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MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF *CARALLUMA* SPECIES

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Abstract

Caralluma a genus of the family *Asclepiadaceae* is an important medicinal and threatened succulent plant in Pakistan. Ethnobotanically, it is being used either to cure diabetes and fat accumulation or as vegetable in different regions of Pakistan. The main objective of present study was to investigate genetic variations within and between two species of *Caralluma* viz., *Caralluma tuberculata* and *Caralluma edulis* collected from different locations. Random primers OPC series was used for genetic characterization. Ten RAPD markers were used and only 6 of them gave reproducible results with all the primers showing polymorphism (79%) among the samples of both the species. The primer OPC5 showed highest number of bands (89). UPGMA cluster analysis of molecular data showed mixed grouping of samples of both the species and failed to differentiate the species at molecular level. Morphological data of both the species was also recorded for comparison with the molecular data. Based on morphological data the two species of *Caralluma* were clustered into two groups, viz., *C. tuberculata* and the *C. edulis*, while molecular data showed a high level of genetic diversity as both species have shown mixed patterns.

Introduction

Caralluma of the family *Asclepiadaceae* is a genus of about a hundred species, distributed in Africa, Spain, Saudi Arabia, Middle East, Pakistan and India. In Pakistan, it is represented by two species viz., *C. edulis* and *C. tuberculata (*Ali, 1983). *Caralluma* is found in dry regions of the world has paramount medicinal importance and has significant anti-inflammatory and antitumor activity (Deepak *et al.*, 1997; Ramesh *et al.*, 1999; Zakaria *et al.*, 2001). The pregnane glycosides of *Caralluma* have been shown to possess antitumor and anti-cancer activities and in some studies *Caralluma* is reported to protect gastric mucosa and have antiulcer properties (Al-Harbi *et al.*, 1994; Zakaria *et al.*, 2002). The juicy stem of *C. tuberculata* is bitter tonic, febrifuge, stomachic and carminative useful in rheumatism and consumed as vegetable especially when cooked with minced meat (Shinwari *et al*., 2006). The plant had been utilized as a traditional anti- diabetic therapeutic agent equally well in both urban and rural population in Pakistan. It was observed that administration of *C. siniaca* in different doses to healthy animals can cause significant decrease in glucose level (Habibuddin *et al.*, 2008). In another report, it was observed that *C. fimbriata*, can be used in weight reduction (Lawrence & Choudhary, 2004). *Caralluma* species have been used for centuries in semi arid areas of Pakistan as emergency foods (Atal *et al*., 1980). *C. edulis* is known for its anti-diabetic properties (Wadood *et al*., 1989) and other *Caralluma* species for their anti-hyperglyceric activity (Venktash *et al.*, 2003). The extracts of *C. attenuata* and *C. edulis* had hypoglycemic properties and provides synergistic effect in combination with the phlorizin extract which beneficially modify glucose transport, blood and urine glucose levels, blood insulin levels and helps in weight loss. Plants of *C. tuberculata* have been extensively used for paralysis and joints pain and fever (Khan & Khatoon, 2008).

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Plowes (1995) reclassified the genus *Caralluma* and according to him Indian *Caralluma* includes three sub-genera. Besides all this, there exists greater difficulty in identification and nomenclature on this genus worldwide. Many of Indian *Caralluma* species are exhibiting more intermediate forms in their habitat due to their inter hybridizable potency. Gravely & Mayuranathan (1931) have described five varieties under *C. adscendens*. One of these varieties have been elevated to the species level (*C. geniculata*) with the help of molecular systematics. *Caralluma* in *Asclepiadoideae* tribe *Ceropegieae* has been divided into seven genera (Meve *et al*., 2001), although their flowers are so similar in many cases that they cannot be keyed out morphologically (Meve, 2002).

