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Authentication of *Costus Pictus*.D.Don A Medicinal Plant by PCR-Based RAPD Marker

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Abstract

Costus pictus D.Don an important medical plant widely used in the management of diabetes to reduce the blood glucose level known as "Insulin plant". Its rhizomatous herb possess many therapeutic properties, belonging to *Costaceae* family, many species under this family share identical morphological characters and difficult to differentiate by external morphology. During formulation of herbal drugs, identification, authentication, quality assurance, safety control being an initial and major step. In surplus use of medicinal plants, authentication plays a unique role, which are often adulterated or substituted with other species. The morphological, anatomical, phytochemical and molecular approach helps in clear distinction of species. Thus our aim of study was to isolate Genomic DNA, amplify with specific primers and RAPD technique for authentication of C.*pictus* from its substituent's four species, *C.stenophyllus*, *C.dubius*, *C.speciosus*, *and C.erythrophyllus*. The PCR reactions among 21 primers, 14 primers showed reproducible amplicons, OPD-02, OPU-18, OPR-08, OPJ-07, OPC-06, OPW-02 show unique banding patterns in C.*pictus*. Polymorphic information content (PIC) was maximum for the primer OPD-02 0.80 followed by other primers least OPJ-07 0.25, clustering analysis by Jaccard Co-efficient index exhibited similarity matrix and distance matrix 0-0.98. The phylogenetic tree was constructed by UPGMA cluster analysis significant variations in the amplified genomic DNA suggest genetic variability of species.

Key words: *Costaceae, Costus pictus, Dendrogram clusters, Genetic diversity, Polymorphism, RAPD Markers*.

1. Introduction

The medicinal plants are exploited as folklore medicine and culinary purposes since pre-historic period for treating many human aliments, traditionally in several forms of medicinal system such as Unani, Ayurveda, and Homeopathy. Plant based derivatives are source of compounds used as a drug across the globe for many chronic diseases, as whole plant or plants parts in developing countries. In developed countries use of herbal preparation also gaining more importance in health care sector due to very less or completely no side effects. In the modern times even the allopathic or alternate medicines are looking for plants or plant based derivatives for synthesis of compounds in drug preparation. In any system of medicine development and formulation of drug, requires a disease free, a pathogen free or plants free from agents causing any adverse side effects is important in terms of good health. As per the WHO nearly 80% of population relies on herbs and other traditional medicine for health care needs, thus maintenance of purity, quality, safety, of the herbal medicinal plants is necessary. The major issue faced in pharmaceutical industries is maintenance of good quality, purity of the herbal products, which are utilized as a raw material for drug synthesis. The most crucial step is proper or correct identification of plant and plant parts, to differentiate between the substituent and adulterant caused due to misidentification of medicinal herbs. The herbal products are assessed at various stages by testing them in different frame work for its application in modern science and technology. As there is a lacuna or bridging gap between traditional and modern scientific tools; research

and development activity is necessary for developing a standardized protocol for bioassay, pharmaceutical evaluation, pharmacological, toxicological, clinical trials on cell lines and animal models in order to establish bio-active compound from the plant extract. Apart from the analysis through morphological, anatomical, biochemical parameters, the amalgamation of molecular approach being important. The molecular tools and technique has proved itself as a valuable tool in the identification of plant varieties belonging to the given species with identical morphological characters (Nagamani & Sabitha, 2018). In the past three decades many molecular tools and techniques like RAPD (Random Amplified Polymeric DNA), RFLP (Restricted Fragment Length Polymorphism), AFLP (Amplified Fragments Length Polymorphism) SSR (Simple Sequence Repeats) IRAP (Inter-retro transposon amplified polymorphism), ISSR (Inter-simple Sequence Repeats), ITS (Internal Transcribed Spaces) (Khan, 2015) being used as molecular tools. RAPD technique is more advantageous allowing random sampling of markers over whole genomic DNA without any previous information of the genome of the organism under investigation (Vakariya et al., 2017). RAPD (pronounced as 'rapid') is more reliable, reproducible, cost effective and less laborious, (Agarwal et al 2008). PCR based markers requires amplification of particular DNA loci with the help of specific or arbitrary oligonucleotide primers and the thermostable DNA polymerase enzyme, it requires a very small amount of DNA with no information of prior sequence. The main objective of this study was to collect the species belonging to same family *Costaceae* showing similar morphological characters and to develop a standard protocol for accurate identification of leaf samples by PCR based RAPD markers.

