Assessment of diversity in *Garcinia indica* (Dupetit-Thouars.) Choisy. using morphological and molecular markers

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Abstract

Garcinia indica (Dupetit-Thouars.) Choisy. is an endemic plant in the southern Western Ghats. Although endemic species show low levels of genetic diversity, morphological variations abound in *G. indica*. Genetic diversity of *G. indica* plants collected from four locations in Maharashtra were evaluated at inter and intra-population levels using morphological traits and molecular markers. Leaf length and plant height showed profound variability. Plants exhibited less diversity within the population when screened by molecular markers like RAPD and ISSR. In case of RAPD primers, the highest diversity was observed for the Sawantwadi population (19.93%), intermediate variation in Dive-agar (8.11%), low variation in Chiplun (5.20%), and least variability in Dapoli populations (4.10%). Similarly, 10 ISSR primers expressed 11.37% variation in Sawantwadi region, 9.20% in Dive-agar, 4.70% in Dapoli, and only 1.20% in Chiplun. There were small pockets, e.g., Dive-agar or Otavane in Sawantwadi in which the plants showed diversity at both morphological as well as molecular levels, implying the need to conserve the germplasm of these populations.

Keywords: Endemism, Inter and intra-population variability, Germplasm conservation.

Introduction

Garcinia indica (Dupetit-Thouars.) Choisy (family: Clusiaceae) is endemic to the southern Western Ghats (Rajashekharan and Ganeshan, 2002). It is a polygamo-dioecious and insect pollinated plant. Fruits of *G. indica* are rich sources of hydroxy citric acid (HCA) and are used as an anti-obesity and anti-cholesterol drug. The fat extracted from the seed is used in cosmetics as emollient. Fruits are also used to prepare a pleasant and attractive beverage, with bilious action (Patil, 2005). Attempts were made to characterize fruit quality, yield, and commercial production of *G. indica* (Haldankar et al., 1993). Tembe and Deodhar (2011) made attempts to identify early fruiting, high yielding, HCA rich clones of *G. indica,* and to standardize a protocol for clonal propagation of elite clones. They also reported variations in height and branching pattern as well as canopy, leaf, and fruit morphology, particularly in locations such as Dive-agar and Sawantwadi in Maharashtra. It is necessary to ascertain whether the observed variability is due to environmental factors or whether it exists at molecular level also. Hence, a molecular study of these populations was undertaken in which RAPD and ISSR primers have been used to assess genetic diversity.

Materials and Methods

Distinct populations were selected from Otavane, Talkat, and Shiroda in Sawantwadi, Sindhudurg district; Narvan, Lote, Hedvi, and Chiplun in Ratna-

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Table 1. Regions of Western Ghats of Maharashtra, India from where *G. indica* populations were collected.

Populations	Latitude	Longitude	Sub-populations	No. of plants collected
Sawantwadi, Sindhudurg district	16°40' N	73°19' E	Otavane	7
			Shiroda	9
			Talkat	8
			Zolimbe	6
Chiplun, Ratnagiri district	17°30' N	73°36' E	Narvan	15
			Hedavi	5
			Lote + Chiplun	8
Dapoli, Ratnagiri district	17°48' N	73°12' E	Orchard of Dr Balasaheb Sawant Agricultural University	15
Dive agar, Raigad district	18°10' N	72°59' E	Bapat's Garden	10
			Joshi's Garden	20

giri district; Dr. Balasaheb Sawant Agricultural University, Dapoli also in Ratnagiri district; and from Bapat's garden and Joshi's garden at Dive-agar in Raigad district (Table 1) in May and June (2009–10). From each location, 5 to 20 plants managed by local farmers in orchards were randomly selected, based on differences in morphological characteristics. Four apical leaves were collected from each plant and cryopreserved using liquid nitrogen until DNA extraction. Morphological characteristics like height of plant, leaf length, breadth, leaf area (graph paper method), and thickness of fruit rind were measured. The data were analyzed using Analysis of Variance (ANOVA). Branching pattern (parallel or drooping), leaf apex (blunt or acute), leaf colour and fruit colour was also recorded for all four populations.

