculated according to Jeffreys and Morton[16] as $P = (1 - 2\bar{S} + 2\bar{S}^2)^{n/\bar{S}}$, where $\bar{S}$ was the mean of S and n was the mean number of isozyme bands per clone.

RESULTS AND DISCUSSION

Genetic Relatedness

Genetic distances and phenetic relationships between *Hevea* clones as determined by isozyme markers[1-3] provide useful information on the relatedness of potential parental clones when these are being selected by breeders for artificial pollination. Pairs of clones picked from the most divergent groupings would be useful candidates for setting up crosses aimed at attaining high heterosis and vigour in the progenies. With the availability of objective guidance from genetic markers, inbreeding depression can be minimised by identifying closely related genotypes and avoiding crosses between them.

Sixty clones were analysed for electrophoretic variation at seven isoenzyme loci representing seven enzyme systems. One polymorphic gene locus was identified for each enzyme and they were controlled by two alleles for Per-1, Aat-1, Sdh-1, Idh-1, Est-1 and by three alleles for Ugp-1 and Pgd-1. (Chevallier *et al.*[1] recorded a total of five alleles for Est-1, but three of these are absent or extremely rare among Wickham clones. They are not considered here.) A total of 16 alleles were scored for the seven loci assayed, giving rise to 23 isozyme genotypes (*Figure 1*). The frequencies of each allele in the sample of clones studied are given in *Table 2*.

In the study, allele 2 of Aat-1 was rarely encountered (*Table 2*), in agreement with the report of Chevallier *et al.*[1] (who referred to the enzyme as glutamate or oxaloacetate transaminase). The 11 homozygotes at Per-1 and Sdh-1, the 22 alleles at Aat-1 and the 33 alleles at Pgd-1 were not encountered among the sixty clones examined. The observed heterozygosity for each of the sixty clones based on the seven enzyme systems are shown in *Table 3*. Mean heterozygosity for all clones was 0.567 with values ranging from 0.286 to 1. In comparison, heterozygosity calculated from the allele frequencies was 0.461. The lower value was not surprising given that the population was the result of selective breeding and genotypic equilibrium was not attained. Both values are higher than the figure of 0.362 given by Chevallier[2] for Wickham clones. This is probably because, unlike Chevallier's sample, the present population is represented by a selection of some of the best yielding cultivars from the Wickham pool. As vigour is a trait selected for high yield in *Hevea* breeding, heterozygosity is favoured. Maintenance of increased heterozygosity is facilitated by the fact that *Hevea* cultivars are multiplied vegetatively without the need for back-crossing to fix desired traits.

Genetic similarity was computed according to Nei[11] between each pair of clones for all combinations of the sixty clones based on allele frequencies at the isozyme loci. F values that were obtained ranged from 0.402 to 1 with a mean of 0.754. The mean genetic distance (1-F) between two clones in the population of 60 was 0.246. A dendrogram depicting phenetic

