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Research Paper

## MOLECULAR CHARACTERISATION OF SOME *HIBISCUS* SPECIES CULTIVATED IN MAURITIUS

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Studies were undertaken for the determination of genetic variation within *Hibiscus* species present in Mauritius using morphological traits and Random Amplified Polymorphic (RAPD) markers. 12 morphological characters were used to characterise seven *Hibiscus* species and greater variations in flower colour, size and shape were observed between species and varieties studied. RAPD amplification successfully generated polymorphisms in all the seven *Hibiscus* species studied. From the present study, RAPD analysis in combination with morphological characters can be used in the identification and determination of the genetic variation between the different varieties and species of *Hibiscus*. RAPD technique can be said to be reliable and promising for the characterisation of the *Hibiscus* germplasm and therefore Sequence Characterised Amplified Regions (SCAR) primers can be easily designed for many of these *Hibiscus* varieties and species.

**Keywords:** Characterisation, *Hibiscus* species, primers, RAPD Marker

### INTRODUCTION

Mauritius possesses the world's most interesting flora due to its level of endemism, which, is estimated, to be as high as 73%. The flora is composed of over 700 species of indigenous plants of which about 300 (about 60%) are endemic (Guého 1988). Over the past 400 years, Mauritius has known large scale forest clearance for agriculture and urban development, and also due to the introduction of invasive plants and animal competitors, a significant proportion of native plants are either extinct or on the verge of

extinction. Among the endangered species there are some *Hibiscus* species which are found in Mauritius.

There are about 250 species of *Hibiscus* in the tropical and subtropical regions (Bossier *et al.*, 1987). Five species of *Hibiscus* are known to be endemic to Mauritius and these are namely *H. boryanus* D C, *H. columnaris* Cav., *H. fragilis* D C, *H. genevii* Bojerex Hook. and *H. ovalifolius* Forssk. Until now in Mauritius, identification and classification of *Hibiscus* have mainly been based on morphology and according to Wachira *et al.*

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(2001) even if these descriptors are useful, they show limited levels of inter and intra-varietal polymorphism and hence, may not account for all the diversity in the species.

Identification and characterization of germplasm is essential for the conservation and utilization of plant genetic resources (Suvakanta *et al.*, 2006). Characterization of plant with the use of molecular markers is an ideal way to conserve plant genetic resources. Molecular characterization helps to determine the breeding behavior of species, individual reproductive success and the existence of gene flow, that is, the movement of alleles within and between populations of the same or related species, and its consequences (Papa and Gepts, 2003). Molecular data improve or even allow the elucidation of phylogeny, and provide the basic knowledge for understanding taxonomy, domestication and evolution of plants (Nwakanma *et al.*, 2003). Information from molecular markers or DNA sequences offers a good basis for better conservation approaches.

Modern molecular techniques have been developed in order to meet the demands of the horticulture industry. Population genetics, genetic linkage map and marker assisted selection techniques have significantly simplified the breeding procedures and overcome some of the agronomic, abiotic and biotic problems, which otherwise would not be achievable through conventional breeding methods. The development and remarkable achievements with molecular biotechnology in ornamental plants made during the three decades have been reviewed. Identifications of variety, cultivar or genotype by molecular markers have been

attempted since the early 1980's (Rout and Mohapatra, 2006).

These 'markers' of genetic variation are generally independent of environmental factors and more numerous than phenotypic characters, thereby providing a clearer indication of the underlying variation in the genome of an organism (Lai *et al.*, 2001; Chamberlain *et al.*, 2001). Identification of plant material using DNA markers has already become a very valuable tool in plant breeding and gene bank management (Nybom, 2001).

In the present study the genetic diversity of *Hibiscus* varieties and species cultivated in Mauritius was characterized using both morphological markers and the RAPD technique.

## **MATERIALS AND METHODS**

### **Morphological Analysis**

Twelve morphological characters were used for the identification and characterization of the different *Hibiscus* species and varieties. These were: height, growth habit, petal colour, flower shape, flower form, diameter of flower, length of staminal column, length of corolla, length of adult leaf, width of adult leaf, leaf shape, and length of petiole. All measurements were taken on five different plants of each species or variety and measurements namely diameter of the flower, length of staminal column, length of corolla, length of adult leaf, width of adult leaf and length of petiole were replicated 3 times to minimize error where possible.

### **Molecular Studies**

All molecular work was carried out in the molecular biology laboratory of the Faculty of Agriculture at the University of Mauritius.