In Pakistan recent morphological characters are supported with various kinds of biochemical and molecular data. Mostly RAPD, RFLP, AFLP etc., were used to study interspecific and intraspecific divergence in Angiosperms. However to higher ranks, DNA sequencing of various genes (*matK, rbcL, rpl16* intron, *rps16* intron, and 3' *trnK* intron) were used to clear taxonomic ambiguities (Shinwari *et al*., 1994a; Shinwari, 1998). Many other members of *Asclepiadaceae* have been genetically characterized by using molecular markers such as *Gymnema sylvestri* R.Br., a natural potential to chemical means of blood sugar regulation (Siddiqui *et al*., 2000). In this particular report, Siddiqui *et al*., (2000), attempted to characterize 18 accessions of *Gymnema*, which were earlier identified as variants based on morphological and biochemical evaluations (Nair & Keshavachandran, 2006) using isozyme and RAPD markers. Study revealed the existence of considerable variations at the molecular level in the *G. germplasm*, which confirms the earlier results based on morphological and biochemical studies. The results could be used for identification of ideal genotypes for extraction of drugs by correlating the molecular fingerprints with desirable morphological and biochemical features. It may also be help full in devising strategies to protect the genetic diversity of this species (Nair & Keshavachandran, 2006).

For efficient conservation and management of such threatened species as *Caralluma* the genetic composition of the species in different geographic location needs to be assessed. Yet there has been no previous report on the use of these methods to characterize the genetic diversity of *Caralluma* species. The objectives of this study was to determine 1) the genetic variation between two species of *Caralluma* and 2) within the individual of each species collected from different locations by using RAPD markers.

Materials and Methods

Plant material: *Caralluma* is distributed in dry areas of Pakistan. Fifteen samples of *C. tuberculata* were collected from Bhawalpur, Karak, Khyber Pass, Mianwali, Nizampur, Quetta and Swabi, while four samples of *C. edulis* were collected from Bhawalpur. The sampling was done in the month of March.

Isolation of genomic DNA: Different methods were used for DNA extraction from fresh leaves. DNA was extracted with the help of modified protocol of Khanuja *et al.,* (1999) and Micheiels *et al.,* (2002). First of all the plant material was washed, dried and weighed (0.6g) and then extracted in a pre-chilled mortar and postel. The plants were crushed in 2% Cetyl trimethyl ammonium bromide (CTAB) and then homogenized material was transferred to eppendorf tubes. The samples were incubated at 65^{o}C for 30 minutes with occasional mixing. An equal volume of phenol was added to an aqueous

DNA sample in eppendorf tube and incubated at 25 ^{o}C overnight period. After overnight incubation the samples were centrifuged at 2500g for five minutes. Supernatant was taken out and treated with 500µl chloroform iso-amyl alcohol. The tubes were inverted gently and then centrifuged at 2500g for 5 minutes. The chloroform iso-amyl treatment was repeated again. The supernatant was treated with chilled iso-propanol and left for 3 hours at 4^{o}C for precipitation of DNA. Then the supernatant was removed and the pellet was washed with 70% ethanol and allowed to be dried. Finally, the pellet was incubated at 37^{o}C in 40µl TE (Tris EDTA) buffer and RNase A to get rid of RNA. The quality and quantity of isolated DNA was determined by gel electrophoresis with 1% agarose in 0.5xTAE (Tris acetate EDTA) buffer, followed by Ethidium bromide staining. The gel was visualized in a gel documentation system (Wealtech Dolphin Doc^{Plus}).

Screening of primers: Random primers (decamers) from OPC series were tested for the amplification of DNA. In total ten primers were used in this study viz., OPC1, OPC2, OPC3, OPC4, OPC5, OPC6, OPC7, OPC8, OPC9 and OPC10. Score able amplification profiles were given by OPC1, OPC5, OPC6, OPC7, OPC8 and OPC9.

PCR amplification with RAPD primers: The PCR reactions were carried out in 25 µl reaction volume containing 20-25ng/µl DNA, 25pM primer, 12.5µl PCR master mix (Fermentas) and 10.5 µl of nuclease free water (Fermentas). The amplification was conducted in a thermocycler (Labnet MultiGene) and programmed for an initial denaturation step of 1 minute at 94°C, followed by 44 cycles of denaturation at 94°C for 30 seconds, primer annealing at 40°C for 1 minute and extension at 72°C for 2 minutes. Final extension was carried out at 72°C for 7 minutes and a hold at 4°C temperature.