Genomic DNA Extraction and RAPD analysis

DNA was extracted by modified CTAB method (Sahasrabudhe and Deodhar, 2010). The quality and purity of extracted DNA were checked using 0.8% Agarose gel with 1X TAE buffer (Tris base 40 mM pH 8.0, Acetate 20 mM, EDTA 1mM pH 8.0) at 50V for 3 h and stained with ethidium bromide (0.1µg ml^{-1} of gel solution). In all, 60 RAPD primers (Kit A, B, C; Operon Technologies, Alameda, CA, USA) were used to assess the genetic diversity within and between populations. PCR amplification reaction was carried out in 25 µl volume containing 40 ng template DNA, 2.5 µl 10X assay buffer (Tris with 15 mM $MgCl_2$), 25 µM each dNTP's, 0.4 µM RAPD primer (Operon Technologies, Alameda, CA, USA), and 1.2U Taq polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplification was carried out in a thermal cycler (Primus 25, Peqlab, Germany). The first cycle consisted of denaturation of template DNA at 94°C for 5 min., and then 40 cycles were repeated with denaturation at 94°C for 1 min, primer annealing step at 52°C for 1 min and extension of newly synthesized DNA strand at 72°C for 2 min. This was followed by a final extension step of 7 min at 72°C. The amplification products were resolved on 2% agarose gel in 1X TAE buffer. The size of the amplicons was determined using standards (100 bp DNA ladder: Bangalore Genei Pvt. Ltd., Bangalore, India). DNA fragments were visualized under UV light after staining with Ethidium bromide (0.1µg ml^{-1} of gel solution) and documented in Gel-Doc (Alpha-innotech, USA).

ISSR analysis

The 17mer primers (UBC-kit 09) obtained from Bangalore Genei Pvt. Ltd were used. The reaction mixture of amplification was the same as in the case of RAPD analysis with little variation in PCR cycles. First denaturation took place at 94°C for 5

min. Forty five cycles were repeated with initial denaturation at 94°C for 30 seconds. Primer annealing temperature varied from 50 to 56°C for 45 seconds and extension at 72°C for 2 min. The final extension step was carried out at 72°C for 7 min. The amplification products were electrophoresed on 2% agarose gel in 1X TAE buffer. The DNA banding pattern was observed in UV light after staining with ethidium bromide (0.1µg ml^{-1} of gel solution) and photographed in Gel-Doc (Alpha-innotech, USA).

Results and Discussion

Morphological characters such as leaf length, breadth, leaf apex, leaf area, stem height, stem girth, fruit colour, and rind thickness showed profound variability (Tables 2 and 3). The tree leaves from the Talkat region in Sawantwadi had broad lamina (5.73 cm) with leaf length of 12.8 cm (Table 2; Fig. 1a) while that of Bapat's garden population were long (16.3 cm) with 4.9 cm breadth (Table 2; Fig.1b). Likewise, the populations from Otavane and Talkat from Sawantwadi region were different from that of Shiroda. The plants were comparatively taller. Leaves had broad lamina and acute apex. The population from Bapat's garden at Dive-agar was also significantly taller with longer dark-green leaf lamina and acute apex. Although endemic species show low levels of genetic variability due to genetic drift in small populations and strong selection of dominant characters in controlled environment, insect pollinated, seed raised progenies of *G. indica* exhibited lots of variability in height, branching pattern, and leaf and fruit morphology. It is necessary to examine whether the morphological diversity in plants at Otavane or at Dive-agar region existed at molecular level or it is mainly due to environmental conditions.

Molecular fingerprinting

The polymorphism generated by the RAPD and ISSR primers in the four populations of *G. indica* is

Table 2. Morphological diversity (mean ± SE) in different populations of *G. indica*.

Location	Leaf length (cm)	Leaf breadth (cm)	Stem girth (cm)	Branching point (m)
Dapoli	11.06 ± 0.95	4.0 ± 0.5	106.0 ± 10.58	1.72
Chiplun	9.9 ± 0.52	3.8 ± 0.51	106.33 ± 7.09	1.92
Bapat's Garden	16.3 ± 0.81*	4.93 ± 0.20*	77.66 ± 9.50*	2.74*
Joshi's Garden	13.2 ± 0.34*	5.16 ± 0.28*	60.66 ± 7.50*	1.82
Otavane	11.26 ± 1.00	4.7 ± 0.1	131.33 ± 10.88*	4.36*
Talkat	12.8 ± 0.69	5.73 ± 0.25*	86.86 ± 10.21	2.13
Shiroda	9.23 ± 0.56*	5.16 ± 0.45*	92.2 ± 4.91	2.02

*Significant at ($p < 0.05$) compared to control samples (n = 3).