## Plant Materials

The young fresh leaf material representing three endemic varieties; '*Hibiscus genevii*', '*Hibiscus boryanus*', and '*Hibiscus fragilis*' were collected from the NPCS Nursery at Robinson, Curepipe. The leaf material of the endemic species *H. columanaris* was collected in the wild at Black River. The different varieties of *Hibiscus rosa-sinensis* were collected in the premises of the University of Mauritius and in the yard of the MSIRI (Mauritius Sugar Industry and Research Institute). The leaves were wrapped between moist tissue paper, placed in a labelled plastic bag and kept in a refrigerator at  $-50^{\circ}\text{C}$  until they were used for DNA extraction.

## DNA Extraction

DNA was extracted from young fresh leaves based on a modified CTAB method (Porebski *et al.* 1997) with some modification such as follows: Preheating the lysis buffer at  $65^{\circ}\text{C}$  for 45 min prior to addition of ground leaves; Inclusion of 2% PVP in the extraction buffer; Addition of 0.3 volumes of 5 M NaCl before precipitation with isopropanol; Phenol treatment followed by addition of chloroform. The pellet obtained was washed with cold ( $4^{\circ}\text{C}$ ) 70% v/v ethanol. Afterwards spectrophotometric analysis was carried out to determine the concentration and purity of the DNA.

## RAPD Analysis

PCR-RAPD reactions were carried out in a volume of 25  $\mu\text{l}$  containing 1X reaction Buffer, 0.2mM  $\text{MgCl}_2$ , 0.25mM dNTP, 0.5 $\mu\text{M}$  Primer, 1 Unit Taq DNA Polymerase, 37.5 ng Template DNA and made up to the final volume with nanopure water using the Applied Biosystems 2720 thermal cycler. However, the thermal profile for RAPD was as follows: 90 s denaturation at  $95^{\circ}\text{C}$ , annealing for 35 cycles for 30 s at  $92^{\circ}\text{C}$ , 1 min at  $35^{\circ}\text{C}$ , 3 min

at  $72^{\circ}\text{C}$ , extension for 10 min at  $72^{\circ}\text{C}$  and 5 min at  $15^{\circ}\text{C}$ . Maximum polymorphism was observed using primers OPA 09, OPA10, OPB12 which gave clear and polymorphic bands for all the species under study.

## RESULTS AND DISCUSSION

### Morphological Characterization

Data of 12 characters from the 3 different *Hibiscus* species and 4 different varieties of *H. rosa-sinensis* were collected. The morphological variation of the *Hibiscus* species and varieties is summarized in Tables 1 and 2, respectively. Greater variations were observed between species and varieties, particularly in the characters of 'flower colour, size and shape'. The quantitative characters in some of the species and varieties of *Hibiscus*, showed large different degree of variation, which made them difficult to be used for characterization.

### Molecular Characterization

#### DNA Extraction

Genomic DNA was extracted using the protocol of Doyle and Doyle (1987) with minor modifications. 0.075 g fresh leaf tissue was ground in liquid Nitrogen and was then transferred into a tube containing 0.75 ml of preheated ( $60^{\circ}\text{C}$ ) CTAB buffer followed by addition of 0.2% (v/v)  $\beta$ -mercaptoethanol and 2% (v/v) PVP. The tube was placed in  $60^{\circ}\text{C}$  water bath for 25-30 min with occasional swirling. Afterwards, 2/3 volume chloroform: isoamyl alcohol (24:1) was added and the tubes were inverted several times and then microcentrifuged at 10,000 rpm for 10 min. This step was repeated until no interface was visible and 2/3 volume of ice-cold isopropanol and 0.5 volume of 5 M NaCl was added. The tubes were left overnight at  $4^{\circ}\text{C}$  to allow further precipitation

