Assessment of genetic variability in Indian Karonda (*Carissa opaca* L.) accessions using DNA based inter simple sequence repeat (ISSR) Markers

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***Abstract*-Genetic phylogenetic relationships in the fourteen accessions of *Carissa opaca* L. have been analyzed using ISSR markers. At the genetic level a lot of heterogeneity has been observed among the accessions of *Carissa* and the similarities varied from 30-86% with the help of cluster analysis. This study is showed PCR based amplification of DNA from the 14 accessions of *Carissa* plant which were collected from the different locations of the India. This study may be helpful in resolving the taxonomic ambiguities in species delimitation in genus *Carissa* L.**

Keywords-Accession, *Carissa opaca*, phylogeny, taxonomic similarity, dendrogram.

I. INTRODUCTION

Carissa opaca is a shrubs or small evergreen trees native to tropical and subtropical regions; it is well grown in all over India with the height of 3 meters. Around 100 species were noticed worldwide and about 12 sps. are found in India. The plants are frequently cultivated for its edible fruits and commonly raised from seeds. The unripe fruits of the *Carissa opaca* is sour in taste and astringent and used in pickles. The ripe fruits are sweets, edible and particularly suitable salads, tarts, pudding jams and jellies. The unripe fruits is reported for the good source of vitamin-C and used as an anti-ascorbic and in bilious complaints. Roots, leaves fruits are well used in Ayurveda since ancient times.

Natural variability has so for been studied only for few species for the horticulture and agriculture importance. Its haves got importance of research since the natural biodiversity is decaying at the alarming rate. Germplasm of *Carissa opaca* was collected from wide range of locations from eight districts of westerns Maharastra, one district from Marathwada and one from Goa.

A microsatellite is a zone of repetitive DNA in which certain DNA motifs (2-10 base pairs) are continual. Microsatellites occur at thousands of locations in the plants genome and they are noteworthy for their high mutation rate and high diversity in the population. Microsatellites and their longer co-part, the minisatellites, together are classified as VNTR (variable number of tandem repeats) DNA. The name "satellite" refers to the early observation that centrifugation of genomic DNA in a test tube separates a prominent layer of bulk DNA from accompanying "satellite" layers of repetitive DNA. Microsatellites are often referred to as short tandem repeats (STRs) by forensic geneticists, or as Inter simple sequence repeats (ISSR) by plant geneticists. ISSR (inter-simple sequence repeat) is a general term for a genome region between microsatellite loci. The complementary sequences to two neighboring microsatellites are used as PCR primers; the variable region between them gets amplified. The limited length of amplification cycles during PCR prevents excessive replication of overly long contiguous DNA sequences, so the result will be a mix of a variety of amplified DNA strands which are generally short but vary much in length.

Sequences amplified by ISSR-PCR can be used for DNA fingerprinting. Since an ISSR may be a conserved or non conserved region, this technique is not useful for distinguishing individuals, but rather for phylogeography analyses or maybe delimiting species; sequence diversity is lower than in SSR-PCR, but still higher than in actual gene sequences. In addition, microsatellite sequencing and ISSR sequencing are mutually assisting, as one produces primers for the other.

The primarily objective and aim of the study is to use certain pre-scanned primers for variability and polymorphism studies and to find out the relationship between different accession of *Carissa*.

II. REVIEWS AND LITERATURES

Carissa opaca a vigorous, spreading, woody shrub with abundant white, gummy sap, the Carissa may reach a height of 4.5-5.5 m and an equal breadth. C. opaca is the handsome, evergreen; opposite leaves is broad-ovate, with 2.5-5 cm long, dark-green, glossy, leathery. Some plants stand flowers that are functionally male, larger than normal and with larger anthers, and stamens much longer than the style. Functionally female flowers have bears stamens the same length as the style and small anthers without pollen. *Carissa opaca* bears round, oval or oblong fruit, and also bears green and rich in latex when unripe. As it ripens, the tender, smooth skin turns to a

bright magenta-red coated with a thin, whitish bloom, and finally dark-crimson. The flesh is tender, very juicy, strawberry-colored and -flavored, with flecks of milky sap. The *carissa* is native to the coastal region of Natal, South Africa, and is cultivated in the land of Transvaal. It is widely cultivated in Israel, flourished and flowered freely but rarely set fruit.

