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Random amplified polymorphic DNA (RAPD) markers reveal genetic homogeneity in the endangered Himalayan species *Meconopsis paniculata* and *M. simplicifolia*

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Abstract Random amplified polymorphic DNA (RAPD) marker-based analysis was carried out to study the extent of genetic polymorphism between populations of the two endangered Himalayan poppy species, *Meconopsis paniculata* and *M. simplicifolia*. Of the 90 primers tested, 38 revealed marked inter-species genetic polymorphism between individuals of the two species from geographically isolated populations. However, intra-species genetic homogeneity was also evident with respect to a number of primers both within and between populations. A comprehensive analysis incorporating data from RAPDs, DNA fingerprinting and isozyme pattern was carried out and, based on the presence or absence of bands, three matrices of similarity indices were estimated. These matrices were subsequently utilized in cluster analysis. In order to compare the three clusters generated using these three different marker systems, a Mantel matrix-correspondence test was carried out on the basis of comparisons of co-phenetic values. The overall representation of relationships by cluster analysis was similar for all three marker systems and this was substantiated by high correlations among the three analyses revealed by the Mantel matrix-correspondence test. Our results point to very low or absence of, genetic polymorphism in *M. paniculata* and *M. simplicifolia*, and are in broad agreement with our previous observations on genetic diversity of *Meconopsis* species which point to a genetic basis for the possible extinction of this economically important genus.

Key words *Meconopsis* species · Himalayan poppy · Genetic diversity · Geographically isolated populations · Cluster analysis · PCR-based genetic markers · RAPD · DNA fingerprinting · Isozymes

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Introduction

Meconopsis paniculata and *M. simplicifolia* are two endangered species of ornamental and medicinal value, confined to specialized habitats and niches at higher altitudes of the Himalayas. Low seed germination and/or seedling recruitment, together with habitat destruction, have threatened and restricted these valuable gene pools to a narrow range of distribution with small population sizes (Sulaiman 1992, 1993).

Isozymes have been used as genetic markers for estimating genetic diversity and evaluating population differentiation. For a number of endangered plant species with small isolated populations such studies showed low genetic variation (Schwaegerle and Schaal 1979; Waller et al. 1987; Crisp 1988). With the advent of molecular biology techniques alternative DNA-based methods (RFLP/DNA fingerprinting) for the detection of polymorphism have been successfully used in demographic studies on natural plant populations, for the estimation of genetic relatedness within populations, and for paternity testing (Weising et al. 1991). Random amplified polymorphic DNAs (RAPDs) serve as better genetic markers for loci within the genome which are not otherwise accessible to RFLP probes due to the presence of repetitive sequences (Williams et al. 1990). In recent years RAPD markers have been extensively used in a number of plant species to solve problems related to genetics, such as clone and cultivar identification, genetic mapping, phylogenetic, pedigree and linkage analysis, population differentiation and the estimation of outcrossing rates (Hu and Quiros 1991; Fritsch and Reissberg 1992; Wilde et al. 1992; Russel et al. 1993; Binelli and Bucci 1994; Campos et al. 1994; Orozco-Castillo et al. 1994). We previously described results based on isozymes and DNA fingerprinting analyses which pointed to the absence of genetic variation, possibly leading to eventual genetic extinction, at both intra- and inter-population levels (Sulaiman 1992; Sulaiman and Babu 1995; Sulaiman and Hasnain 1995; Sulaiman

et al. 1995). In the present communication we describe the use of RAPDs in assessing the extent of genetic diversity among the members of geographically isolated populations of *M. paniculata* and *M. simplicifolia*, and compare the genetic relatedness estimates based on RAPD markers with isozyme analysis (Sulaiman 1992; Sulaiman and Babu 1995) and DNA fingerprinting (Sulaiman and Hasnain 1995; Sulaiman et al. 1995). These results further re-inforce our earlier observations pointing to genetic homogeneity in these species.

