Merits of characterization of cacao by botanical descriptors and isozyme analysis

Felicia Hosein

Two methods of characterization of *Theobroma cacao* L. accessions, morphological characterization and isozyme analysis, were compared. A common group of 34 accessions was studied. Twelve morphological flower descriptors were used. Starch gel electrophoresis was employed to obtain isozyme phenotypes for 5 enzyme systems. According to our analysis, flower descriptors more effectively distinguished the 34 cacao accessions than did isozyme analysis; however the latter may represent more definitive descriptors. The advantages and disadvantages of both methods are discussed.

Introduction

The purpose of a germplasm collection is to make available to users a representative sample of the genetic diversity of a particular plant species. Users must be able to identify accessions of potential use and therefore require adequate characterization and evaluation of the material held in the collection.

Characterization as defined by IBPGR consists of recording those characters which are highly heritable, can be seen by the eye and are expressed in all environments. Characterization for the purpose of classification or identification also includes characters which cannot be seen by the eye, such as isozyme data (Brown *et. al.* 1989).

One of the objectives of the Cocoa research Unit (CRU) is to characterise the primary germplasm of International Cocoa Genebank, Trinidad (ICG,T). This characterization data may be useful in the management of ICG,T by allowing the rationalization of the collection. That is, allowing the minimization of accessions maintained in the collection, without the erosion of the genepool.

Two methods of characterization have been routinely employed at CRU: morphological characters and isozyme markers. A third method, the use of RAPD technology to generate molecular markers, is presently being set up at the CRU.

The purpose of this report is to compare the results we have obtained using the first two methods.

Materials and Methods

Thirty-four accessions maintained in the ICG,T were analyzed (Table 1).

Table 1 *Theobroma cacao* accessions analyzed for morphological flower descriptors and isozyme phenotypes

AMAZ 15	IMC 59
CRUZ 7-14	IMC 60
EET 162	IMC 65
ICS 70	IMC 68
IMC 2	IMC 73
IMC 3	IMC 96
IMC 6	IMC 105
IMC 12	NA 342
IMC 14	NA 534
IMC 33	NA 746
IMC 36	NA 935
IMC 41	P 2B
IMC 42	P 7A
IMC 51	PA 121
IMC 54	PA 169
IMC 55	SCA 6
IMC 57	SPEC 138-11

Morphological descriptors:

Twelve morphological flower descriptors were used (Table 2). Flowers were sampled according to Bekele, (1992). A total of 15 flowers were randomly collected for each accession from an average of four trees.

To allow the comparison with the isozyme data, each of the 12 morphological descriptors were artificially transferred into discrete classes by calculating the mean value from all the data collected. Accessions were then separated into those which showed a value greater than the mean; these were assigned a code 1, and those which showed a value less than the mean, assigned a code 0.

For any particular accession, the combined results for the 12 descriptors was taken as the "morphological" fingerprint for that accession.

Isozyme analysis:

Starch gel electrophoresis was employed to obtain isozyme phenotypes for 5 enzyme systems (Table 3). 300 mg of flush leaf material were used for enzyme extraction, apart from peroxidase (PRX) which was extracted from bark tissue. The tissue was ground with the aid of liquid nitrogen, in 1 ml extraction buffer, (0.5M Tris-HCl pH 8.0, 0.3M Ascorbic acid. 0.1M EDTA tetrasodium salt, 2% w/v DTT, 400 uL Troton X-100 and 300 mg PVPP). Extracts were centrifuged at 8000 rpm for 10 min. and the supernatant absorbed onto filter paper wicks (1.5 mm * 0.5 mm Whatman No.1). Samples were loaded onto 10% starch gels and electrophoresis conducted at 4^{o}C, using the following buffer systems.

- Acid phosphatase (ACP) and Malate dehydrogenase (MDH), tank buffer 0.04M citric acid adjusted to pH 6.7 with N-3(3 aminopropyl morpholine). Gel buffer, a 1:19 dilution of the tank buffer.

- Isocitrate dehydrogenase (IDH), tank buffer 0.4M citric acid (trisodium salt) pH8.0. Gel buffer 0.005M histidine HCl pH8.0.

- Glucophosphoisomerase (GPI), buffers the same as for IDH but with the pH adjusted to 7.0

- Peroxidase (PRX) tank buffer, 0.029M lithium hydroxide and 0.19M boric acid. Gel buffer, a 1:9 dilution of tank buffer.

For the separation of ACP and MDH a constant voltage of 150 V (33-35 mA) was used. For IDH the voltage used was 100 V (25-27 mA). In these systems electrophoresis was performed for 16 h. For GPI a constant voltage of 170 V (40-45 mA) was used for 7h. For PRX a voltage of 170V (40-45 mA) was used for 4 h.