In pomegranate (*Punica granatum* L.), it has been study and described the genetic diversity across natural populations of Indian pomegranate based on inter-simple sequence repeat (ISSR) markers. In this report it was noted that forty-nine accessions representing eight populations from two regions were analyzed using ISSR. Seventeen ISSR primers resulted in 268 polymorphic bands, with 87.01% polymorphism throughout the accessions. Pair-wise population genetic distances ranged from 0.05 to 0.45, with a mean of 0.25 between populations. AMOVA and Nei's genetic diversity analyses revealed higher genetic variation within populations than among populations. A higher genetic differentiation (G_{ST}) was observed between the spatially distant populations, indicating a low level of genetic exchange (Nm) among these populations. However, clustering of populations was not in accordance with their geographical affiliations in the tree (Narzary et al., 2010).

In *Calibrachoa caesia* it has reported that thirteen ISSR primers has been produced a reproducible banding pattern, with 701 amplified loci and 98% of polymorphism. The ISSR primers 5'CT, 5'CA, 5'GA, 5'GACA, 3'CAC, 3'TG and 3'TC has been generated 100% polymorphic patterns. The Rp values has noted ranged from 23.20 to 10.29 for 5'GACA and 3'AG primers, respectively, while the average values for MI and PIC were found 0.367 and 0.231, respectively. The more informative primers were found 5'GACA and 5'GA, and the less informative was reported 3'AC. Simple matching coefficient of similarity has been varied from 0.8875 to 0.6659, indicated high levels of genetic similarity among the genotypes studied (de la Torre et al., 2012). In Mulberry it has been mentioned that molecular markers are highly polymorphic, more in number, stable across different developmental stages, neutral to selection and least influenced by environmental factors. Among the PCR based marker techniques, ISSR is one of the simplest and widely used techniques, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. Because of these properties ISSR markers have recently been found using extensively for finger printing, pohylogenetic analysis, population structure analysis, varietal/line identification, genetic mapping, marker-assisted selection, etc. In mulberry (Morus spp.), ISSR markers has been used for analyzing phylogenetic tropical and temperate mulberry, for solving the vexed problem of identifying taxonomic positions of genotypes, for identifying markers associated with leaf yield attributing characters. As ISSR markers are one of the cheapest and easiest marker systems with high efficiency in generating polymorphism among closely related varieties, they would play a major role in mulberry genome analysis (Vijayan et al., 2005).

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III. MATERIALS AND METHODS

The leaves of different Carissa accessions were collected from different part of country (Table-1). Leaves were frozen immediately in liquids nitrogen and were stored at -80ºC till the further use of DNA extraction.

Table-1: Accessions studied and their locations

S. N.	Accession	Locality	Characters
1.	MPG1	Madhya Pradesh	Small fruits
2.	MPG2	Madhya Pradesh	Small fruits
3.	HPSN1	Himanchal Pradesh	Small fruits
4.	*C. opaca*	Ooty	Small fruits
5.	CoO1	Coimbatore	Small fruits
6.	CoA1	Coimbatore	Small fruits
7.	CoOS1	Coimbatore	Small fruits
8.	OMS1	Ooty	Small fruits
9.	KBM1	Karnataka	Small fruits
10.	TSD1	Tamil Nadu	Small fruits
11.	TSD2	Tamil Nadu	Small fruits
12.	TSD3	Tamil Nadu	Small fruits
13.	DAK1	Dang forest	Small fruits
14.	NID2	Maharashtra	Small fruits

The search for a more efficient means of extracting DNA of both higher quality and yield has lead to the development of a variety of protocols, however the fundamentals of DNA extraction remains the same. DNA has been purified from cellular material in a manner that prevents degradation. Because of this, even crude extraction procedures have been adopted to prepare a sufficient amount of DNA to allow for multiple end uses. DNA extraction from plant tissues have been depended on the material by mechanical means of breaking down with liquid nitrogen. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allowed access to DNA while harmful cellular enzymes and chemicals remain inactivated. Once the tissue has been sufficiently ground, it was then be re-suspended in a suitable buffer (CTAB). In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other material are separated through mixing with chloroform and centrifugation. DNA has been precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA is then re-suspended and stored in TE buffer or sterile distilled water. By this method we have obtained intact

genomic DNA from plant tissue. To check the quality of the extracted DNA, a sample was run on an agarose gel, stained with ethidium bromide, and visualized under UV light.