Materials and methods

Plant materials

Seeds of *M. paniculata* and *M. simplicifolia* were collected from Sikkim (Eastern Himalaya) and stored at −20 °C. The code for the populations sampled from Sikkim, along with altitude and sampling locality, are described in Table 1.

Genomic DNA extractions

Cold-stored seeds were allowed to germinate (Sulaiman 1993) and high-mlecular-weight genomic DNA was extracted from the 15–20-day-old seedlings following the method of Walbot (1988). DNA was quantitated by spectrophotometery and gel electrophoresis.

Primers

The arbitrary decamer oligonucleotides were obtained from GENOSYS (USA) and used for the amplification of random DNA sequences. The nucleotide sequence of each primer is shown in Table 2.

DNA amplification

The amplification reactions were performed following the protocol of Williams et al. (1990) with minor modifications. The final reaction volume was 25 µl containing 50 ng of genomic DNA, 250 µM each of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim, Germany), 30 ng of primer (GENOSYS, USA), 1 × *Taq* units of polymerase buffer, 0.5 units of *Taq* polymerase (Genei, India) and 2.5 mM of $MgCl_2$. Each reaction mixture was overlaid with 50 µl of mineral oil (Sigma, USA). DNA amplification was carried out for 44 cycles, each comprising de-naturation (1 min, 92 °C), annealing (1 min, 37 °C) and elongation (2 min, 72 °C), in a Perkin Elmer Cetus Thermal Cycler (PCR, Model 4800). One additional cycle of 7 min at 72 °C was used for final extension. The PCR product were analyzed by electrophoresis on a 2.0% agarose gel in 1 × TBE, visualized after EtdBr staining and photographed using a UVP gel documentation system (UK).

Table 1 Seed collection data on 11 natural populations of *M. simplicifolia* and *M. paniculata* from Sikkim Himalaya

Species	Place of collection	Altitude (m)	Population code
M. simplicifolia			
	Kupup	4240	MS1
	Mamenchu Lower	3578	MS2
	Sherathang	4080	MS3
	Thangu	4200	MS4
M. paniculata			
	Nathang	4000	MP1
	Mamenchu Lower	3488	MP2
	Mamenchu Upper	4000	MP3
	Sherathang	3878	MP4
	Thegu	3394	MP5
	Kyangnosla	3090	MP6
	Thangu	3750	MP7

Data analysis

Isozyme analysis (Sulaiman and Babu 1995), DNA fingerprinting (Sulaiman and Hasnain 1995; Sulaiman et al. 1995), and RAPD data (present work) were analyzed separately. A direct comparison among the data was made by estimating the number of loci with allele differences revealed by these three methods. The fragment data were entered in a computer file as binary matrices where 0 scored for the absence of a band and 1 coded for the presence of a band (allele) in an individual sample. Data analyses were conducted using NTSYS-pc, version 1.70 (Exeter Software, New York, USA) Similarities between individuals were estimated using Jaccard's distance coefficients of similarity (Rholf 1992), and the resulting pairwise similarities were expressed as distance matrices. Similarity matrices based on different estimators (Jaccard) and different marker types (isozyme, DNA fingerprinting, RAPD) were compared using the Mantel matrix-correspondence test (Mantel 1967, NTSYS-pc instruction manual). Cluster analyses were carried out on similarity estimates using the unweighted pair group-method arithmetic averges (UPGMA) and the resulting clusters were expressed as dendrograms. In order to obtain estimates of magnitudes of differences among dendrograms, cophenetic values were computed for each dendrogram and a cophenetic matrix for each genetic marker system was constructed. The co-phenetic matrices were compared by the Mantel matrix-correspondence test using the MAXCOMP program in NTSYS-pc (Rholf 1992).

