TAXONOMIC, MOLECULAR AND PHYSIOLOGICAL EVALUATION OF *COSTUS PICTUS* D. DON PLANTS ORIGINALLY OBTAINED FROM DIFFERENT PARTS OF KERALA, INDIA

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Abstract: *Costus pictus* D. Don is known to have antidiabetic property, and its leaves are traditionally consumed (2-3, twice a day) for the management of diabetes. The plant is popularly known as "insulin plant" and it was introduced in India only recently. However, it is already cultivated in home gardens throughout Kerala. The objectives of this study were to identify and characterize the newly introduced *Costus pictus* on the basis of a large number of morphological, molecular (RAPD analyses), and selected physiological (gas and water vapour exchange) characteristics. The results of this study confirm that the three accessions of *Costus pictus* originally collected from different populations growing in south, middle and north Kerala belong to the same gene pool. These findings will be useful marker for the further exploitation of *C. pictus* scientifically.

Keywords: *Costus pictus*; insulin plant; gas and water vapour exchange; morphological characters; RAPD analyses

1. Introduction:

Costus pictus D. Don was recently (in 2000) introduced in India [1], probably from Mexico, and is now widely grown in home gardens as an ornamental, especially in Kerala [2-4]. Though, the antidiabetic property of *C. pictus* (family Costaceae, recently separated from Zingiberaceae) [5] has not been clinically proven in humans, people traditionally consume 2-3 leaves of this plant twice a day for the management of diabetes. This has resulted in it being popularly named as "insulin plant" [6] In spite of its antidiabetic property, only limited information is available on the agronomy, propagation, physiology, molecular, phytochemical and pharmacological characteristics of *C. pictus* [7-11]

The medicinal, ecological, social and economic importance of this plant on one hand and need for extensive propagation in India on the other necessitates systematic evaluation of the plant. Though morphological characterization is important for studying diversity, its limitations for ascertaining genetic or environmental induced variations [12] are well known. Therefore, in the present study gas and water vapour exchange characteristics and DNA based markers have been used, in addition to a large number of morphological parameters, to examine the genetic diversity, if any of *C. pictus* [13], collected from three different agro climatic regions of Kerala.

2. Material and Methods:

2.1. Plant material:

The rhizomes of *Costus pictus* were collected from populations growing in three different agro-climatic regions of Kerala, *i.e.*, south (Thiruvananthapuram), middle (Ernakulam) and north (Kannur) (Figure 1). The propagules were planted in large earthen pots containing a mixture of garden soil & FYM (4:1) and kept in the campus of Sacred Heart Degree College at Sitapur, U.P.,India. For gas and water vapour exchange studies and RAPD analyses plants were grown at G. B. Pant Institute of Himalayan Environment and Development, Kosi-Katarmal, Alora, Uttarakhand, India inside a green house for six months, and were allowed to acclimatize in the

open shady place for three weeks before taking measurements. The plant specimens have also been deposited in the Dr. Fr. Joseph Herbarium established at The Department of Botany in the college.