Materials: CTAB buffer, microcentrifuge tubes, Mortar & Pestle, liquid nitrogen, absolute ethanol (ice cold), 70% ethanol (ice cold), 7.5 M ammonium acetate 55°C water bath chloroform: iso amyl alcohol (24:1), water (sterile), agarose 6x, loading buffer 1x TBE solution, agarose gel electrophoresis system and ethidium bromide solution.

CTAB buffer: 100ml 2.0 g CTAB (Hexadecyl trimethyl-ammonium bromide), 10.0 ml 1 M Tris pH 8.0, 4.0 ml 0.5 M EDTA pH 8.0 (EthylenediaminetetraAcetic acid Di-sodium salt), 28.0 ml 5 M NaCl, 40.0 ml H2O, 1 g PVP 40 (polyvinyl pyrrolidone (vinylpyrrolidine homopolymer) Mw 40,000), Adjusted all to pH 5.0 with HCl and make up to 100 ml with H2O. 1 M Tris pH 8.0 Dissolve 121.1 g of Tris base in 800 ml of H2O. Adjusted pH to 8.0 by adding 42 ml of concentrated HCl. Allowed the solution to cool to room temperature before making the final adjustments to the pH. Adjusted the volume up to 1 liter with H2O. Sterilized was done using an autoclave.

5x TBE buffer: 54 g tris base, 27.5 g boric acid, 20 ml of 0.5M EDTA (pH 8.0) maked up to 1liter with water. Maked a 0.5x working solution, Made a 1:10 dilution of the concentrated stock.

1% Agarose gel: 1 g of agarose was dissolved in 100 ml TBE buffer.

Procedure for isolation of DNA; Ground 200 mg of plant tissue to a fine paste and added 500 µl of CTAB buffer. Transfered CTAB/plant extracts mixture to a microcentrifuge tube. Incubated the CTAB/plant extract mixture for about 15 min at 55ºC in a re-circulating water bath. Centrifuged the CTAB/plant extract mixture at 12000 g for 5 min to spin down cell debris. Transferred the supernatant to clean microfuge tubes. Added 250 µl of chloroform: iso amyl alcohol (24:1) in each tube and mixed the solution by inversion. After mixing, sppined the tubes at 13000 rpm for 1 min. Transferred the upper aqueous phase only (DNA) to a clean microfuge tube. Added 50 µl of 7.5 M ammonium acetate in each tube and followed by 500 µl of ice cold absolute ethanol. Inverted the tubes slowly several times to precipitate the DNA. DNA was seen after the precipitated out of solution. The DNA was pipetted off by slowly rotating/spinning a tip in the cold solution. The precipitated DNA sticks to the pipette and is visible as a clear thick precipitate. To wash the DNA, transfer the precipitate into a micro centrifuge tube containing 500 µl of ice cold 70 % ethanol and slowly inverted the tube. DNA was spined the tube at 13000 rpm for a minute to form a pellet. Removed the supernatant and wash the DNA pellet by adding two changes of ice cold 70 % ethanol)). After the wash, spined the DNA into a pellet by centrifuging at 13000 rpm for 1 min. removed all the supernatant and allowed the DNA pellet to dry. Re-suspend the DNA in sterile DNase free water (50µl H2O). RNase A (10 µg/ml) was added to the water prior to dissolving the DNA to remove any RNA in the preparation. After re-suspension, the DNA was incubated at 65ºC for 20 min to destroy any DNases. Agarose gel electrophoresis was done of the DNA. Concentration of DNA was measured with spectrophotometry.