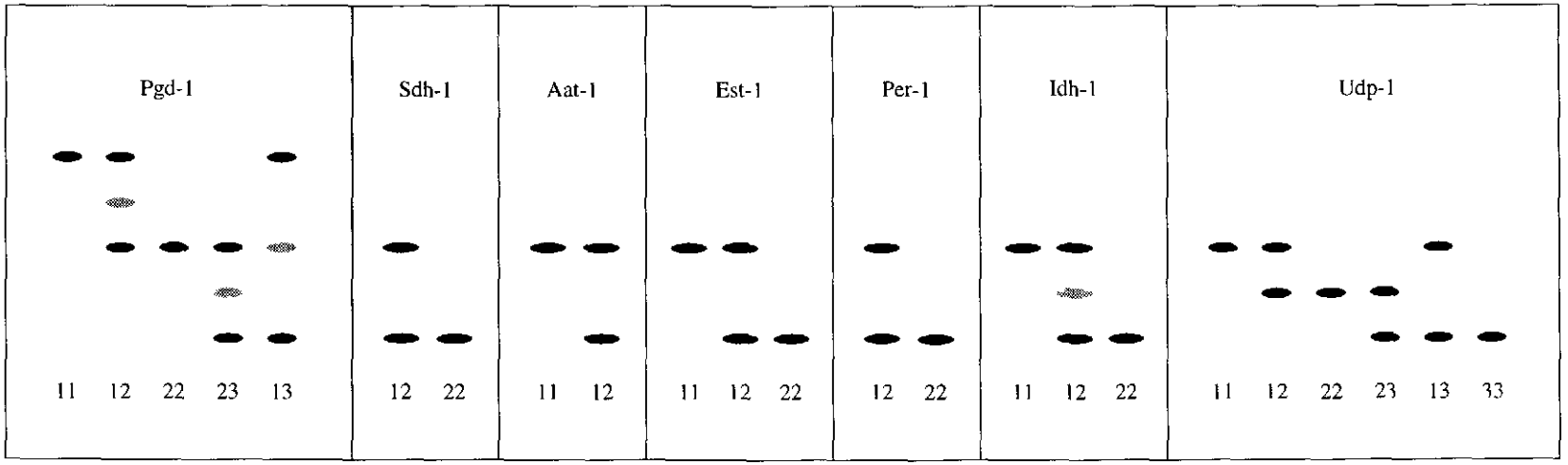

Figure 1 Diagrammatic representations of 23 isozyme genotypes derived from 16 alleles scored for seven loci. Pgd-1 and Idh-1 are dimeric enzymes. In the heterozygous state a heterodimer appears as an intermediate band (shaded) between the two parental homodimers. For Idh-1 the homozygous allozymes (11 or 22) sometimes appear as doublets.

TABLE 2. ISOZYME ALLELE FREQUENCIES

Locus	Per-1		Aat-1		Sdh-1		Idh-1		Est-1		Ugp-1			Pgd-1		
Allele	1	2	1	2	1	2	1	2	1	2	1	2	3	1	2	3
Freq.	.40	.60	.95	.05	.33	.67	.45	.55	.61	.39	.24	.33	.43	.16	.54	.30

TABLE 3. MEAN HETEROZYGOSITY OF CLONES OVER SEVEN GENETIC LOCI

Clone	Mean heterozygosity	Clone	Mean heterozygosity
BPM 1	0.714	PR 302	0.286
BPM 21	1.000	PR 305	0.571
BPM 24	0.429	PR 306	0.429
BPM 26	0.286	PR 307	0.571
GT 1	0.429	RRIC 100	0.429
NAB 17	0.571	RRIC 101	0.714
PB 28/59	0.714	RRIC 102	0.714
PB 86	1.000	RRIC 110	0.714
PB 217	0.714	RRIM 528	0.571
PB 230	0.714	RRIM 600	0.857
PB 235	0.429	RRIM 623	0.571
PB 254	0.571	RRIM 701	0.429
PB 255	0.714	RRIM 702	0.286
PB 260	0.714	RRIM 703	0.857
PB 265	0.286	RRIM 712	0.571
PB 280	0.714	RRIM 713	0.429
PB 290	0.714	RRIM 717	0.429
PB 310	0.714	RRIM 728	0.429
PB 311	0.714	RRIM 729	0.571
PB 312	0.714	RRIM 805	0.571
PB 330	0.429	RRIM 809	0.286
PC 1	0.429	RRIM 901	0.286
PC 96	0.429	RRIM 905	0.429
PC 119	0.714	RRIM 916	0.571
PM 8	0.571	RRIM 921	0.286
PM 10	0.857	RRIM 926	0.571
PR 107	0.429	RRIM 936	0.714
PR 255	0.429	RRIM 937	0.571
PR 258	0.429	RRIM 938	0.714
PR 261	0.286	RRIM 939	0.714

relationships among the sixty *Hevea* clones was constructed based on the genetic similarity data using the unweighted pair-group method with arithmetic averaging (*Figure 2*).