Table 3. Variations in morphological characteristics of *G. indica* populations at different locations in Maharashtra, India.

Attributes	Dapoli	Chiplun	Dive-agar		Sawantwadi		
			Bapat's Garden	Joshi's Garden	Otavane	Talkat	Shiroda
Type of branching	Drooping	Drooping	Parallel	Parallel	Parallel	Parallel	Parallel
Leaf Apex	Blunt	Blunt	Acute	Blunt	Acute	Acute	Blunt
Leaf Colour	Green	Yellow green	Dark green	Light green	Dark green	Light green	Dark green
Fruit Colour	Red	Red	Dark red	Dark red	Dark red	Dark red	Red
Rind Thickness	Thin	Thin	Thick	Thick	Thick	Thick	Thin

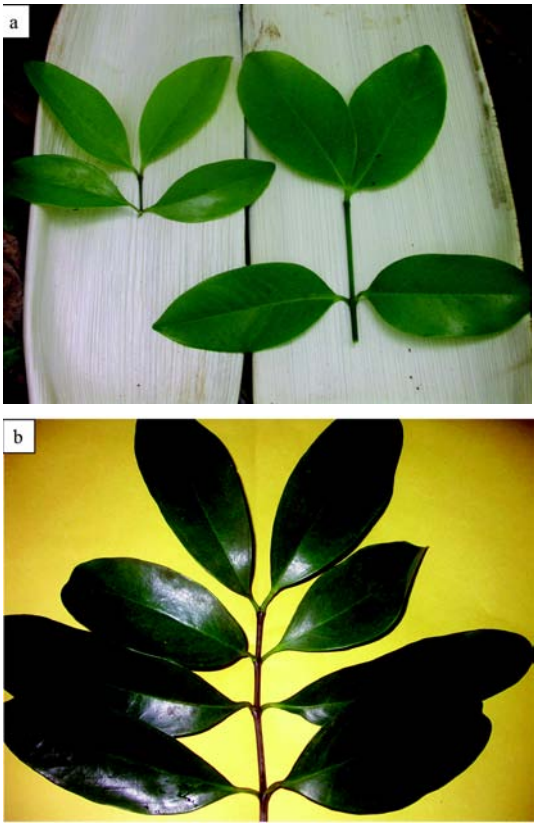

Figure 1. Variations in leaf morphology of *G. indica* populations from Maharashtra state: (a) normal leaves (left) compared with leaves from Otavane (right) and (b) leaves from Bapat's garden at Dive-agar.

given in Table 4. The lowest variation was observed in Dapoli and Chiplun populations by RAPD and ISSR markers respectively. Figure 2 represents the amplification pattern generated by OPA-18 at various locations. Only one polymorphic band of 1300 bp was present in all plants collected from Otavane and Talkat as indicated by the arrow in Fig. 2a. Although this band was found in four out of six plants from Otavane, it was absent at Shiroda, but four of the five plants collected from Talkat showed the occurrence of band 1300 bp. The amplification pattern produced by primer OPA-18 in plants collected from various regions of Dive-agar (Fig. 2b) indicates that the 1300 bp band was absent in plants from Bapat's garden (Lane numbers 1 to 9), but this band was present in the population from Joshi's garden of Dive-agar (lane numbers 10 and 12 to 21). As can be seen from Fig. 2c (amplification pattern produced by primer OPA-18 in various regions of Chiplun), the 1300 bp band was present in Hedvi, Narvan, and Lote populations.

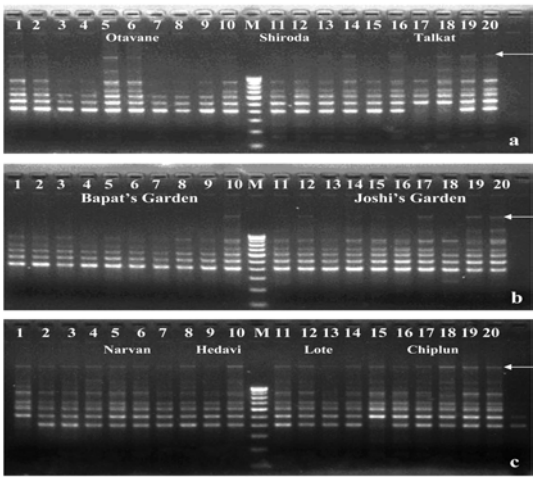

Figure 2. Amplification pattern produced by OPA-18 in *G. indica* plants collected from various locations (a) Sawantwadi population, (b) Dive-agar population, and (c) Chiplun population from Maharashtra, India.