DNA quality confirmation: Prepared a 1 % solution of agarose by melting 1 g of agarose in 100 mL of 0.5x TBE buffer in a microwave for approximately 2 min. Allowed to cool for a couple of minutes then added 2.5 µl of ethidium bromide, stirred to mix. Casted a gel using a supplied tray and comb. Allowed the gel to set for a minimum of 20 min at room temperature on a flat surface. Loaded the DNA into separate wells with 10 µl 1kb ladder and 5 µl sample + 5 µl water + 2 µl 6x Loading Buffer. Run the gel for 30 min at 100 V. Exposed the gel to UV light and took photograph. Confirmed the DNA quality.

Agarose gel Electrophoresis: Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, microbial genetics, and clinical chemistry to separate a mixed population of DNA or proteins in a matrix of agarose. The proteins may be separated by charge and/or size (isoelectric focusing) agarose electrophoresis is essentially size independent), and the DNA fragments by length. Biomolecules are separated by applying an electric field to move the charged molecules through an agarose matrix, and the biomolecules are separated by size in the agarose gel matrix. Agarose gels are easy to cast and are particularly suitable for separating DNA of size range most often encountered in laboratories, which accounts for the popularity of its use. The separated DNA may be viewed with stain, most commonly under UV light, and the DNA fragments can be extracted from the gel with relative ease. Most agarose gels was used are between 0.7-2% dissolved in a suitable electrophoresis buffer.

Quantification of DNA: Used 1X TE buffer as a solvent to suspend the nucleic acids (DNA), and placed each sample in a quartz cuvette. Make zero the spectrophotometer reading with buffer. The optical density was set at 260 nm (OD_{260}) with the 50 µg/mL solution of DNA sample. Calculated the OD_{260}/OD_{280} ratio to get indication of nucleic acid purity.

PCR:

Table-2, composition of PCR reaction mixture

S.N.	Component	Volume (µl/25µl reaction)
1.	Double sterile water	12.93
2.	*Taq* buffer	2.5
3.	dNTPs	2.5
4.	Spermidine (0.01M)	1.0
5.	Formamide	0.5

6.	*Taq* polymerase enzyme	0.17
7.	Primer	0.5

Stages of PCR, ISSR PCR protocol: The following PCR protocol has been proposed to amplified collected DNA. (a) Initial denaturation at 94C for 3 min (b) strand separation at 94C for 30 sec (c) annealing at 51C for 45 sec (d) extension at 72 C for 2 min (e) repeated step b to d for 44 times, total no of cycles was 45 (f) final extension at 72 C for 5 min (g) maintained 4 C forever.

DNA samples have been amplified in PTC 200 Thermal Cycler (MJ research, USA) using the selected primers. The amplifications were repeated two to three to ensure the reproducibility. The amplified products were run on agarose gel (1.5%) to obtained a pattern of bands. The gel images were documented using a gel documented system and were also photographed. Only reproducible bands were scored from the photographed.

Scoring of bands: The ISSRs are reliable markers for detecting polymorphism in the difference of the position and number of bands on agarose gel. The degree of polymorphism is expected to vary directly with genetic relatedness of the two varieties compared, implying greater differences in ISSR patterns relatives. A dendrogram constructed based on similarity or differences in band pattern can be used to inter the genetic relatedness as well as the phylogenetic distance between the component varieties.

How to read a gel? One genotype per well is run on the gel. A number of bands are observed at various positions across the whole length of the gel depending on the size of the amplification product. The marker ladder gives an idea about approximate fragment size represented by the band positioned. Thus a single genotype may have region of different length complementary to the primer reflecting in varying fragment size i.e. band on different positions.

Depending on the genetic makeup, each genotype will exhibited a particular pattern of bands on agarose gel corresponding to the genomic regions amplified. If all these genotype are run simultaneously along with a marker ladder (λ DNA/Hind III digest. We have been obtained a pattern for that particular prime used. Each primer is tested for as many times as is needed to obtained the reproducibility in the pattern. Usually ISSR primers give satisfactory results in two or three such profiles.

These profiles are then compared to mark the bands common to both. The results are recorded in such a way that a band is marked as being present (denoted by '+' or the number '1') only if all the profiles show its presence. Otherwise it is scored as being absent denoted by '-' sign or simply by '0'. This is considered due to chance amplification. The representative records are shown below:

Table-3, matrix of similarity among genotypes

Band	Profile 1	Profile 2	Profile 3
1st	Present	Present	+ or 1
2nd	Present	Absent	- or 0
3rd	Absent	Absent	- or -
4th	Absent	Present	- or 0

The above data is tabulated in the following manner;

Genotype	G1	G2	G3
Band Number			
B1	+	+	-
B2	-	+	+
B3	-	-	+

A matrix of similarity between genotype is constructed based on Dices similarity coefficient.