Table 2 Classification of 5 *T. cacao* accessions into morphological fingerprints using 12 flower characters

CHARACTERS	MEANS	AMAZ 15	CRUZ 7-14	EET 162	ICS 70	IMC 2
Sepal length	7.12	0	1	1	0	1
Sepal width	2.43	0	0	0	0	1
Ligule length	2.869	0	1	1	0	1
Ligule width	2.491	0	0	0	1	1
Ribbon length	3.166	1	1	1	1	1
Guideline length	3.585	0	1	1	0	0
Staminode length	6.46	1	0	0	0	1
Ovary length	1.339	0	0	0	1	0
Ovary diameter	1.069	0	0	0	0	1
Ovule number	49	1	1	1	1	1
Style length	2.093	1	1	1	0	1
Pedicel length	14.88	1	0	0	1	1

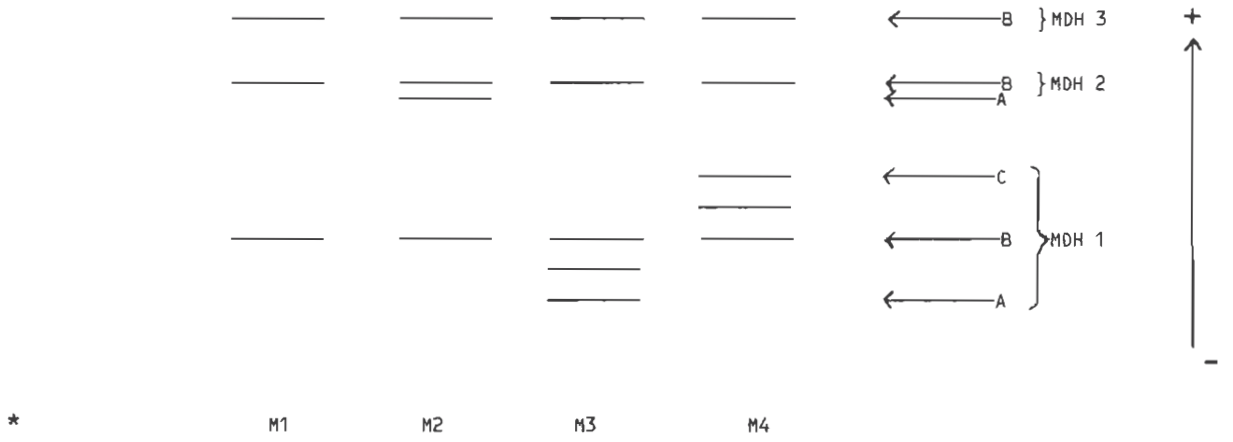
Table 3 Enzyme systems used in the characterization of *T. cacao* accessions.

ENZYME	SOURCE
Peroxidase (PRX)	Bark tissue
Acid Phosphatase (ACP)	Flush leaves
Isocitrate dehydrogenase (IDH)	Flush leaves
Glucophosphoisomerase (GPI)	Flush leaves
Malate dehydrogenase (MDH)	Flush leaves

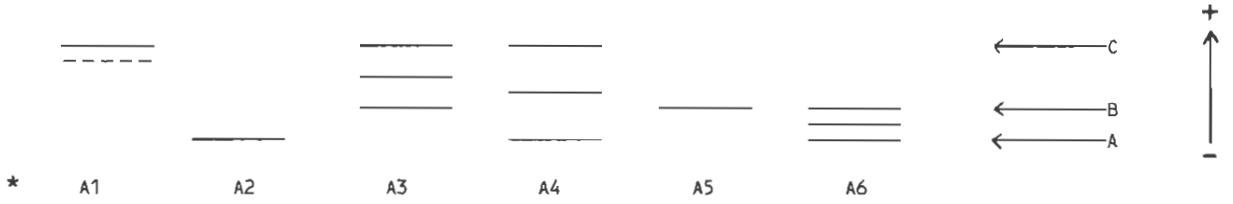
As far as possible, zymogrammes (Fig. 1) were scored for isozyme phenotypes, according to the published genetic models for the enzyme systems in cocoa (Lanaud, 1986). Each phenotype was assigned an identifying code.

The combination of isozyme phenotypes displayed by a particular accession for the 5 enzyme systems was designated the "isozyme fingerprint" for that accession. Accessions were separated into groups based on their isozyme fingerprint.

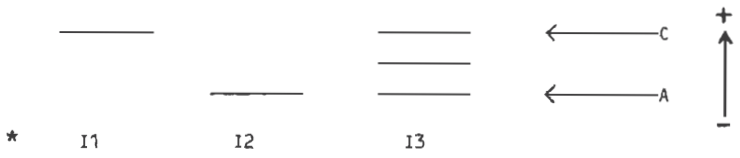
Figure 1 Diagrammatic representation of isozyme phenotypes of cacao accessions obtained by starch gel electrophoresis, for enzyme systems, MDH, ACP and IDH.