The Wickham gene pool was derived from a small number of plants originally sourced from one collection area in Brazil[7]. Despite the fact that the present day Wickham clones can be said to represent an inbred population, a fair amount of genetic diversity was discernible. This diversity has enabled the rubber yield output of *Hevea brasiliensis* to be increased six-fold in modern clones as compared with the early unselected seedlings[17]. There is even further diversity in indigenous wild *Hevea* populations in South America. The average genetic distance between the Wickham clones in the present study, estimated at 0.246 is about half of the value of 0.490 previously calculated for the genetic distance between recent germplasm sourced from two geographically separated Brazilian states, Acre and Mato Grosso[1].

The principal component analysis revealed interesting trends in genetic diversity among the sixty clones studied. *Figure 3* shows a depiction of the relationships among the clones on the two axes that best explain the clonal variability. The Axis 1/Axis 2 plane represents 34.7% of the total variability. All eight PR clones are clustered on the negative side of Axis 2. On the other hand, the majority of the fifteen PB clones are clustered on the positive side of Axis 1, or close to it. PB clones are absent in the quadrant encompassed by the negative co-ordinates of Axes 1 and 2. These trends suggest a prevalence of common ancestry or restricted gene pools among the PR clones (of Indonesian Javanese origin) and the PB clones (of Malaysian origin), respectively. There could have been a deliberate strategy of selecting for specific traits to which some of the isozymes might be linked. Unlike either the PR or PB clones, the twenty-five RRIM-bred clones (including the three PC clones) in the study sample are evenly distributed, being represented in all sectors of the Axis 1/Axis 2 plane (*Figure 2*). This reflects the RRIM's *Hevea* breeding policy that employed a generally wider genetic base from among the Wickham clones.

Clonal Identification

The vast majority of rubber trees grown in Malaysia are vegetatively propagated as selected high yielding clonal cultivars. Presently in Malaysia, the reliable identification of clones in the field rests on trained clonal inspectors who compare various morphological characters of the tree, such as the branching habit, leaf shape, seed coat pattern, *etc.* Even for them, clonal identification is reliable only for mature trees. Young budded plants that have not set in their distinctly recognisable morphological characters are not always readily identifiable. The ability to identify the diverse clones is important to regulate and monitor planting of the correct cultivars appropriate to the location. Clonal purity is even more important in source bush nurseries from where vegetative buds for clonal propagation are obtained. Any identification error in source bush nurseries would be multiplied when they are disbudded for clonal propagation. Identification of source bush nurseries by clone inspectors is made difficult by the fact that the plants do not attain their natural habit because they are cut back constantly to induce the sprouting of new shoots. Hence, additional identification parameters are required for both immature budded plants as well as source bush nurseries