**Table 1: Results for Morphological Characterisation Between *Hibiscus* Species**

Specie Name	<i>Hibiscus genevii</i>	<i>Hibiscus boryanus</i>	<i>Hibiscus fragilis</i>	<i>Hibiscus rosa-sinensis</i>	<i>Hibiscus columnaris</i>
Height	2.3 ± 0.3 m	7.9 ± 0.9 m	2.3 ± 0.6 m	4.7 ± 0.4 m	8 ± 1.4 m
Growth habit	Shrub	Shrub	Shrub	Shrub	Shrub
Flower colour	Pink purple with red centre	Red orange, deep red or pink with a purple centre	Bright red to pink	Colours in addition to white are Red, orange, purple and yellow	Yellow to dark yellow with a pale brown centre
Flower shape	Funnel shaped	Funnel shaped	Funnel shaped	Reflexed shaped	Saucer shaped
Flower form	Single petal	Single petal	Single petal	Single petal	Single petal
Diameter of flower	11.6 ± 0.7 cm	7.5 ± 0.9 cm	11.1 ± 0.9 cm	15.6 ± 0.4 cm	10 ± 0.7 cm
Length of staminal column	7.1 ± 1.1 cm	7.6 ± 0.4 cm	4.6 ± 0.4 cm	6.4 ± 0.3 cm	6 ± 1.2 cm
Length of corolla	7.1 ± 0.7 cm	3.5 ± 0.4 cm	5.6 ± 0.3 cm	8.6 ± 0.3 cm	6 ± 0.7 cm
Length of adult leaf	6.7 ± 0.9 cm	6.6 ± 1.1 cm	6.6 ± 1 cm	7.5 ± 1.6 cm	17 ± 0.6 cm
Width of adult leaf	4.1 ± 0.8 cm	6.7 ± 0.7 cm	5.2 ± 0.8 cm	6.2 ± 1.5 cm	12 ± 0.8 cm
Leaf shape	Elliptical with rounded tip	Elliptical/ovate	Elliptical	Elliptical with rounded tip	Elliptical with rounded tip
Length of petiole	2.9 ± 0.9 cm	3.4 ± 0.7 cm	1.6 ± 0.3 cm	5 ± 1.1 cm	10 ± 1.5 cm

**Figure 1: *Hibiscus genevii*    *Hibiscus boryanus*    *Hibiscus fragillis*    *Hibiscus columnaris***

of DNA and then the tubes were spun in a microcentrifuge for 30 minutes at 13,000 rpm. The supernatant was discarded and washed with 70% alcohol. The pellet was air dried under the hood and re-dissolved in 50µl sterile distilled water. The DNA was purified by incubation with RNase and a phenol treatment.

The modified CTAB protocol for DNA extraction that was used gave relatively good, clear and translucent DNA indicating that polyphenolics, RNA and other contaminant were successfully

eliminated by the use of PVP, 2 β-Mercaptoethanol, phenol, chloroform, and ethanol accompanied by the phenol and RNase treatment. This was reflected when the purity of the DNA was obtained from the spectrophoto-meter which ranged from 1.67-1.78.

### RAPD - PCR Analysis

The Random Amplified Polymorphic DNA technique has been successfully used in a variety of taxonomic and genetic diversity studies (Jain

**Table 2: Results for Morphological Characterisation within *Hibiscus rosa-sinensis***

Variety name	<i>Hibiscus rosa-sinensis</i>			
	(White single)	(Pink double)	(Red single)	(Yellow single)
Height	4.7 ± 0.4 m	1.8 ± 0.4 m	1.7 ± 0.3 m	1.8 ± 0.3 m
Growth habit	Shrub	Shrub	Shrub	Shrub
Flower colour	White to pale pink with dark pink to red centre and pink vertical stripes along corolla	Pink to purple pink with red to dark red centre	Pink purple to bright red with dark red centre and pale pink vertical stripes along corolla	Lemon to Pale yellow
Flower shape	Reflexed shaped	Reflex shaped	Reflexed shaped	Saucer shaped
Flower form	Single petal	Double petal	Single petal	Single petal
Diameter of flower	15.6 ± 0.4 cm	18.2 ± 0.7 cm	13.6 ± 0.4 cm	12 ± 0.5 cm
Length of staminal column	6.4 ± 0.3 cm	5.6 ± 0.3 cm	5.3 ± 0.2 cm	3.6 ± 0.4 cm
Length of corolla	8.6 ± 0.3 cm	6.8 ± 1.7cm	6.6 ± 0.3 cm	6.4 ± 0.3 cm
Length of adult leaf	7.5 ± 1.6 cm	7.2 ± 1.4 cm	8.7 ± 1.9 cm	4.7 ± 1.1 cm
Width of adult leaf	6.2 ± 1.5 cm	5 ± 1.1 cm	6.3 ± 1.8 cm	5.6 ± 0.3 cm
Leaf shape	Elliptical with rounded tip	Elliptical with pointed tip	Elliptical with rounded tip	Elliptical with rounded tip
Length of petiole	5 ± 1.1 cm	1-3 cm	5.6 ± 0.3 cm	1.7 ± 0.2 cm