PCR products were resolved on 1.5% agarose gel, in 0.5xTAE buffer at 70 V for 3 hours and then stained with ethidium bromide (0.5µg/ml). Gel with amplification fragments was visualized and photographed by using gel documentation system (Wealtech Dolphin Doc^{Plus}).

Data analysis: The amplification profiles of all the samples were compared with each other and presence of band was represented by “1” and absence by “0”. In this way all the amplified profiles from all the primers used were scored and integrated together to form a data matrix for the estimation of genetic distance between the samples collected from different areas. Cluster analysis was performed based on the similarity coefficient between samples based on morphological and molecular data using Numerical Taxonomy and Multivariate Analysis System (NTSYS) pc version 2.01 (Rohlf, 2002).

Results

Morphological analysis: Morphological data of 19 individuals (15 from *C. tuberculata* and 4 from *C. edulis*) was observed. The data was transformed into numerical form and was analyzed by NTSYS. While studying *Caralluma* genus, 20 characters were taken under consideration in which the characters like stem length, stem width, stem surface, stem ornamentation presence of ridges, presence of scars, leaf shape leaf color and leaf type were observed. The plants of *C. tuberculata* showed slight morphological differences such as variation in stem length, stem color, length of leaves but as they were collected from different areas which have different environmental conditions, these changes are exceptional.