Costaceae family belonging to pantropical rhizomatous monocot herb placed under the order Zingiberales, commonly grown as ornamental purpose in horticulture for their attractive branching and brightly coloured cones and flowers. It found to be spread across South America and Africa and recently introduced to parts of India as it possesses various therapeutic properties. *Costus* taxa recorded initially in Hortus malabaricus by Hendrick Anadariann Van Rheede (1678-1693). It is perennial, terrestrial, epiphytic herbs, aerial erect pseudo stem arising from submersed rhizome, spirally arranged leaves, branching through the leaf sheath with monistichous phyllotaxy, inflorescence open or closed dense globose or ellipsoidal, occurs terminally on the leafy pseudo stem or no a separate leafless shoot (Justin R. Nayagam, 2015). Bracts are imbricate arranged in the form of cone bearing a single or multiple flowers. *Costaceae* family being a major group of Angiosperms consists of 7 plant genera includes 308 scientific plant names of species ranked under the family, out of which 137 are accepted species. The leaves of *Costus* are identical in morphological features; it is difficult to distinguish between the plants. The herbal drugs when released into market in the form of powders, not been identified correctly, and usage of such products leads in ineffective or adverse condition in the patients. Thus proper authentication should be done before harvesting of the plant material and utilization of plant and plant products, till the formulation of final herbal product.

Costus pictus .D.Don (Syn *Costus igneus* nak, *Costus mexicanus* Liebm ex, Peterson or *Costus congenitus* Rowle) commonly known as medicinal ginger or Insulin plant, leaves are arranged spirally on pseudo stem thus called spiral flag, fiery *Costus*, Step ladder. C.*pictus* leaves consumed daily to believe to reduce the blood glucose level (Al-Romaiyan et al., 2010; Annadurai et al 2012; Shiny et al., 2013). Many experimental studies carried out by treating the diabetic rats induced with alloxon, streptozotocin rats and treated with methanol extract to reduce blood glucose level (Jayasri et al., 2008; Gireesh et al., 2009), anti-bacterial (Beena & Jyothi, 2010, Sulakshana et al 2013; Sardessai et al., 2014, Ramya, 2016), anti-cancer (Nadumane et al., 2011, Malairaj 2012), anti-helminthetic (Thomas & Devi 2013, Raj & Kalaivani, 2016), anti-oxidant (Kavitha, 2013, Prakash et al., 2014, Prejeena et al., 2017) aqueous extract of C.*pictus* leaves show diuretic effect (Camargo et al., 2006).

The major objective of this experiment was to obtain a good quality and quantity of DNA, for the isolation of DNA from leaf tissue, the extraction technique need to be simple, raid, inexpensive, effective, reproducible and efficient with intact DNA for the molecular analysis. A distinct PCR based amplification of genomic DNA with specific primers was necessary (Skolnick & Wallace, 1988). The method involves a modified CTAB procedure of Doyle & Doyle, 1990). The DNA obtained through this method was highly pure and proved to be good in restriction digestion with endonuleases and also useful for polymerase chain reaction (PCR) with random primers. The RAPD random amplified polymeric DNA involves the usage of single "arbitrary" primers in the polymerase chain reaction (PCR) and results in the

amplification of several discrete fragments (Welsh, 1992). The fragment obtained from the genomic region consists of opposite complementary stands to the primer and sufficiently get close together for the amplification (Wilde et al., 1992).

2. Materials and methods:

Plant Material for DNA Isolation:

The plant materials were collected from Calicut University, Calicut Kerala and maintained in green house Department of Botany, Bangalore University, Bangalore. The fresh and young leaves were harvested from 5 samples and washed with sterile distilled water followed by swabbing with 70% alcohol.