**Figure 2: Variety *H. rosasinensis***

**White Single**

**Pink double**

**Red single**

**Yellow single**



*et al.*, 1993). Its popularity stems from the ease, cost effectiveness and applicability of the technique. Only a small quantity of DNA is required to generate a great amount of genetic markers, even without cloning, sequencing, or any knowledge of the genome being examined (Bassam *et al* 1991). Genetic variation among different *Hibiscus* species and varieties was determined using the RAPD method

**Morphological Analysis**

The most important characters which helped in characterizing the different species or varieties of *Hibiscus* was the ‘flower color, shape and form’. The quantitative results obtained were not effective to differentiate between the species or the varieties. For example same height (2.3 ± 0.3 and 2.3 ± 0.6) was obtained between the species *H. genevii* and *H. fragilis* and this character could

not be used to differentiate between species. The same result was obtained for many of the quantitative data especially those for the varieties. It can thus be deduced that the shape and size of the leaves varied according to the physiological state of the plant, the environment such as light intensity, water, and others, by pest and diseases and also by the cultural practices undergone on the shrub such as pruning.

Despite the difficulties of characterization based on the morphology of the plants, morphological characterization remains a quick and easy method of identification (Zhou *et al.*, 2002). Data from this study demonstrate that morphological analysis seems to be useful for a rough classification and indication of relationship between the different varieties and species of *Hibiscus*.

### RAPD-PCR Analysis

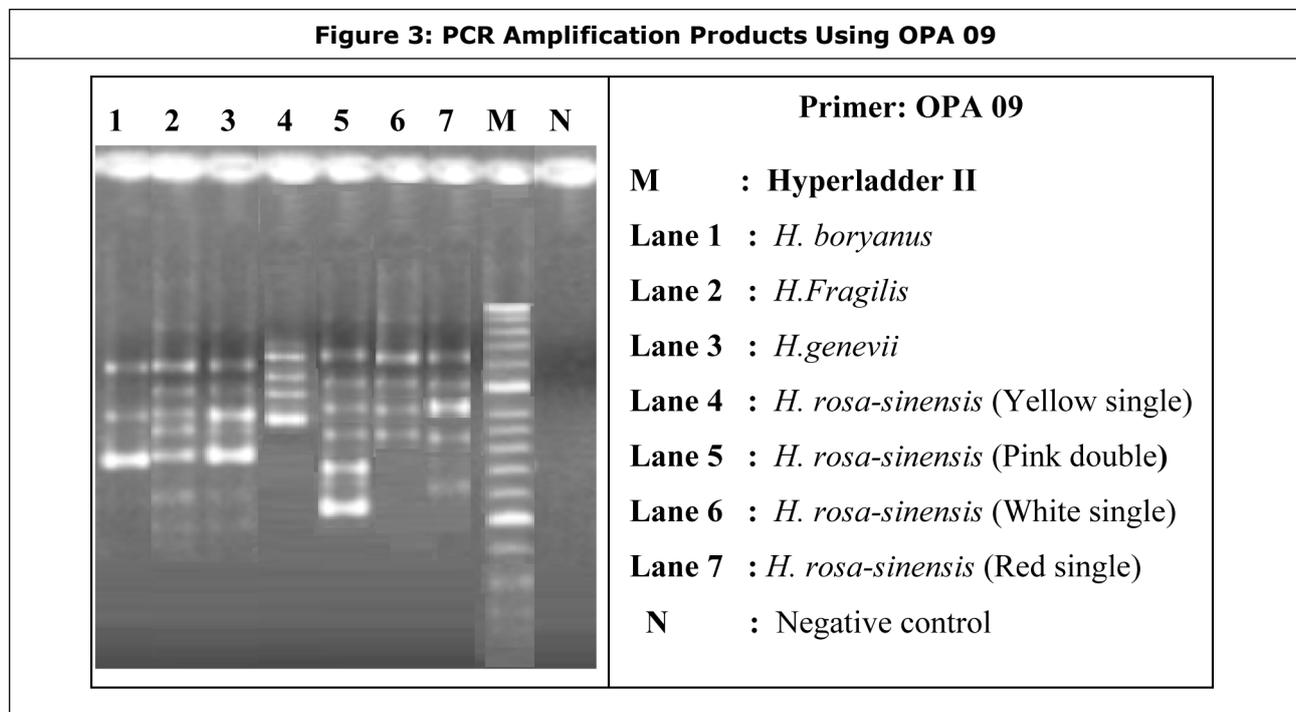
Polymorphic bands were successfully generated from the seven DNA of different species and

varieties of *Hibiscus* when the primers OPA 09, OPA 10 and OPB12 were used.