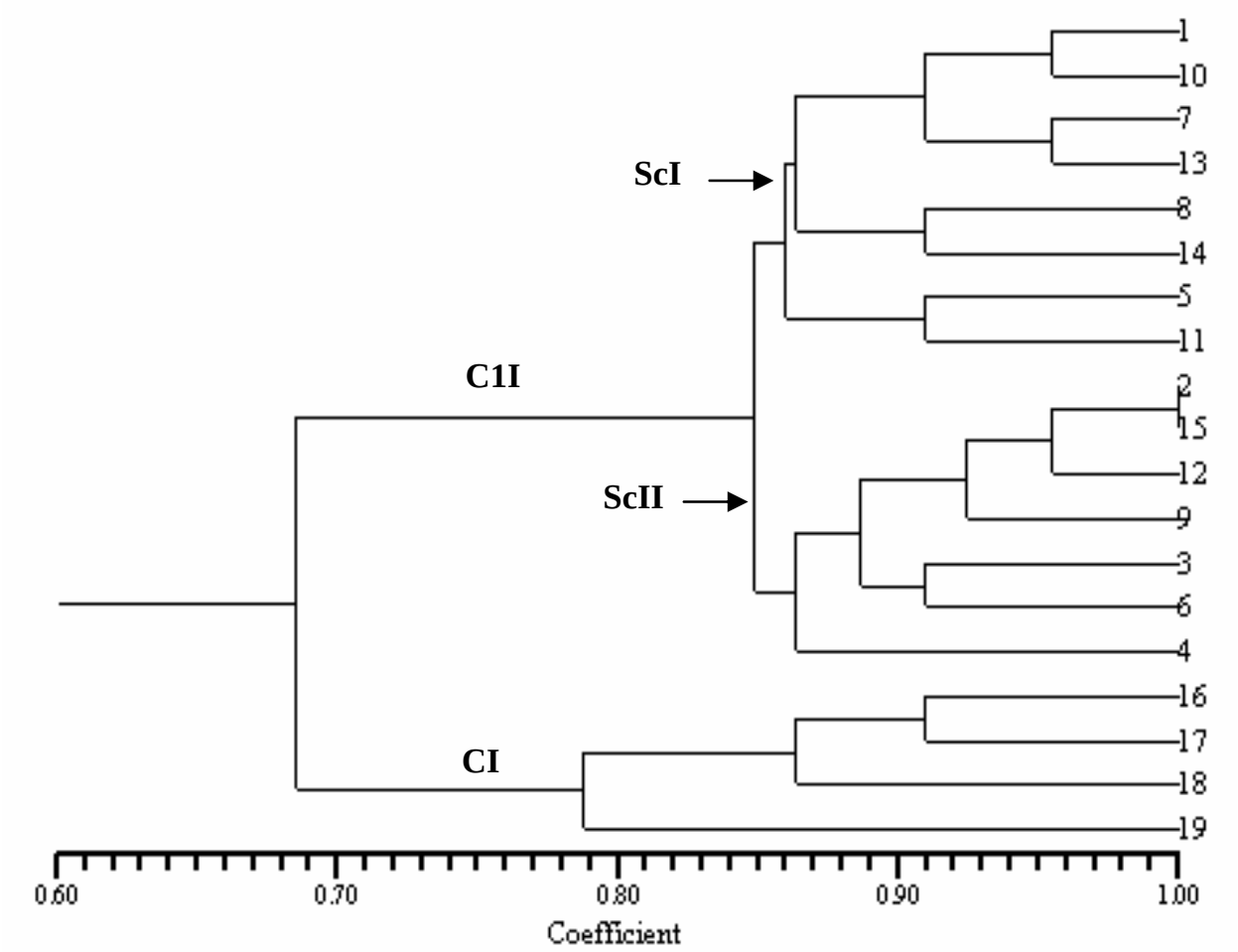

Fig. 1. UPGMA cluster analysis showing morphological diversity among the samples of *Caralluma* species. *C. tuberculata*: 1, 2, 3, 4 (Mianwali) 5, 6, 7, 8 (Nizampur) 9, 10, 11, 12 (Swabi) 13 (Khyber Pass), 14 (Karak) and15 (Quetta). *C. edulis*: 16, 17, 18 and 19 (Bhawalpur).

The NTSYS analysis revealed that the UPGMA cluster can be divided into two clusters, cluster I (CI) and Cluster II (CII) at 69% similarity level. The cluster I had samples of *C. edulis* and cluster II had samples of *C. tuberculata*. Cluster I had four samples (16, 17, 18 and 19) of *C. edulis* collected from Bhawalpur and it was further divided into two sub clusters. Sub cluster I had three individual (16, 17 and 18) while sample number 19 remain unresolved and showed 79% similarity with the other three samples of the same species. Cluster II showed high level of diversity and was further divided into two sub clusters. Sub cluster I (ScI) had eight samples (1, 10, 7, 13, 8, 14, 5 and 11) from various sites. Subcluster1 showed 85% similarity with the sub cluster II (ScII). Sub cluster II was found to contain seven samples (2, 15, 12, 9, 3, 6 and 4) collected from different areas. In the sub cluster II sample number 2 and 15 showed 100% similarity. Morphologically the samples of *C. tuberculata* and *C. edulis* have formed two distinct groups (Fig. 1).

Genomic DNA isolation polymerase chain reaction: High quality DNA was isolated, which was used in PCR reactions and good quality of amplification was observed (Fig. 2). Ten random primers were used for the RAPD analysis, in which only six of them gave satisfactory results. The six random decamer primers (OPC1, OPC5, OPC6, OPC7, OPC8 and OPC9) generated a total of 395 amplified products, out of which 314 were found to be polymorphic and have shown 79% polymorphism. The size of the amplified products was varied from 150 bp to 1200 bp (Table 1). Highest number of bands (89) was

generated by OPC5 and revealed 89% polymorphism, while OPC8 showed highest genetic variation (98%) across the samples. The results showed that all the samples had different banding composition (Table 1).

UPGMA cluster analysis of all primers: Genetic similarities were measured through the analysis of data produced by RAPD primers across the samples of understudied *Caralluma* species. The dendrogram separated the samples into three main clusters, cluster I (CI), cluster II (CII) and cluster III (CIII) at 63% similarity level. Interestingly, cluster I appeared separately from other two clusters (CII, CIII), which have same origin, showing high level of genetic variation. Cluster I had four samples (3, 5, 6 and 9) of same species *C. tuberculata*, while Cluster II had five samples of which four (14, 16, 18 and 19) belongs to *C. edulis* and two samples (13) from *C. tuberculata*. Cluster II showed 68% similarity with cluster III. Cluster III had ten samples, out of which nine (2, 4, 7, 9, 10, 11, 12, 15 and 17) were from *C. tuberculata* and sample number 17 belong to *C. edulis*. Further, Cluster III was observed to be divided into three sub-clusters and had showed high level of genetic diversity. Sub cluster I had two samples (7 and 11) and showed 100% similarity, while sub cluster II had 6 samples in which sample number 9 and 10 appeared in a group with 100% similarity. It was seen that sub cluster III had two samples 1 (*C. tuberculata*) and 17 (*C. edulis*) with 76% similarity level.