Dice coefficient is calculated as follows:

D= 2*n11/{(2*n11)+n01+n10)}

Here n01= No of bands absents in first genotype and present in the second genotype.

N10= No of bands presents in first and absent in the second genotype.

Similarity values were calculated for each pair of genotypes and the data obtained was subjected to cluster analysis.

Dendrogram: (Cluster Analysis): The highest similarity value is looked for in the matrix the n*n table showing similarity between any two pairs of genotypes, where n is the total no of genotype and the construction of dendrogram is initiated. Formation of clusters is a stepwise process. On xy scale, genotypes are plotted on y-axis while x-axis bears similarities values. Genotypes are jointed at respective similarities values, forming individual clusters, till all clusters are ultimately joined together. Here it should be noted that one does not have to plot all values for all clusters to be jointed in one big cluster. In the present study all these operations were carried out using the software NTSYS pc 2.1.

IV. RESULTS AND DISCUSION

Screening of primers for polymorphism:

Based on the earlier amplification data, a set of 17 primers was selected for amplifying DNAs of all the selected accessions with small pea sized fruits. The results are tabulated in Table 1. Primers showing monomorphic pattern i.e. identical band profiles were not selected for the further analysis. While primers showing polymorphic band profile were selected for the further analysis and to check the reproducibility of amplified products.

Table 4: **Polymorphism data for ISSR Primers**

S.N.	Primer	Repeat	Amplification	Polymorphism
1.	807	(AG)nT	Amplified	Polymorphic
2.	808	(AG)nC	Amplified	Polymorphic
3.	809	(AG)nG	Amplified	Polymorphic
4.	810	(GA)nT	Amplified	Polymorphic
5.	811	(GA)nC	Amplified	Polymorphic
6.	812	(GA)nA	Amplified	Polymorphic
7.	824	(TC)nG	Amplified	----------
8.	825	(AC)nT	Amplified	----------
9.	827	(AC)nG	Not amplified	----------
10.	843	(CT)nRA	Amplified	Polymorphic
11.	844	(CT)nRC	Amplified	Polymorphic
12.	846	(CA)nRT	Amplified	----------
13.	850	(GT)nYC	Amplified	Polymorphic
14.	855	(AC)nYT	Not amplified	----------
15.	857	(AC)nYG	Amplified	Polymorphic
16.	859	(TG)nRC	Amplified	Polymorphic
17	881	(GG)nTG	Amplified	Polymorphic

Codes: D=(A/G/T); H=(A/C/T); V=(A/C/G); Y=C or T; R=A of G.