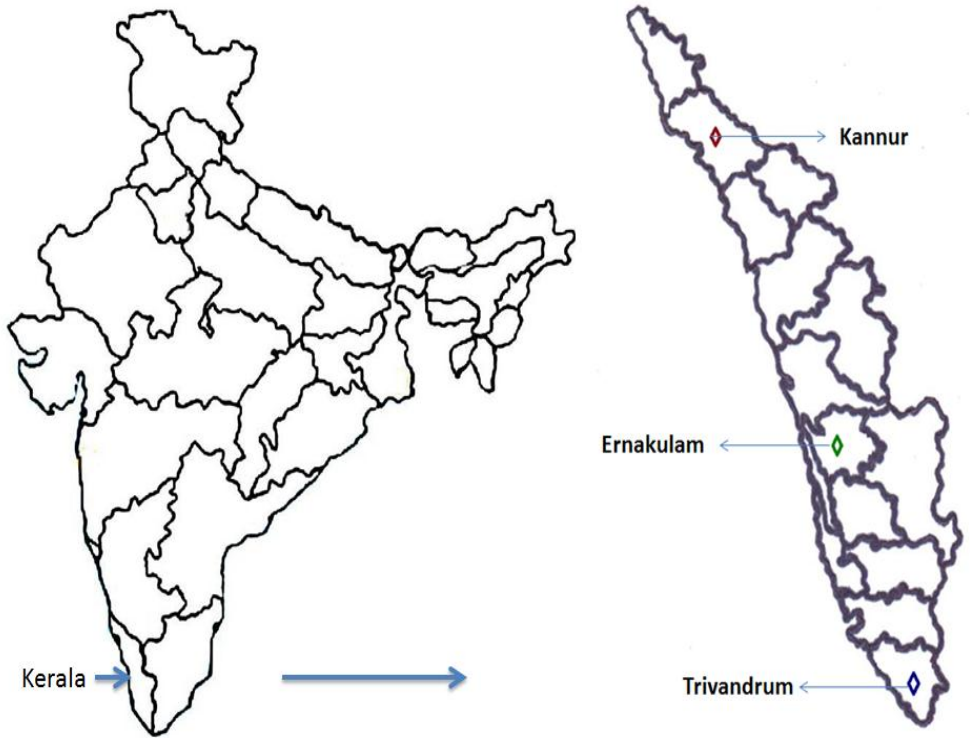

Figure 1: Map of India showing Kerala state and the collection sites of *Costus pictus* in Kerala.

2.2. Random Amplified Polymorphic DNA fingerprinting:

Genomic DNA from the young leaves was isolated following CTAB method [14]. A total of forty random decamer oligonucleotides (Operon Technologies Inc., Alameda, California, USA) were used as single primers for the amplification of RAPD fragments. PCR was carried out in a final volume of 25µl containing 20ng template DNA, 200µM deoxynucleotide triphosphate, 20ng of decanucleotide primers, 1.5 mM $MgCl_2$, 10 mMTris-HCl, 50mM KCl, 0.1% Triton X-100 and 0.5U Taq DNA polymerase (Bangalore Genei, India). Amplification was achieved in a Thermocycler (Biometra, Germany) programmed for a preliminary 5min denaturation step at 94^0C, followed by 35 cycles of denaturation at 94^0C for 30 sec, annealing at 36^0C for 1 min and extension at 72^0C for 1 min, and finally at 72^0C for 10 min.

Each polymorphic band was considered as a binary character and was given *C. pictus* a score of 1 (presence) or 0 (absence) for each sample and assembled in a data matrix. Only intensely stained, unambiguous, and reproducible bands were scored for analysis. Similarity index was estimated using the Dice coefficient of similarity [15]. A dendrogram was constructed using the Unweighted Pair Group Method with Arithmetical Averages (UPGMA) using Gene Profiler 1-D Phylogenetic analysis & Data basing Software.

2.3. Gas and water vapour exchange measurements:

Gas and water vapour exchange measurements (*viz.* net photosynthesis, stomatal conductance, intercellular CO_2 concentration, transpirational water loss, etc.) were carried out with a closed potable photosynthesis system, namely Infra Red Gas Analyser (Model LI-6400, LI-COR, Lincoln, Nebraska USA), and for this the youngest fully expanded and healthy leaves were used at a photosynthetic photon flux density of 900 µM $m^{-2}s^{-1}$, generated through an artificial light source (Model LI-6400-02; light emitting silicon diode, LICOR), fixed on top of the leaf chamber; and ambient temperature (25 ± 3^0c). The rate of air flow (500µ mol s^{-1}), co_2 concentration inside the leaf chamber (350 ±5ppm) and relative humidity (55±5%) were kept constant throughout the measurements [16]. Since steady state photosynthesis is reached within 30-45 min, the leaves were kept for 45 min in the leaf chamber irradiated with chosen light intensity before taking the observations (3 measurements per plant). Chlorophyll 'a' fluorescence was measured using a Plant Efficiency Analyzer (Hansatech, UK).

Fluorescence measurements were made after a period of dark adaptation (30 min), at a wave length of 680 nm with 40% light intensity. Dark adaptation of 30 min and 40 per cent light intensity were previously found to be optimal for obtaining maximum values under shade conditions.