Malate Dehydrogenase (MDH)



Acid phosphatase (ACP)



Isocitrate Dehydrogenase (IDH)

*Identifying code assigned to isozyme phenotypes.

Results and Discussion

As reported by Bekele (1991), flower descriptors were found to be better discriminators than other morphological characters, the former were fairly easy to observe and were more environmentally stable. Using this analysis, unique morphological fingerprints were found for 30 of the 34 accessions under study (Table 2).

Only accessions NA 935, P 2B, IMC 51 and IMC 54 could not be uniquely described using these morphological flower descriptors.

A total of 24 isozyme fingerprints was obtained (Table 4). Of the 34 accessions studied, 16 were uniquely described using the 5 enzyme systems.

Based on this analysis, it would seem that the 12 morphological flower descriptors are more effective for classifying cacao germplasm compared to isozyme analysis. There are, however, some points to consider.

The morphological fingerprints described above, were based on mean values calculated for continuous variables. These mean values will therefore not be constant but will depend on the number and type of accessions being analysed. Also morphological characters are strongly influenced by environmental changes, the calculated means will be influenced by this factor, and thus fingerprints may change between environments. Morphological fingerprints may therefore not be considered as constant.

On the other hand, isozyme phenotypes are discrete characters, being determined almost entirely by the genotype of the plant, and enzyme systems, which are not influenced by the environment, can be used for characterization. Isozyme fingerprints may therefore represent more definitive descriptors than morphological descriptors.

Isozyme analysis can be obtained from sexually immature seedlings whereas older, flowering plants are needed for morphological descriptors when flower characters are used.

Isozyme analysis may also be less time consuming than morphological characterization.

These factors may make isozyme analysis more valuable for the characterization of cacao. However, the collection of morphological data, for example those of agronomically economic traits, may help in the use of the available germplasm in breeding programmes. Such characters, for example bean size, can presently only be recorded in terms of their phenotypic expression. In the future, these may be aided by biochemical or molecular markers.

It can be concluded, therefore, that isozyme data may prove to be more definitive descriptors for cacao characterization than morphological descriptors, however the latter may be useful for germplasm evaluation.

One characteristic of isozyme analysis, is that each of the possible genotypes for a particular enzyme system is not equally represented within a population. This may be due to selection pressures, which may result in certain genotypes being favoured over others within a population, or to founder effects. The result is that a larger number of enzyme systems are needed for the unique description of a group of accessions than might be expected from the theoretical estimation of the number of possible isozyme fingerprints.

Table 4 Classification of *T. cacao* accessions into isozyme fingerprints using enzyme systems; PRX, ACP, IDH, MDH and GPI

ISOZYME FINGERPRINT P. A. I. M. G.	ACCESSIONS
1 1 2 1 11	IMC 14, IMC 96
2 1 3 1 11	IMC 12, IMC 65
2 1 3 1 2	IMC 51, IMC 59
2 1 2 1 11	IMC 42
1 1 3 1 9	SPEC 138-11
1 1 1 1 11	IMC 55, IMC 60
2 5 3 1 2	IMC 41, NA 342, NA 746, P 2B
2 3 3 1 2	P 7A, PA 121
1 3 3 1 9	IMC 73
1 1 2 1 9	IMC 105
2 1 1 1 2	IMC 2
2 1 3 3 2	IMC 36
1 1 3 1 2	IMC 6
1 3 1 1 9	IMC 54
1 5 3 1 10	CRUZ 7-14, NA 935
2 5 3 1 9	PA 169
2 1 3 1 10	ICS 70
1 3 3 2 2	IMC 3
2 1 2 1 2	IMC 33
2 1 2 4 2	IMC 68
1 1 1 1 5	SCA 6
1 5 2 4 9	AMAZ 15
2 5 2 1 2	EET 162
2 5 1 1 2	NA 534

This limitation might be overcome by the use of the RAPD technique. This technique is used to detect polymorphisms in the DNA molecules through the use of randomly primed PCR reactions. Since variations in both the non coding and the coding regions of the genome are detected, RAPD markers may prove to be more highly discriminatory than isozyme fingerprints.

RAPD markers may be less influenced by environmental effects, and can also be applied to juvenile material. RAPD analysis is presently being set up at CRU.

Acknowledgements

Research on the application of isozyme analysis for the characterization of *Theobroma cacao* is financed by the Biscuit, Cake, Chocolate and Confectionery Alliance, that of the morphological descriptors by the European Economic Community.

Morphological data were collected and compiled by:
Frances Bekele, Arlene Drakes, Charmela Jagroop, Sandra Ramnarine, Gillian Seow, and Roy Waheed.

Isozyme analysis was done by: Yvette Christopher,
Felicia Hosein, Elizabeth Johnson and Susanne Wharwood. This project was supervised by Dr. John Warren.

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