A comparison of the amplification pattern produced by UBC-822 in plants from Sawantwadi (Fig. 3a) showed two polymorphic bands of size 1200 bp and 1300 bp (see the arrow) in the Otavane and Talkat populations. In Otavane, this band was found in four out of six random plants. Whereas at Shiroda, this band was absent in all plants. At Talkat, four plants out of five showed this band. Another band of 1100 bp was found in the Shiroda population (lane numbers 5 to 11). However, this band was seen only in two plants from Otavane (lane numbers 1 and 3) and few plants from Talkat (lane numbers 13, 15, 16, and 17). Both bands 1200 bp and 1300 bp were present in all the plants at Dive-agar (Fig 3b). Likewise, the 1300 bp band was present in almost all samples from Narvan and Lote (Chiplun: Fig. 3c). This band, however, was absent in plants from Hedvi and in two plants from Lote (lane numbers 10 to13). But the monomorphic band of 1200 bp was present in all the plants collected from various regions of Chiplun.

Table 4. Polymorphism observed in different populations of *G. indica* using molecular markers.

Location	RAPD markers Total number of bands	ISSR markers Number of polymorphic bands	% polymorphism	Total number of bands	Number of polymorphic bands	% polymorphism
Dapoli	268	6	4.10	170	8	4.70
Chiplun	269	14	5.20	166	2	1.20
Dive-agar	262	26	9.92	163	15	9.20
Sawantwadi	291	58	19.93	167	19	11.37

Given that *G. indica* is insect pollinated (Karnik, 1978), occurrence of less polymorphism in some populations like Dapoli and Chiplun supports the view that most of the plants are derived from apomixis and not by cross pollination. Similar observations were made by Pangsuban et al. (2009) in *G. atroviridis*, endemic to Peninsular Malaysia. Although very less diversity exists at the molecular level in case of *G. indica*, there are certain plants in Otavane region of Sawantwadi showing consistent polymorphism (Fig. 4). For instance, there were

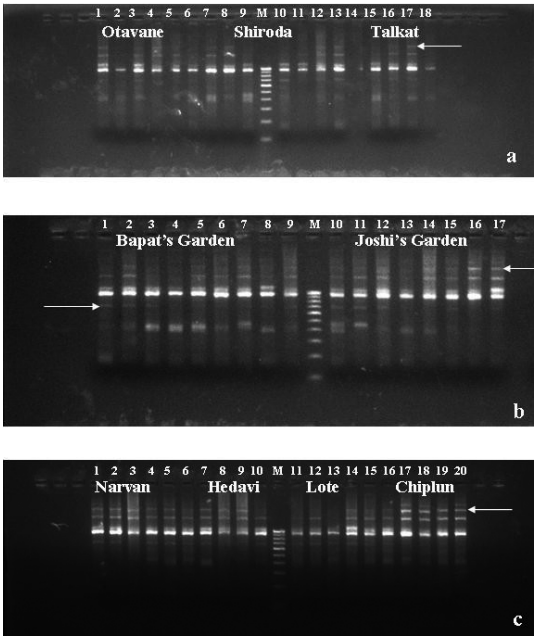

Figure 3. Amplification pattern produced by UBC-822 in *G. indica* plants collected from various locations: (a) Sawantwadi population, (b) Dive-agar population, and (c) Chiplun population.

21 RAPD and 13 ISSR primers, which showed polymorphic bands. Fig. 4 (OPA-04: Sawantwadi population) illustrates the absence of 1300 bp band for plants in lane numbers 3 and 4 but it was present in all plants of Sawantwadi. Similarly, for Sawantwadi population, 1100 bp band produced by primer OPA-08 was missing in these two plants (lane no. 3 and 4). In lane numbers 3 and 4, OPA-12 produced a 1400 bp band in almost all plants from Talkat and Shiroda but was absent in these two plants of Otavane (lane numbers 3 and 4). Similarly, in the case of ISSR primers, primer UBC-848 generated two polymorphic bands, *viz.,* 1100 and 1300 bp, which were absent in two plants from Otavane (lane numbers 3 and 4). Another primer UBC-811 produced 1100 bp and 1200 bp band in almost all plants from Sawantwadi region but were absent in two plants from Otavane (lane numbers 1 and 2).