3. Results:

The present study revealed that the representative samples of *Costus pictus* collected from three different geographical regions of Kerala belong to the same genetic stock as they did not show any significant variation in terms of morphological characteristics, gas-exchange parameters and following RAPD analyses.
The samples of *Costus pictus* initially collected from three different regions of Kerala, when examined did not reveal any significant difference in their vegetative and reproductive characters. *C. pictus* is a herbaceous monocot, perennial, rhizomatous, vigorously growing plant and loves shady conditions. This particular form has broad, dark green leaves and beautiful solid red and fleshy stems, which grow with an outstanding spiral effect (hence the common name: spiral ginger). The stem is green at the apex; internode length varies between 2-5cm. The large, smooth, dark green leaves (light green when immature) of this tropical evergreen plant are lanceolate to oval in shape, have light purple undersides and are spirally arranged around stem, forming attractive, arching clumps arising from underground root stocks (Figure 2). The leaf base is red in colour, leaf sheathing is with joined margins, acuminate leaf apex, and conspicuously ligulate, pinnate parallel venation. Plant attains the height of about two to two and a half meters. Beautiful, 1.5 inch diameter, showy, yellow with red striped flowers are produced during warm months, appearing on cone like heads giving a beautiful effect sitting atop of the stems. The plant is viviparous and the propagules appear on old inflorescence.

Figure 2: Morphological and reproductive characters of *Costus pictus* D.Don.. **2a:** Perennial, rhizomatous herbaceousplant. **2b:** Alternate, spiral, simple, glossy, thick green leaves. **2c:** Red painted fleshy stem. **2d:** Yellow flowers with red streaks. **2e:** Bracts, red at base with green apex. **2f:** Cone like inflorescence at the apex of the stem. **2g:** Viviparous mode of multiplication

Morphological characters of *Costus pictus* D. Don. collected from three different agroclimatic regions of Kerala and cultivated at Sitapur, U.P. are briefly described below.
Habit: Perennial, rhizomatous herbaceous, upright, spreading and semi succulent
Trunk and Branches: Typically multi-trunked or clumping stems
Stem: Stems hirsute and green near apex, glabrous and purple toward base, 2-2.5m ht (maximum), 2- 2.5cm dia. and hirsute also at the base, fleshy, internode length 2-5cm, number of nodes 10-40
Leaf: Alternate, spiral, simple, entire, oblong, smooth surface, pinnate parallel venation, conspicuously ligulate (red coloured), light green when tender and dark green when mature, evergreen, 10 -34 cm length, 2 -6 cm width

with red leaf base, apex accuminate. Leaf sheath with joined margins, leaf is rolled at initiation, leaf pose erect, apex with down turn, absence of leaf pubescence and waxy coating.
Petiole: Reddish green and purple at margins, approx. 8mm long
Inflorescence: Globose to ovoid, terminal spike on a leafy stem, sessile, 3-8 cm long, 3-4 cm dia.
Bract: Green, deep scarlet in covered part, margins entire, thick coriaceous, 2-4cm long, 2-4cm dia.
Bracteole: Red, 1.6 - 2.1 cm long
Flower: Yellow in colour with red stripes, Zygomorphic, bracteolate, trimerous, cyclic arrangement, flowering during Spring- summer, fall after 2-3 days of blooming.
Perianth: Distinct calyx and corolla, 6, free, 2 whorled
Calyx : 3, 1 whorled, gamosepalous, blunt lobed, unequal, imbricate, with the median member anterior, red, 4 – 10 mm long, lobes 2 - 3.5 mm long
Corolla: 3, 1 whorled, gamopetalous, unequal, the median lobe larger and often upcurved, yellow, glabrous, 4-7 cm long
Androecium: 1 (ostensibly) or 6 (theoretically, but scarcely recognizable as such). Androecium includes staminodes. Staminodes supposedly 5, petaloid. Stamens 1, reduced in number relative to the adjacent perianth, petaloid. Anthers adnate, non versatile.
Labellum: Longer than corolla, 6 cm long and 5 cm wide, yellow with red streaks, the lip yellow and pubescent, recurved, and several-lobed.
Stamen: Anthers 7 – 8 mm long, yellow, apex red.
Gynoecium: 3 carpellery, syncarpous, epigynous ovary, trilocular, axile placentation, ovules many per locule. Style 1, apical. Stigma 1 wet type, sticky, papillate.
Fruit: Inconspicuous and not showy