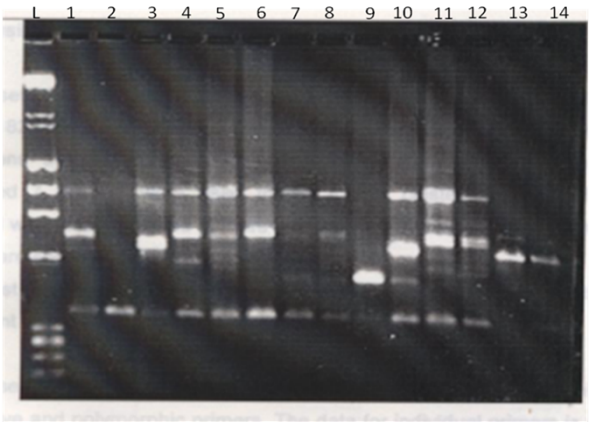

Fig. 1, Amplification profile obtained with ISSR primer 810

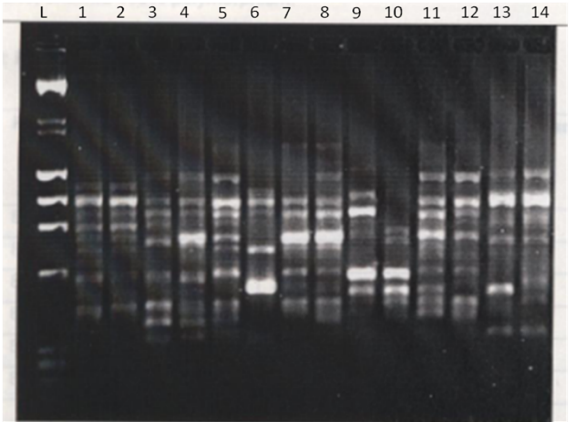

Fig. 2, Amplification profile obtained with ISSR primer 807

Analysis of polymorphism in *Carissa* accessions: A set of 9 polymorphic primers was used to amplify all the 14 DNAs. Two primers 823 and 825 did not have produced scorable amplification with all the accessions and hence were not used further. Finally amplification profiles generated 7 primers were used in the further cluster analysis. The results obtained with these selected primers are shown in the Table and the representative profile is shown in figures as above.

Most of the polymorphic primers contained dinucleotide repeats. This is consistent with the earlier reports in several other plants. Based on the amplification profiles, total 102 bands were obtained by 7 informative and polymorphic primers. The data for individual primers is presented in Table 4. Primer 843 showed the lowest number of bands (5) while the maximum number of bands (22) was obtained with primers 809. All the primers except 843 showed 100% polymorphism.

Table-5; **Polymorphism data for primers selected for the cluster analysis.**

S.N.	Primer	Total no of bands	No of polymorphic bands	Polymorphism (%)
1.	807	17	17	100
2.	808	17	17	100
3.	809	22	22	100
4.	810	21	11	100
5.	843	5	4	80
6.	844	14	14	100

7.	881	15	15	100

Cluster analysis: Based on the scoring data of 177 bands, the similarity matrix was obtained which was further used to derive the dendrogram using UPGMA algorithm. The dendrogram obtained with and Dice coefficient ids shown in figures 5.

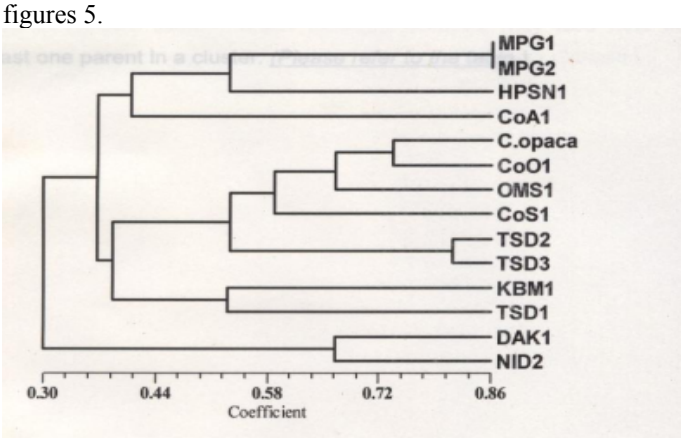

Fig. 3, Genetic relationship between *C. opaca* accessions.

The salient observations based on the cluster analysis:

1. The similarities between the different accessions varied from 30 to 86%.
2. Three main clusters are obtained. Two accession NID2 and DAK1 separated initially from the rest. In the remaining accessions, two main clusters were observed one consisting of four and another of eight.
3. Accessions MPG1 and MPG2 showed maximum 86% similarity incidentally both these accessions were collected from Madhya Pradesh.
4. In the cluster of 8 accessions, two sub-clusters were obtained. First was consisting only 2 and remaining six.
5. Grouping is in the close relation to parentage; the clusters obtained confirm close relation between parents and hybrids. Hybrids are usually grouped together along with at least one parent in a cluster (please refer to Table).

SUMMARY AND CONCLUSION

Genetic relationships in a set of Carissa L. accessions with small pea sized fruits were analyzed using ISSR markers. All the accessions have small pea sized fruits which is characteristic of species *C. opaca*. However, at the genetic level a lot of heterogeneity was observed among the accessions and the similarities varied from 30-86%. The variability needs to be studied further in detailed to understand its genetic basis. Further in depth analysis may also help in resolving the taxonomic ambiguities in species delimitation in genus *Carissa* L.

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