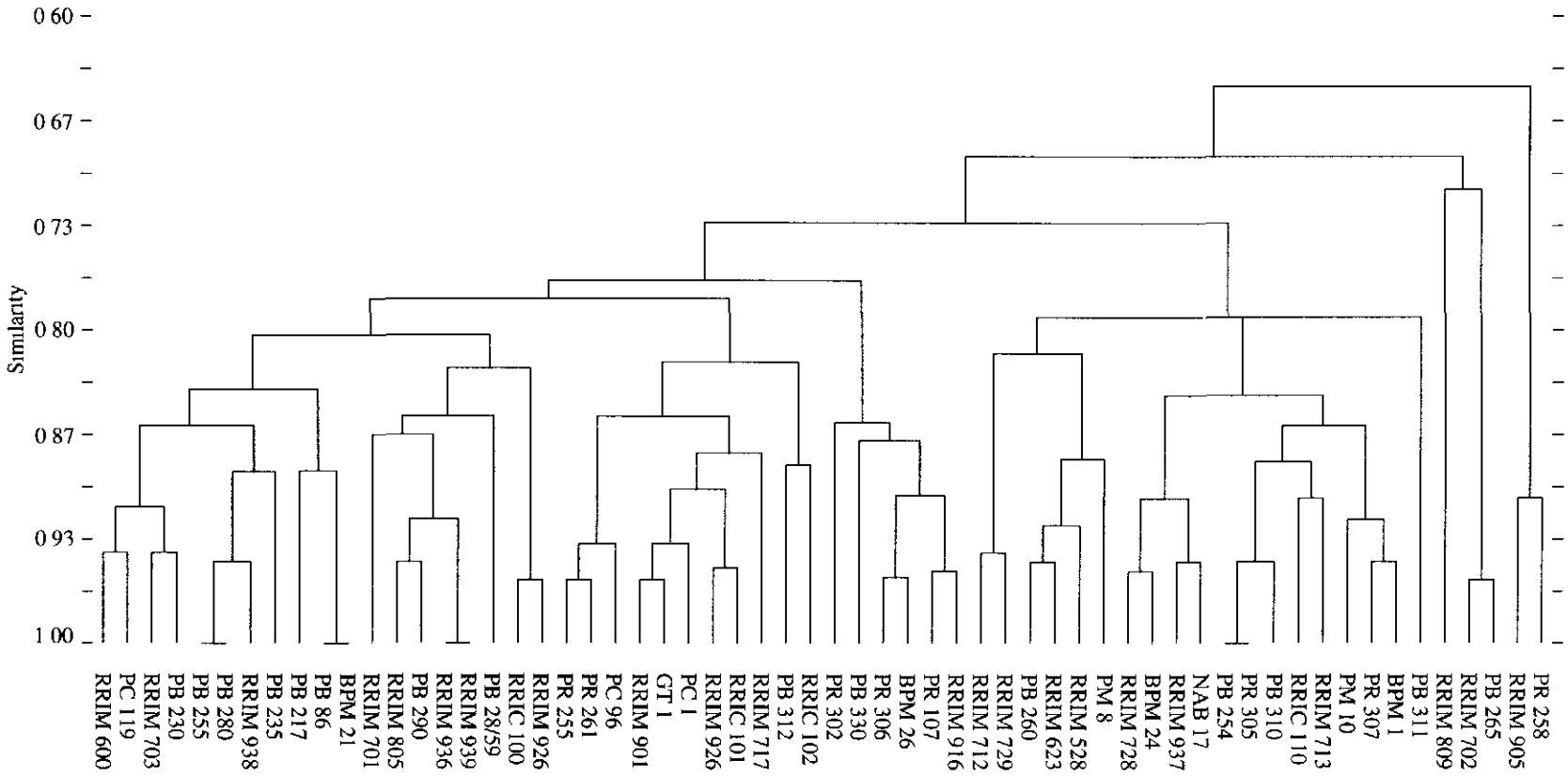

Figure 2 Dendrogram depicting phenetic relationships among sixty Hevea *clones constructed from genetic distance data based on allele frequencies in the clones Dendrogram construction was by the unweighted pair-group method with arithmetic averaging*