Required solutions:

An extracting buffer consists of 2% CTAB (Hexa decyl thimethyl ammonium bromide), 1M Tris pH 8.0, 0.5 M EDTA pH 8.0, 5M NaCl , PVP (Poly vinyl pyrolidone), 0.2% β-merceptoethanol, chloroform, iso-amyl alcohol (24:1), 95% ethanol, TE buffer consists of 1M Tris HCl pH 8.0, 0.5M EDTA pH 8.0, Absolute ethanol (Ice cold), 70% ethanol, Agarose, loading buffer, 1X TBE solution, Agarose gel, ethidium bromide solution.

DNA Isolation:

Fresh leaves were surface sterilized with ethanol, 200mg of plant tissue was weighed and finely crushed, and the homogenized tissue sample was washed with 500µl of 70% ethanol centrifuged at 10,000rpm for 5 minutes. The ethanol after washing was discarded and 15 ml of 2% CTAB with plant extract mixture were transferred to the tube and mixed thoroughly, 20µl of beta merceptoethanol was added and mixed and incubated for 90 minutes at 60^0C in water bath. After incubation the mixture was cooled at room temperature and centrifuged at 10,000 rpm for 15 minutes. The supernatant was carefully decanted into fresh tubes and equal volume of mixture of chloroform: iso-amyl alcohol (24:1) was added. The mixture were mixed well for 10 min at room temperature by inverting tubes intermittently and centrifuged at 10,000 rpm for 15 minutes. The aqueous phase was transferred to the fresh tube and the DNA was precipitated by adding equal volume of ice-cold iso-propyl alcohol and centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded and DNA pellet was washed with 0.5ml of 70% alcohol. The pellet was dried at 37^0C for 10 min and re-suspended in 500µl of 1x TE buffer.

Purification of DNA:

The crude DNA obtained was treated with 1µl RNase (10mg/ml stock) for 15mins at 37^0C and equal volume of the mixture of iso-amyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 2mins. To the aqueous layer equal volume of iso-propanol were added and stored at -20^0C for 10minutes and the mixture was centrifuged at 10,000 rpm for 2 minutes. The supernatant was decanted carefully. The pellet obtained was washed with 70% alcohol and dried at 37^0C and dissolved in 50µl 1x TE buffer.

Quantification and Restriction of DNA:

The yield of DNA obtained per gram of fresh tissues, determined using a UV-Vis spectrophotometer. The purity of DNA was determined by calculating the absorbance ratio at 260nm at 280nm. The purity of DNA was verified by restriction digestion of the purified DNA samples with EcoR1 (4units per µg) for 5 hours incubation periods and restricted DNA eluted on agarose gel stained with ethidium bromide.

PCR amplification:

The PCR amplification helps to check the quality of isolated DNA from the leaf tissue of five *Costus* species selected PCR reaction programmed for 40 cycles which initial denaturation at 94^0C for 2 min, annealing at 45^0C for 1 min, extension at 72^0C, amplification reaction carried out with 21 random primers PCR products loaded on to agarose gel stained with ethidium bromide as mentioned in table 1.

Table No.1 PCR Cycle Condition

Temperature	Time	No. of cycles
94^0C	2minutes	1
94^0C	30 second	40
45^0C	1 minute	
72^0C	1.30 minute	
72^0C	7 minute	1

RAPD Analysis:

Amplified RAPD fragments from the five genomic DNA samples was carried out by the total volume of 38µl of aliquots of 5 different loaded PCR vial with 2µl of different templates DNA added to the PCR mixture. The master mix of 40µl and 200µl consists of 1x components with 2x PCR buffer 10mM Tris HCl, 50mM KCl. 1.5mM $MgCl_2$ pH 8.0, 1µl of Random primers and 1.5µl Taq DNA polymerase fragments, distilled water was added to the complete volume of the reaction mixture with 4µl for 5x components 2x PCR

master mix of 100µl, 5µl random primers, 10µl of templates was added and final volume of 200µl was made up by sterile distilled water of 85µl from this two RAPD mixtures 38µl was aliquoted for PCR reactions.