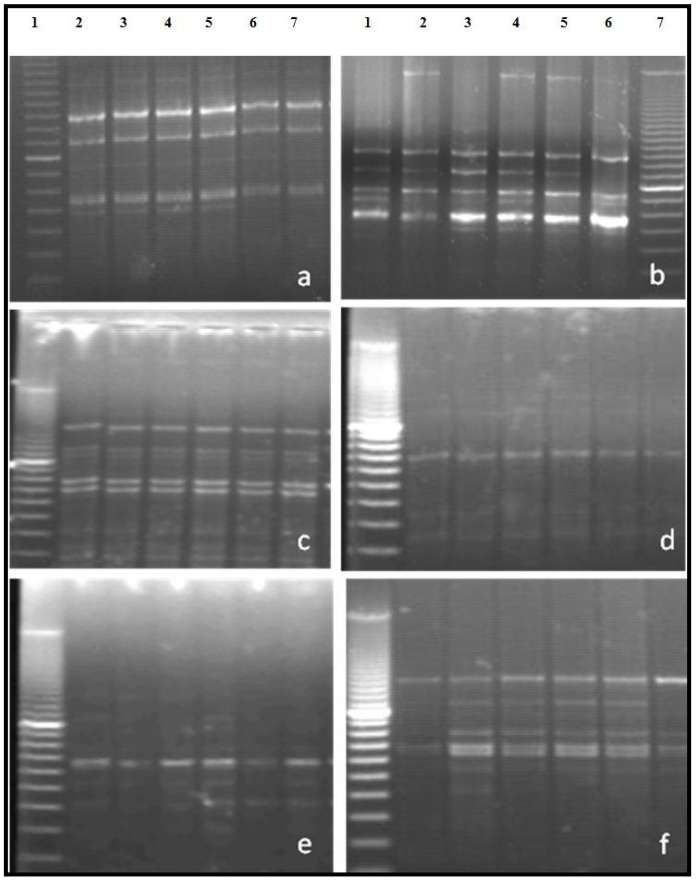

Figure 3: RAPD patterns of *Costus pictus* using various primers. **Lane 1 (Figs. 3a, 3c- 3f) and Lane 7 (fig 3b): Molecular markers. Lanes 2 & 3, 4 & 5 and 6 & 7(Figs. 3a, 3c-3f) and lanes 1 & 2, 3 & 4 and 5 & 6 (Fig. 3b) represent samples of *C. pictus* from 3 different parts of Kerala (south, middle and north, respectively) Primers used were OPA2 (Fig 3a), OPA 3 (Fig 3b), OPA 7 (Fig 3c), OPA 10 (Fig 3d), OPC 5 (Fig 3e) and OPC 16 (Fig 3f).**

In RAPD analyses, during the primer-screening step, out of a total of 40 primers used, 10 produced clear and scorable amplification products when used with different samples of *C. igneus*. The amplification profiles of total genomic DNA with ten primers selected out of preliminary screening produced a total of 44 fragments ranging in size from 0.3-2.9 kb, out of which 4 (9.09%) were polymorphic (Table 1). The number of fragments produced by a primer ranged from 2 (OPA10 &OPC 19) to 6 (OPA 3, OPA 11 &OPC 18; Figure 3). Primer OPA 2 produced 4 amplified products (Figure 3a); none of these were polymorphic.