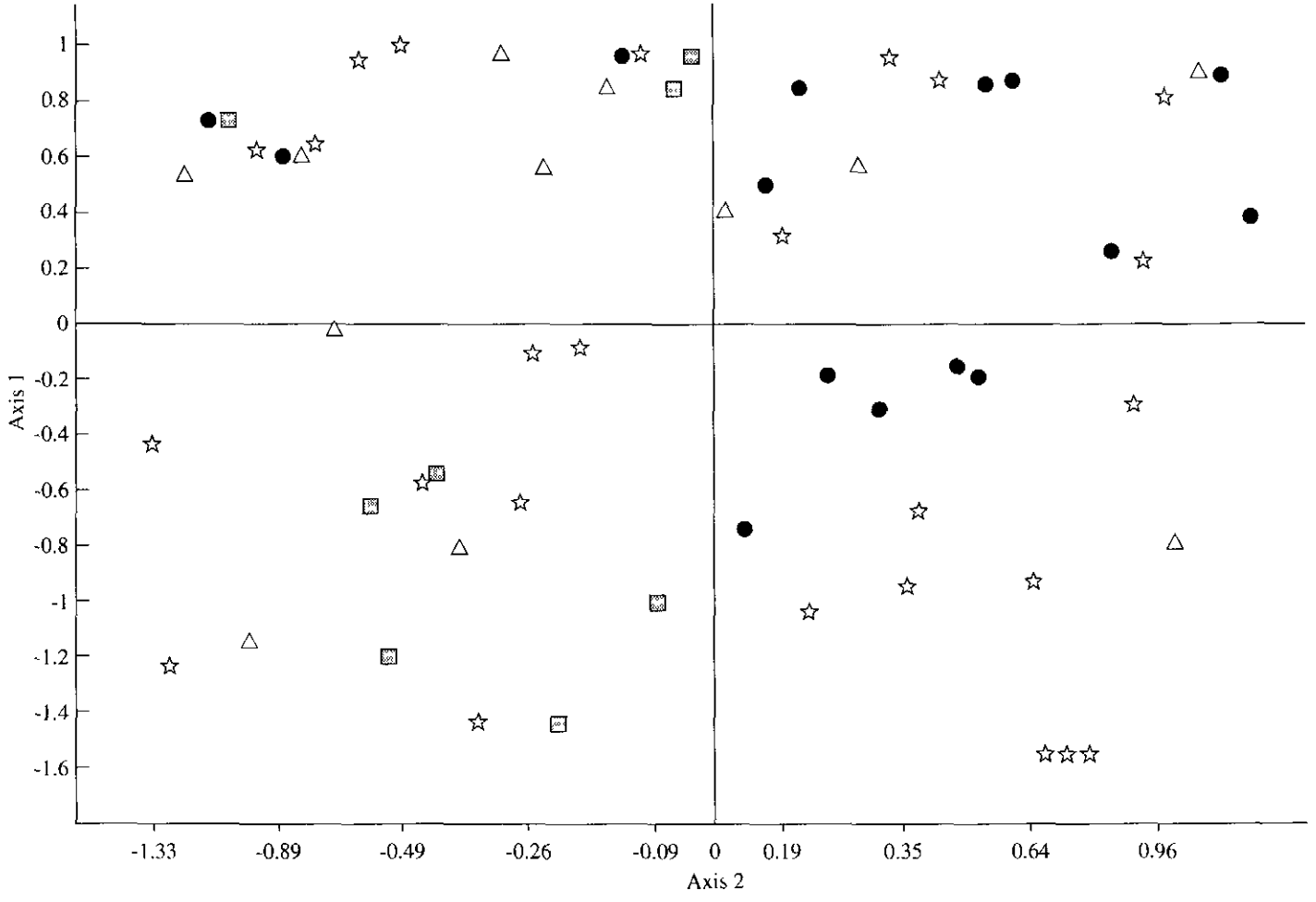

Figure 3. Principal component analysis of the genetic relationships among sixty Hevea *clones based on isozyme data. The first and second axes represent 21.1% and 13.6%, respectively of the total genetic variation. Overlapping points are positioned side by side.*

☆ RRIM clones ● PB clones ⊡ PR clones △ Other clones

to supplement the inadequacies of identification by morphological traits. It is here that isozyme markers are especially useful for clonal identification[6].