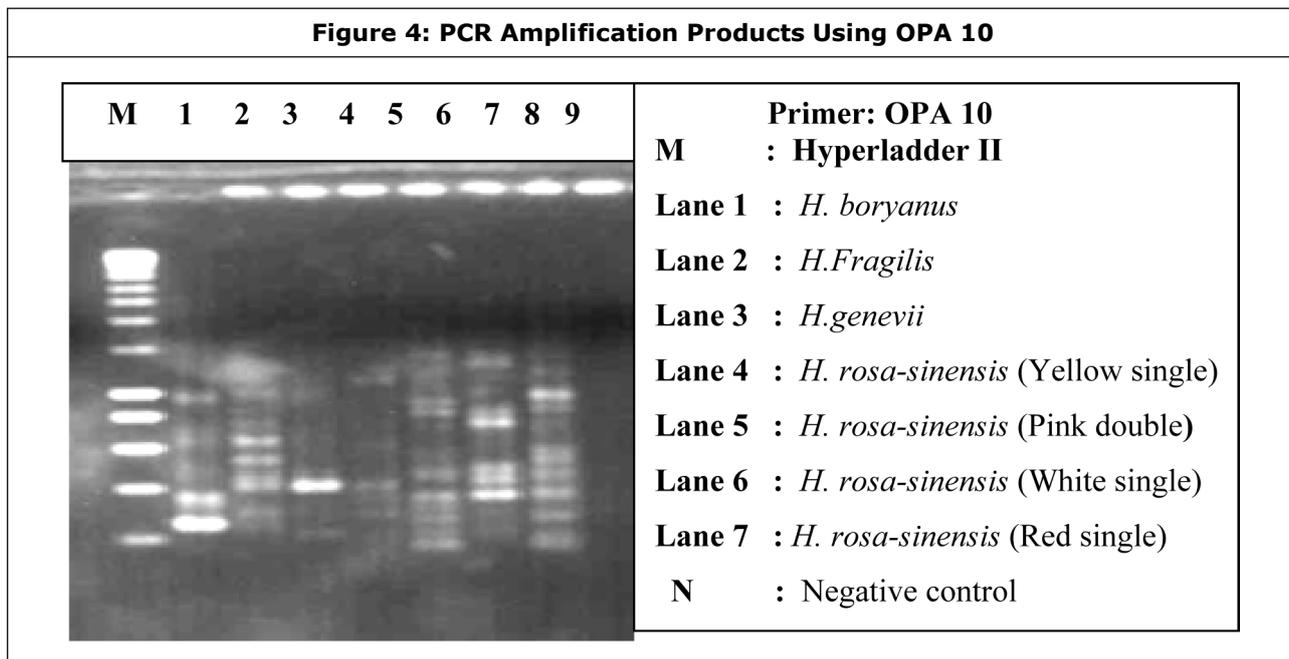
The present finding showed that there was not much variations within the varieties *H. rosa-sinensis* (White single), *H. rosa-sinensis* (Red single), *H. rosa-sinensis* (Yellow single) and *H. rosa-sinensis* (Pink double), as it could be observed that they all generated almost similar profiles. Even though all the varieties of *H. rosa-sinensis* have the same DNA profile, there was somehow some bands that were different from the other. Apart from the first four bands generated by all the varieties, variety 'pink Double' generated two more distinct bands when primer OPA 09 and OPB 12 was used. This could be attributed to the difference in the morphology of variety 'Pink Double', which has double petals form and larger bloom size.

Close relationships could be found between the species using the RAPD primers. For instance, *H. genevii* and *H. fragilis* was seen to