Table No. 2 PCR Templates

	Master mix	
Components	1x	5x
Double Distilled water	17µl	85µl
2x PCR master mix	20µl	100µl
Random primers	1µl	5µl
Template	2µl (100ng)	10µl
Total volume	40µl	200µl

Data Analysis:

DNA binding patterns obtained by primers of RAPD were scored as 1 for the presence of band and 0 for its absence. All the RAPD assays were performed for 5 species and only the reproducible bands were scored. The percentage of polymorphism of all primers was calculated by the formula mentioned below.

$$\%\text{Polymorphism} = \frac{\text{No of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Table 3 indicates the sequence pattern of primers 5' -3' these data were used to construct dendrogram for cluster analysis based on Unweighted Pair Group Method with arithmetic mean (UPGMA) with jaccard co-efficient and similarity matrix of 5 species. Jaccard similarity coefficient, also known as Tanimoto co-efficient as mentioned in table 4&5, It measures the similarity between two sets of binary data. It is defined as the size of the intersection divided by the size of the union of the sample sets. It gives a value between 0 and 1. Jaccard similarity coefficient: S AB= c a+b−c a is defined as the number of bits set to "1" in A, b as the numbers of bits set to "1" in B and c as the numbers of bits that are "1" in both A and B as depicted in the figures.

$$S_{AB} = \frac{c}{a+b-c}$$

Table No.3 Sequence information of RAPD oligonucleotide primers used for amplification

SL. No	RAPD primer	Sequence 5' -3'
1	OPD-02	TGCCGAGCTG
2	OPAA-09	AGATGGGCAG
3	OPA-17	GACCGCTTGT
4	OPA-11	CAATCGCCGT
5	OPA-02	TGCCGAGCTG
6	OPU-18	GAGGTCCACA
7	OPK-08	GAACACTGGG
8	OPR-08	CCCGTTGCCT
9	OPB-08	GTCCACACGG
10	OPH-05	AGTCGTCCCC
11	OPF-02	GAGGATCCCT
12	OPD-02	GGACCCAACC
13	OPJ-07	CCTCTCGACA
14	OPC-07	GTCCCGACGA

Table No. 4 Sequence information of RADP oligonucleotide primers used for amplification and polymorphism studies in 5 medicinal plants

SL.No	RAPD primers	Total Bands	C.*pictus*	C.*stenophyllus*	C.*dubius*	C.*speciosus*	C.*erythrophyllus*
1	OPD-02	15	12	9	5	5	11
2	OPAA-09	20	6	6	9	14	6
3	OPA-17	14	9	4	10	2	9
4	OPA-11	22	6	7	10	8	12
5	OPA-02	14	8	8	6	8	8
6	OPU-18	16	8	5	10	8	8
7	OPK-08	21	5	4	12	10	5
8	OPR-08	24	11	7	10	8	5
9	OPB-08	19	4	4	9	9	8
10	OPH-08	10	6	1	6	2	2
11	OPF-02	16	1	6	6	7	4
12	OPD-02	15	8	5	7	8	10
13	OPJ-07	8	2	1	2	2	1
14	OPC-07	12	4	1	6	4	4

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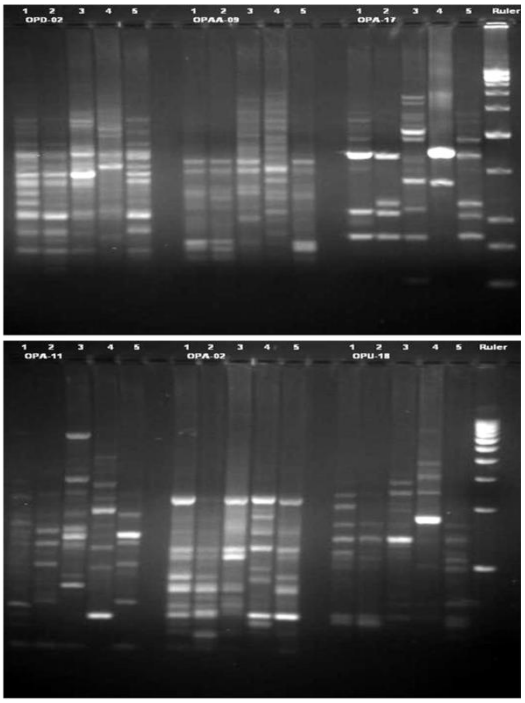
Table No.5 the polymorphic information content in 5 species of *Costus*