The seven isozymes – Est-1, Ugp-1, Aat-1, Per-1, Pgd-1, Skd-1 and Idh-1 – have been adopted to discriminate among sixty commercial *Hevea* clones. Out of the seven isozyme systems, a combined total of sixteen isozyme bands were consistently scorable and a mean of 10.93 bands could be scored per clone. A total of 56 genotypes were recorded among the 60 clones evaluated. It was possible to identify 52 out of the 60 clones outright using these seven isozymes, while 4 pairs of clones shared common zymograms with one another. The chance occurrence that any two clones should have an identical isozyme profile was calculated as 0.0017 according to Jeffreys and Morton[16]. Hence, there is about 0.2% chance of clonal identification error in this manner based on the seven isozyme systems. In practical terms, there is about 0.2% probability of identification error due to the chance occurrence that a rogue plant in a nursery would have an isozyme profile identical to that of the purported clone. Among the sixty clones in this study, there are $^{60}C_2$ combinations, or a possible 1770 ways by which pairs of clones can be combined. Therefore, the four pairs of clones having identical isozyme profiles to one another represent 0.0023% or 0.23% of the possible combinations, which is very close to the predicted figure of 0.0017.

In the present study, only the allelic bands of loci that were identified were used in the scoring. Had other reproducible bands not belonging to specific identified loci been included in the scoring, even better discrimination between clones would have been possible. Obviously, adding more isozymes to the seven presently used in the analysis would further increase the discriminatory power of the technique for greater accuracy in clonal identification.

Pedigree Verification

Plant breeders tracing the lineages of specific clones turn to breeding records for pedigree information. Where carefully compiled, such information is useful and reliable. Even so, infrequent errors do occur and it would be very useful if genetic markers are available to verify the presumed pedigree.

The seven isozymes used for clonal identification were used to validate parent-progeny relationships in breeding experiments where crosses had been made by hand-pollination. Isozyme analysis was carried out on leaves harvested from source bushes of 24 clones and the data were examined for consistency with the expected allelic inheritance behaviour (*Table 4*). The allozymes of the 24 clones were found to be consistent with those of their assigned pedigrees, assuming concurrence to Mendelian genetics, with the exception of RRIM 936. The genotype of this clone was not conciliable with the genotypes of its purported parents, GT 1 and PR 107 for the isozyme loci, Est-1, Ugp-1 and Pgd-1. With these three isozyme systems, RRIM 936 was shown to have some alleles uniquely different from those of either of its purported parents (*Table 5*).

CONCLUSIONS

The following main conclusions may be drawn from the results of the study:

TABLE 4. LIST OF CLONES CHECKED FOR THEIR PEDIGREE BY ISOZYME ANALYSIS

Female parent	Male parent	Progeny
PB 5/51	RRIM 600	RRIM 901
		RRIM 902
		RRIM 903
		RRIM 904
		RRIM 905
		PB 310
Tjir 1	PB 49	RRIM 604
		RRIM 605
		RRIM 607
		RRIM 632
RRIM 605	PB 49	RRIM 714
RRIM 600	PB 235	PB 311
		PB 314
		PB 350
PB 5/51	RRIM 703	RRIM 937
		RRIM 938
PB 5/51	RRIM 623	RRIM 906
		RRIM 907
		RRIM 909
PB 28/59	PR 107	PB 359
PB 325	PR 107	PB 355
Tjir 1	PB 86	RRIM 600
GT 1	RRIM 623	RRIM 728
GT 1	PR 107	RRIM 936

TABLE 5. DISCREPANT ISOZYME GENOTYPES BETWEEN RRIM 936 AND ITS PURPORTED PARENTS

Isozyme loci	GT 1 (purported female parent)	PR 107 (purported male parent)	RRIM 936
Est-1	11	11	12
Ugp-1	33	33	13
Pgd-1	23	23	12

- The population of sixty *Hevea brasiliensis* clones studied had an observed heterozygosity of 0.567.
- The mean genetic similarity between the clones was 0.754.
- PR and PB clones formed distinct clusters based on genetic similarity whereas RRIM clones, in general, were genetically diverse.
- Isozymes can be used for *Hevea* clonal identification and validation.

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