Discussion

Caralluma is an important medicinal succulent plant and threatened in Pakistan represented by two species, *C. tuberculata* and *C. edulis*. Nineteen samples of *Caralluma* species were collected from different locations of Pakistan. Fifteen samples belonged to *C. tuberculata* and four representatives of *C. edulis* were analyzed at morphological and molecular level collected from different locations in Pakistan. Morphologically, 20 characters of each sample were studied and the data was transformed to numerical form for cluster analysis through UPGMA. The cluster analysis divided all the samples in to two clusters, which showed clear, splitting up of both the species of *Caralluma*. It has been reported to classify *Caralluma* on the basis of floral characters because flowers had similarity in many cases and it is difficult to key out the taxa morphologically (Plowes, 1995). While Meve & Liede (2002) through molecular based technique supported by stem and leaf characters and keyed out taxa from genus *Caralluma*.

Formerly, morphological analysis and sometimes cytogenetic and chemical analysis were used in order to study the plant diversity (Volis *et al*., 2001). Applying molecular markers and recognition of polymorphic nucleotide sequences dispersed throughout the genome have provided new possibility for evaluating genetic diversity and determining of inter and intra specific genetic relationships (Gostimsky *et al*., 2005). Measurement and characterization of genetic diversity had always been a primary concern in population and evolutionary genetic studies.

The main emphasis of present study was to analyze the genetic diversity at inter specific and intra specific level and differentiation of species at molecular level. RAPD molecular markers used in this particular study, is one of the most popular DNA marker system and highly suitable for quick fingerprinting and for the analysis of genetic relationships. All the primers showed polymorphism (79%) among the samples of both the species. The variations in the number of bands amplified by different primers were influenced by variable factors such as primer structure, template quantity and less number of annealing sites in the genome (Kernodle *et al.,* 1993). It was observed that, amplification through OPC9 produced ten unique bands and could be more informative in classification.