SL.No	RAPD primers	Amplicons	C.*pictus*	C.*stenophyllus*	C.*dubius*	C.*speciosus*	C.*erythrophyllus*
1	OPD-02	15	0.80	0.60	0.33	0.33	0.73
2	OPAA-09	20	0.30	0.30	0.45	0.70	0.30
3	OPA-17	14	0.64	0.28	0.71	0.14	0.64
4	OPA-11	22	0.27	0.31	0.45	0.36	0.54
5	OPA-02	14	0.57	0.57	0.42	0.57	0.57
6	OPU-18	16	0.50	0.31	0.62	0.50	0.50
7	OPK-08	21	0.23	0.19	0.57	0.47	0.23
8	OPR-08	24	0.45	0.29	0.41	0.33	0.20
9	OPB-08	19	0.21	0.21	0.47	0.47	0.42
10	OPH-05	10	0.60	0.10	0.60	0.20	0.20
11	OPF-02	16	0.06	0.37	0.37	0.43	0.25
12	OPD-02	15	0.53	0.33	0.46	0.53	0.66
13	OPJ-07	8	0.25	0.12	0.25	0.25	0.12
14	OPC-07	12	0.33	0.08	0.50	0.33	0.33

Fig. No.1 Banding patterns of 5 *Costus* species using 14 primers



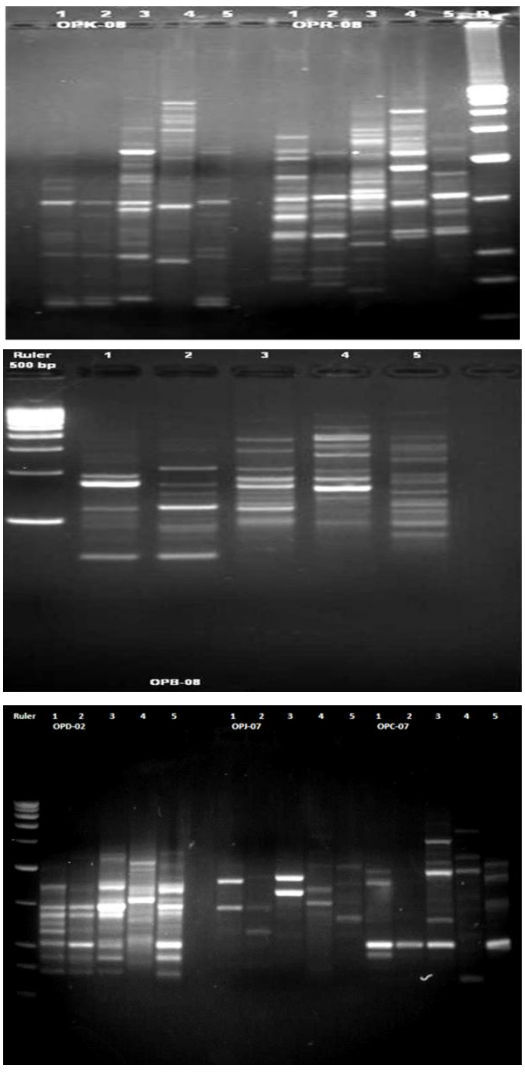
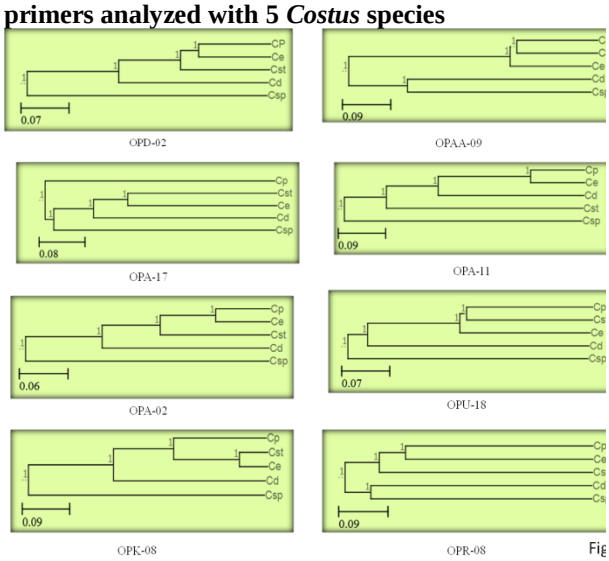