Table 1. Total number and size range of amplified fragments and number of polymorphic fragments generated by 10 random primers by RAPD analyses of samples of *Costus pictus*

Primers	**Primer sequence (from 5' to 3'end)**	**Total no. of amplified products**	**No of polymorphic bands**	**Size Range(Kb)**	**% polymorphic bands**
OPA 2	TGCCGAGCTG	4	0	0.3-2.4	0
OPA 3	AGTCAGCCAC	6	2	0.6-2.9	33
OPA 7	GAAACGGGTG	4	0	0.5-2.6	0
OPA 10	GTGATCGCAG	2	0	0.7-1.5	0
OPA 11	CAATCGCCGT	6	0	1.0-2.4	0
OPC 5	GATGACCGCC	4	0	0.5-0.8	0
OPC 13	AAGCCTCGTC	5	0	0.4-1.2	0
OPC 16	CACACTCCAG	5	1	0.5-1.8	20
OPC 18	TGAGTGGGTG	6	1	0.4-1.9	16.6
OPC 19	GTTGCCAGCC	2	0	0.3-2.4	0
Total	----	44	4	0.3-2.9	9.09

RAPD fragment patterns produced by OPA 3 and OPA 7 have been shown in Figure 3 b & c, respectively. Decamer primer OPA 3 produced 6 amplified fragments out of which 2 bands were polymorphic; size of these fragments ranged between 0.6 to 2.9 kb. Primer OPA 7 produced 4 bands after PCR analysis; none of these were polymorphic and ranged in size from 0.5 to 2.6 kb. Primer OPA 10 produced only two monomorphic bands (Figure 3 d). RAPD pattern generated by primer OPC 5 has been presented in Figure 3 e. Among the primers used for RAPD analysis, primer OPC 5 produced 4 amplified products without any polymorphic bands. Size of the amplified products ranged between 0.5 to 0.8 kb. OPC 16 produced a total of 5 amplified products among these 1 was polymorphic. The size range was from 0.5 to 1.8 kb (Figure 3 f).

Similarity coefficient ranged from 0.96-1.0 in the samples of *Costus pictus* accessions examined in the present study. Data obtained from RAPD analyses indicate that the samples collected from three different parts of Kerala are genetically similar. Dendrogram analysis (not shown) did not show any difference between the representative plants, originally obtained from three different geographical locations of Kerala, used in this study.

Table 2. Net photosynthesis, Stomatal conductance, Transpiration rate, Internal CO_2 concentration and Fv/Fm (variable fluorescence and maximum fluorescence) ratio of *Costus pictus* plants originally collected from three different regions of Kerala

Gas exchange parameters	**Accessions**		
	South Kerala	**Middle Kerala**	**North Kerala**
Net Photosynthesis (mmol $m^{-2}s^{-1}$)	4.44±0.046	4.75±0.029	4.72±0.044
Stomatal conductance (m mol $m^{-2}s^{-1}$)	0.070±0.001	0.078±0.001	0.826±0.001
Transpiration rate (m mol $m^{-2}s^{-1}$)	0.738±0.0013	0.745±0.0005	0.775±0.0015
Internal CO_2 concentration (ml/L)	274.83±1.32	259.17±1.81	226.83±1.28
Fv/Fm ratio	0.808±0.001	0.815±0.001	0.800±0.001

The results obtained in terms of net photosynthesis, stomatal conductance, transpiration rate and PS II efficiency measured by chlorophyll fluorescence (*i.e.*Fv/Fm ratio) did not show any marked difference in the representative samples collected from three different locations of Kerala (Table 2). The net photosynthesis was 4.44 mmol $m^{-2}s^{-1}$, 4.75 mmol $m^{-2}s^{-1}$& 4.72 mmol $m^{-2}s^{-1}$ for plants from the south, middle and north Kerala, respectively. The minimum stomatal conductance was observed for plants from south Kerala (0.070 m mol $m^{-2}s^{-1}$) while the maximum value was recorded for the plants from north Kerala (0.826 m mol $m^{-2}s^{-1}$); plants from

middle Kerala exhibited medium value (0.078 m mol $m^{-2}s^{-1}$). Transpiration rates for the plants from south, middle & north Kerala were observed to be 0.738m mol $m^{-2}s^{-1}$, 0.745 m mol $m^{-2}s^{-1}$ and 0.775 m mol $m^{-2}s^{-1}$, respectively. A little variation was observed in photosynthetic efficiency in terms of internal CO_2 concentration ranging from 226.83 (ml/L) for the plants from north Kerala to 274.83 (ml/L) and 259.17 (ml/L) for plants from the south and middle Kerala regions respectively. Fv/Fm ratio, an indicator of PSII efficiency was found to be very close to 0.80 (*i.e.* 0.808, 0.815 and 0.80 for the plants of three regions) in all cases, which is suggestive of a healthy and normal (unstressed) state of the plant [17].