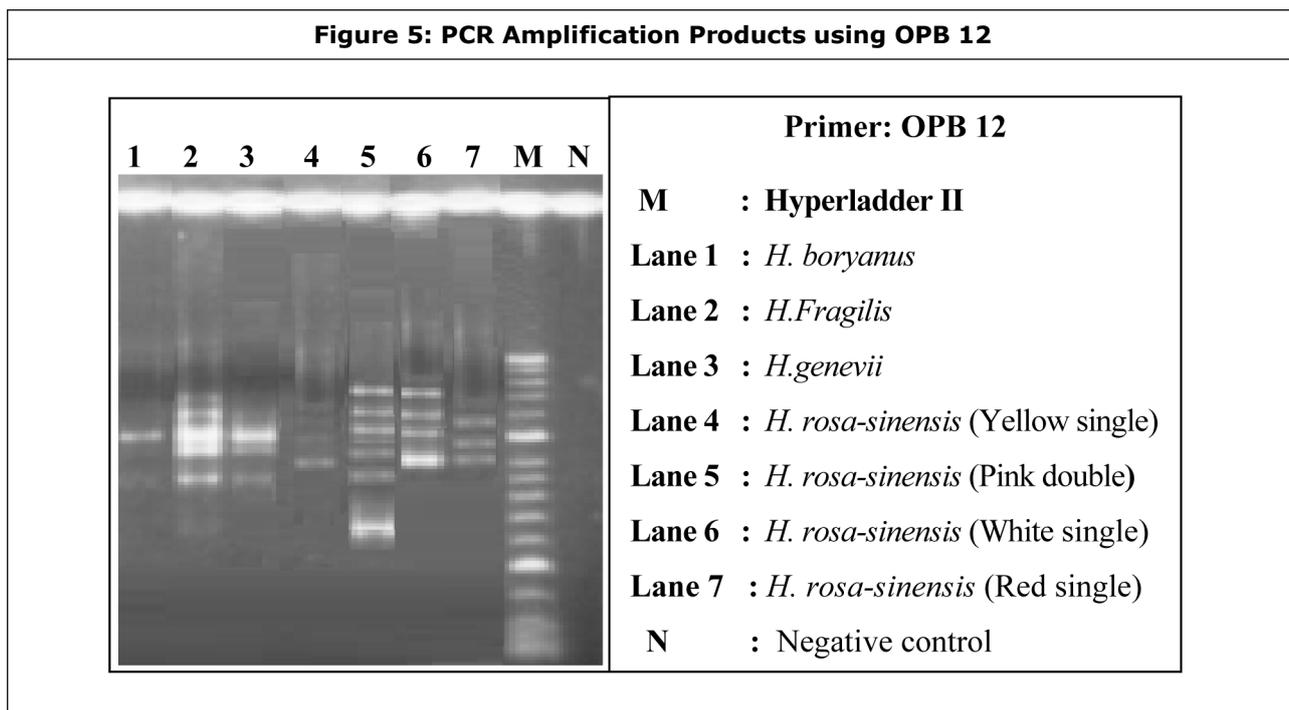
**Figure 3: PCR Amplification Products Using OPA 09**



**Figure 4: PCR Amplification Products Using OPA 10**



**Figure 5: PCR Amplification Products using OPB 12**



be more related to others compared to *H. boryanus*. Moreover the variety "Yellow single" was found to be more related to the species *H. genevii* with two bands of same base pair in common. However, the RAPD profile obtained for

*H. boryanus* and *H. rosa-sinensis* (Yellow single) were different from the other showing that there may be a distant relationship of this species and variety with the other species and varieties studied.

Among the molecular markers, Randomly Amplified Polymorphic DNA markers are simple, versatile, and relatively inexpensive (Crawford *et al.*, 1993) and was able to detect minute genetic differences in different *Hibiscus* species and varieties.

This study showed that morphological characterization provided a rapid and satisfactory means to differentiate between certain *Hibiscus* species. Identification of some species and varieties of *Hibiscus* can be problematic, since flower color, shape and form are the only characters which can be used to discriminate between the species. Due to the influence of environment, the quantitative traits obtained using descriptive statistics proved to be unreliable as they vary a lot between species.

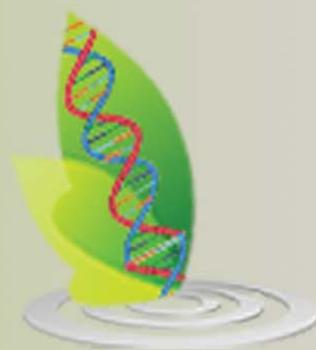
The protocol described by Porebski *et al.* (1997) together with some modifications to deal with protein and polysaccharides, yielded good quality DNA and was amenable to molecular analysis as confirmed by the spectrophotometric analysis. RAPD was successfully carried out with three species and four varieties of *Hibiscus* using the primer OPA 09, OPA 10 and OPB 12.

Data from this experiment demonstrated that morphological analysis together with RAPD markers are useful for classification and indication of relationships among the different *Hibiscus* and can be useful for *Hibiscus* breeding program. Sequence Characterised Amplified Regions (SCAR) primers can be easily designed for many of these *Hibiscus* varieties and species.

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