Fig 2-3. Jaccard similarity index of all 14 primers analyzed with 5 *Costus* species



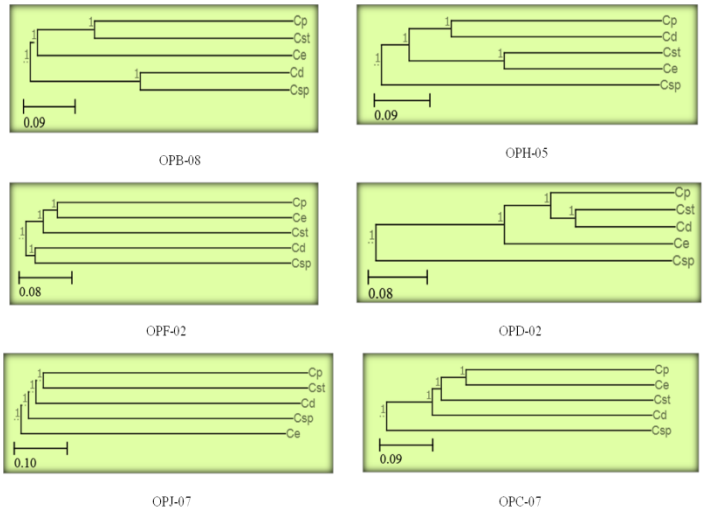


Fig-3

3. Results and Discussion:

As per the general guidelines of WHO for standardization of research methodologies and evaluation of traditional form of medicinal practices, the utilization of medicinal herbs has to be assured initially on the basis of good quality, safety and efficacy with correct identification and authentication of plants. Medicinal plants constitute as major source in traditional and modern medicine or alternate medicine, the raw material required are adulterated or substituted with other similar appearing species, thus adulteration of sample a greatest drawback in consumption of herbal products. The screening of therapeutically important guinea species is important to control intentional or unintentional adulteration of medicinal plants (Sree Lakshmi et al., 2017). The study demonstrated that genetic makers and detection of unique primers amplification helps to clearly distinguish among the species. The polymorphic information content (PIC) monomorphic and polymorphic banding was observed by ethidium bromide staining on agarose gel and documented through gel documenting unit. The isolated DNA from five *Costus* species was compared with random decamers primers the following 14 primers OPD-02, OPAA-09, OPA-17, OPA-11, OPA-02, OPU-18, OPK-08, OPR-08, OPB-08, OPH-05, OPF-02, OPD-02, OPJ-07, and OPC-07. The highest amplification was obtained in produced by 226 bands, the decamers primers were selected for the RAPD analysis which produced by 445 amplification with an average of 8 amplicons and highest 24 amplicons OPR-08, the highest polymorphism in OPD-02 0.80 and least 0.06 in OPF-02 respectively, the overall polymorphism 50.7% were observed. The RAPD markers banding pattern of C.*pictus* can be used to distinguish correct genuine species as well as substituent/ adulterated samples, OPD-02, OPU-18, OPR-08, OPJ-07, OPC-06, OPW-02 show unique banding patterns in comparison that other species. Based on the dendrogram analysis similarity matrix and distance matrix was computed by jaccard co-efficient. The observation of clear unique bands in 6 primers further facilitates wide range scope of research and selection and converting into SCAR markers and DNA bar coding.

RAPD markers are well suited for genetic mapping for plant and animal breeding application and for DNA fingerprinting, with particular utility for studying population genetic and genomic mapping. A molecular marker plays an important role in authentication and identification of medicinal plants by RAPD-PCR technique in Embelica species were analyzed by analyzing the genetic distance by calculating using Jaccard similarity Co-efficient. The RAPD analysis has been widely used for differentiating a large number of medicinal species from their closely related species or adulterants or substituent's, or misidentification in Glycyrrhiza glabra Lin and Cuscuta reflexa (Salim Khan et al., 2009) Croton liglium Linn (Acharya et al., 2017) also

differentiated studies between two common plants using in management of diabetes Khaya senegalensis and Azadirachta indica (Mawobi, 2018). In the past three decades RAPD random amplified polymorphic DNA technique working based on the PCR (polymerase chain reaction) has been one of most commonly used molecular technique developed applicable in various fields (Senthi & Gurusubramanian, 2011).

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