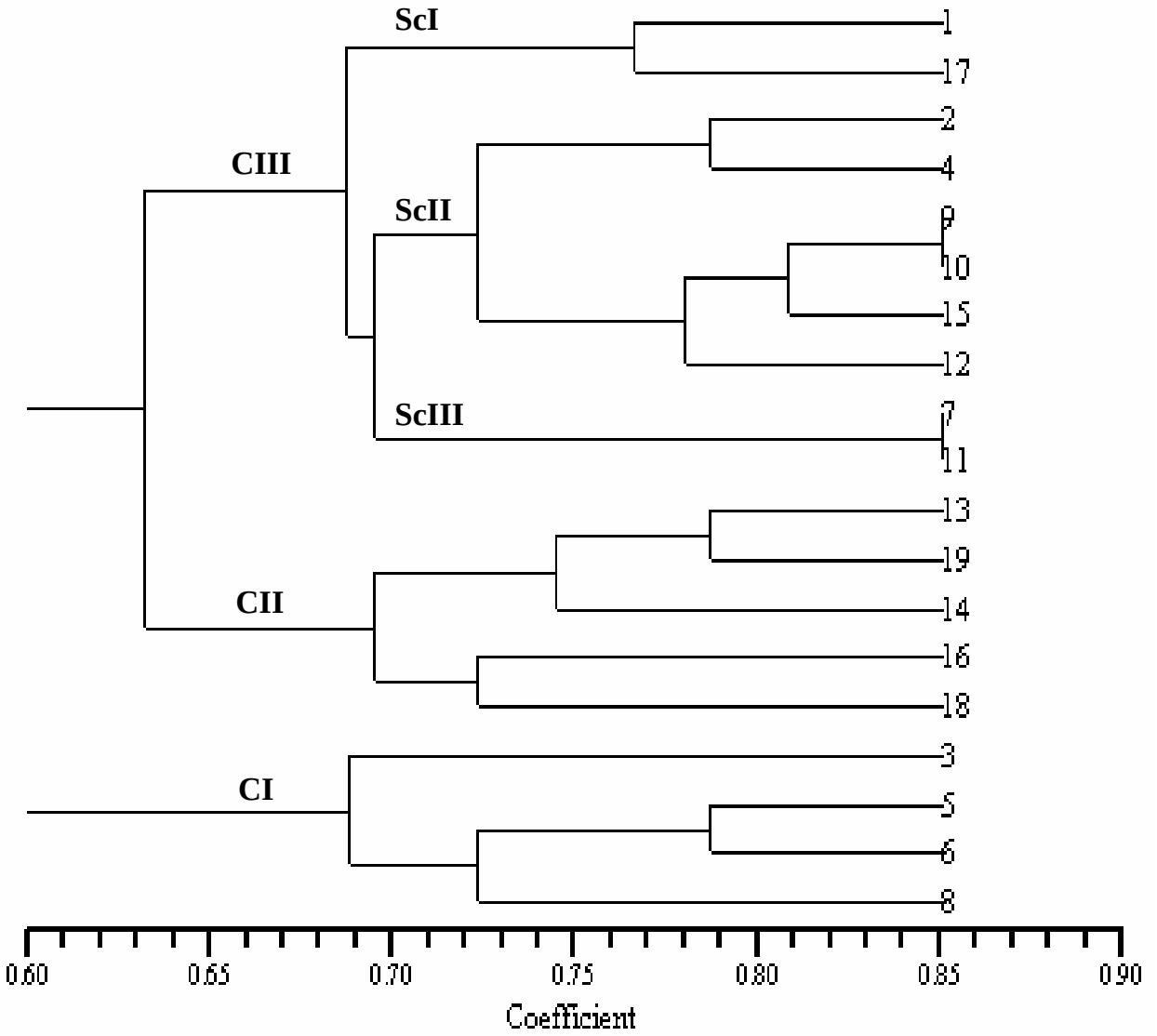

Fig. 2. UPGMA cluster showing diversity among samples of *Caralluma* species by all primers. *C. tuberculata*: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15. *C. edulis*: 16, 17, 18 and 19.

UPGMA cluster analysis of molecular data showed mixed grouping of samples of both the species and failed to differentiate the species at molecular level (Fig. 2). Meve (2002) reported that many of the *Caralluma* species exhibited more intermediate forms in their habitat due to the inter-hybridizable potency. It had also been well documented that geographical distribution and ecological niches exhibit the different genetic characterizations and had strong effects on the organization of diversity (Loveless & Hamrick, 1984).

Conclusion

The results of both morphological and molecular data showed diversity at inter specific and intra specific level. Morphological data grouped two species of *Caralluma* in to two main clusters, whereas at molecular level the samples of both the species showed mixed pattern and demonstrated high level of diversity within the species and between two species. It was also observed that both the species were not clearly differentiated at molecular level by using studied RAPD primers. Both the species are required to be analyzed by using more molecular markers for clear differentiation. Further studies (e.g. *rbcL* sequence data) are needed to clarify ambiguities as earlier reported by Shinwari *et al.,* 1994a and Shinwari *et al.,* 1994b.

Table 1. Percentage polymorphism and size of fragments generated by different RAPD primers.

S. No.	Primers	Total number of bands	Polymorphic bands	% Age of polymorphism	Fragment size (bp)	% Age polymorphism in *C. tuberculata*	% Age polymorphism in *C. edulis*
1	OPC1	69	43	84	200-900	84	57
2	OPC5	89	60	90	300-950	90	74
3	OPC6	37	12	80	250-580	80	75
4	OPC7	61	50	98	220-1000	98	70
5	OPC8	75	60	98	150-550	98	98
6	OPC9	64	35	83	350-1200	83	50
	Total	395	314	79			

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