At Dive-agar, the plants from Bapat's garden were particularly tall with dark green leaf lamina. Leaf length of these plants was higher than other populations (Fig. 1b). There were five RAPD markers which could differentiate the entire plant population of Bapat's garden from the plants collected in other regions of Dive-agar. This polymorphism is clearly given in Fig. 4. The amplification pattern produced by primer OPA-04 shows that 1300 bp band was absent in all the plants collected from the Bapat's garden (lane numbers 1 to 10). A band of 1400 bp was seen in plants collected from other regions of dive-agar (lane numbers 10 to 20) which was absent in the entire Bapat's garden population

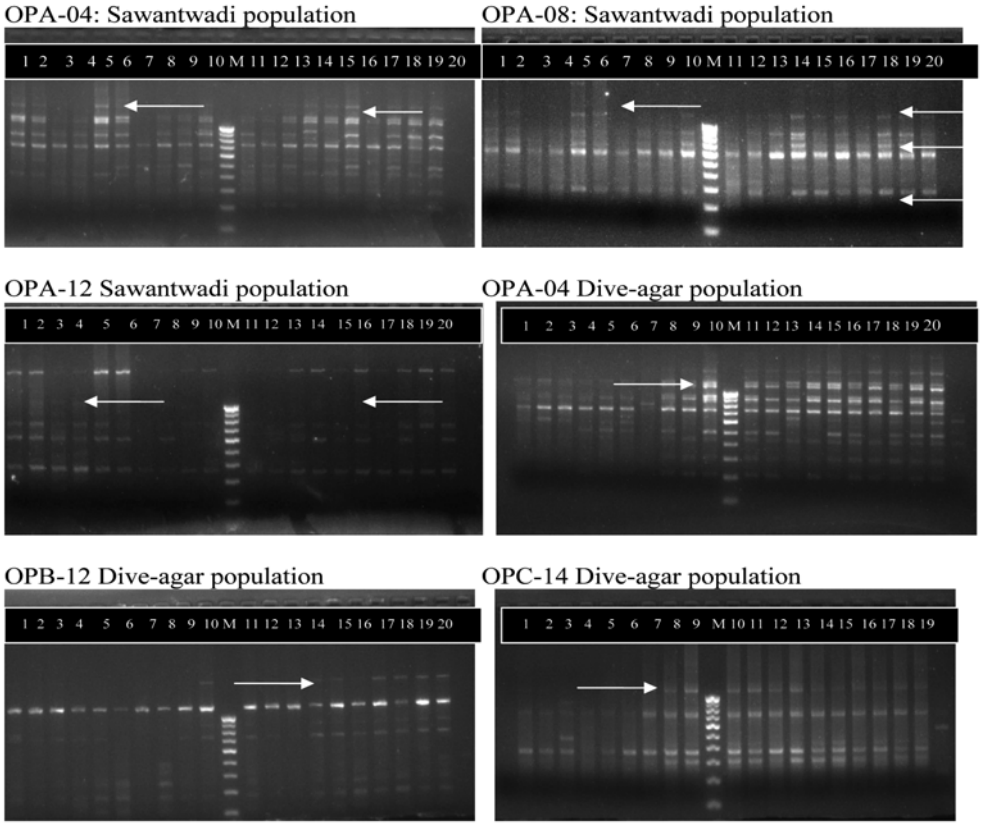

Figure 4. Amplification pattern produced by various RAPD primers in Sawantwadi and Dive-agar population

(lane numbers 1 to 9). There were two polymorphic bands which were absent in all the plants collected from Bapat's garden (lane numbers 1 to 7). A band of size 300 bp and 1100 bp was seen in all the plants from the other regions of Dive-agar.

The low genetic diversity in *G. indica* will threaten its own existence. It is extremely important to detect even the low level of polymorphism present in small populations of *G. indica*. In this context, diversity observed in populations like Otavane and Bapat's garden at Dive-agar is very important because extinction of any one population would reduce the total genetic variability. Certain plants from Otavane or Dive-agar region showed consistent polymorphism with a number of molecular markers. This indicates that the plants are different from rest of the populations at molecular level. Such different ecotypes need to be conserved to maintain the evolutionary potential of the species.

RAPD and ISSR analyses reveal that very less genetic diversity existed in different populations of *G. indica*. But, there were small populations, which showed high polymorphism at both morphological as well as molecular levels. Such small pocketed populations of *G. indica* need to be conserved as germplasm.

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