4. Discussion:

Morphological, biochemical and more recently molecular characters have been used for plant identification [18, 19]. In the present communication, detailed and comprehensive data on various morphological characters, viz. leaf, branch, clump, nodes, inflorescence, flower, bracts, stamens and corolla, etc. have been examined and complemented with important physiological (gas and water vapour exchange) and molecular data for comparison of *C. pictus* plants originally collected from three regions of Kerala and grown under identical conditions at Sitapur, U.P. (for morphological and molecular studies) and at Kosi-Katarmal, Almora (for gas and water vapour exchange studies). In an earlier report [20] the morphological, anatomical and proximate features of this plant grown in a nursery at Vallam, Tamilnadu have been briefly described.

The phylogenetic relationships of Costaceae, a tropical monocotyledonous family, sister to the gingers (Zingiberaceae), were investigated by [21] with a combination of two chloroplast loci and one nuclear locus (ITS1–5.8s–ITS2).

In the present study, RAPD markers were used to assess genetic diversity; analyses did not reveal any genetic differences between the samples of *C. pictus* originally collected from three different regions of Kerala. The similarity within plants was found to vary from 0.96 to 1.0; high level of similarity observed between the plants is also due to their vegetative mode of propagation**.** The study suggests that though the decamer primers are small in comparison to the large genome of *Costus pictus*, they produced appreciable amplicons, sufficient to examine the three accessions. The dendrogram also confirmed genetic relatedness among different accessions. The RAPD pattern of *Costus speciosus* was investigated by [22]. RAPD–PCR analyses involving 12 decamer random primers were used to assess genetic variation at the genomic level. Four primers showed appreciable intra-species variation or molecular polymorphism at amplicon levels. Despite morphological identity, a great deal of polymorphism was observed among the accessions. Concurrently, it is also shown that the entries that were found to be similar in taxonomical classification based on morphological characters do exhibit divergence at the level of DNA. Work carried out on *C. speciosus* shows that this method is capable of revealing appreciable levels of polymorphism in plants of *Costus* species. The studies with *C. speciosus* clearly indicate that if assay conditions are carefully controlled, the RAPD methodology provides a relatively cheap, rapid and effective means to evaluate the genetic diversity among plant populations and helps to devise strategies to complement classical morpho-agronomic descriptors.

The results of physiological analyses pertaining to gas and water vapour exchange studies suggest that if a good correlation between growth and unit rates of photosynthesis and respiration can be established, than the effect of environment or heredity on growth may be easily determined [23]. The increased stomatal opening is a biophysical necessity for increasing the rate of diffusion of carbon dioxide from air to the inside of plant leaves [24]. Typically, net photosynthetic rates increase when stomata open and decrease when they close [25, 26]. Hence, changes in stomatal conductance may cause changes in photosynthetic rates [27].

Although photosynthetic and stomatal physiology are key traits that control the uptake of carbon (by way of CO_2) and loss of water in plants; genetic variation for these traits has not been measured frequently. Significant heritable genetic variation for photosynthetic and stomatal traits has been detected in a few, primarily selfing species [28, 29]. There is no consensus on the potential for the quantitative genetics of photosynthetic and stomatal traits to constrain or facilitate their adaptive evolution in natural populations, especially in out crossing species [28-30]. The results obtained on the gas and water vapour exchange parameters suggest that the three accessions do not differ, and that no genetic or ecotypic variation is indicated. It implies that the three accessions may have originated from the same stock which is very likely as the plant was introduced only recently in India.

Acknowledgement:

The authors are grateful to Indian Academy of Science, Bangalore, India for the summer research fellowship. The authors thank the Director, G.B. Pant Institute of Himalayan Environment & Development, Kosi- Katarmal, Almora, India for providing necessary facilities and support.

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