Results and discussion

In the present work 90 primers were tested to study genetic diversity in individuals belonging respectively to seven geographically isolated populations of *M. paniculata* and four of *M. simplicifolia*. GC-rich sequences have been shown to hybridize to various higher-plant genomes (Weising et al. 1991) and generate a large number of amplified bands (Rowland and Levi 1994); hence primers were selected with a GC content ranging from 50–80%. Out of the 90 primers tested, 38 revealed a scorable polymorphism between the two species of *Meconopsis*. To ensure that the amplified bands were reproducible, the 38 primers were used in a second replication. Only those DNA fragments which were consistently amplified in both replicates were considered for analysis. A total of 2617 fragments (860 for four individuals from four geographically isolated populations of *M. simplicifolia* and 1757 for seven individuals from seven geographically isolated populations of *M. paniculata*) were amplified; of these, 2261 fragments showed polymorphism (86.4%). However, the range of the percent polymorphism for these 38 primers varied from 71.4% to 100% (Table 2). The number of amplification products generated by each primer within these two species varied from 2 to 13 (Table 2). The size of DNA bands ranged from 300 bp to 3 kb. A monomorphic DNA banding pattern was, however, observed

Table 2 Primers utilized in RAPD analysis to score polymorphism between individuals representing *M. paniculata* (MP1) and *M. simplicifolia* (MS2)

Primer	Oligo name and nucleotide sequence (5′ to 3′)	Number of amplification products		Number of polymorphic products (c)	Percent polymorphism (c/a + bx100) (d)
		M. simplicifolia (a)	*M. paniculata* (b)		
03	50-03-AGGATACGTG	3	3	6	100.0
05	50-05-CGGATAACTG	5	6	10	90.9
10	50-10-CCATTTACGC	3	5	8	100.0
13	50-13-TACACTAGCG	6	2	7	87.5
15	50-15-CTACTAGGGT	4	3	7	100.0
18	50-18-TTTACGGTGG	4	3	5	71.4
19	50-19-ATGGTGTAGC	6	7	11	84.6
21	50-21-ACGCTACATC	9	10	17	89.4
22	50-22-CGAAACAGTC	11	13	20	83.3
27	50-27-CCTATCCGTT	9	8	15	88.2
28	50-28-GATTGCGTTC	5	9	12	85.7
30	50-30-TGCTGTGAAC	2	6	8	100.0
35	60-05-GTCCTCAACG	10	7	14	82.3
36	60-06-CTACTACCGC	4	3	5	71.4
45	60-35-CTAGCTCTGG	7	6	12	92.3
46	60-36-GTAGCCATGG	3	4	5	71.4
48	60-38-CGATGAGCCC	2	4	6	100.0
50	60-40-CTAGGTCTGC	7	8	13	86.6
51	70-01-CATCCCGAAC	3	7	9	90.0
55	70-05-GAGATCCGCG	4	2	5	83.3
56	70-06-GGACTCCACG	3	8	8	72.7
58	70-08-CTGTACCCCC	5	7	10	83.3
62	70-32-GGACCGACTG	10	12	17	77.2
65	70-35-CATGTCCGCC	5	8	12	92.3
66	70-36-GCACGTGAGG	10	12	19	86.3
67	70-37-CTATCGCCGC	8	4	11	91.6
68	70-38-GAGAGGGAGG	8	5	12	92.3
69	70-39-CCGGGGTTAC	5	4	7	77.7
72	80-02-CGCCCAAGCC	5	10	12	80.0
74	84-04-CGCCCGATCC	5	9	11	78.5
75	80-05-ACCCCAGCCG	5	4	9	100.0
77	80-07-GCACGCCGGA	6	10	14	87.5
78	80-08-CGCCCTCAGC	4	10	12	85.7
79	80-09-GCACGGTGGG	3	5	6	75.0
80	80-10-CGCCCTGGTC	8	8	15	93.7
81	80-31-CACCCTGCGC	2	9	8	72.7
83	80-33-CGCAGTGGGC	4	7	11	100.0
86	80-36-GGCCTCCACG	8	7	14	93.3

within the members of the seven populations of *M. paniculata*, as shown in Fig. 1. As expected, the pattern of DNA bands varied with the primer used. This was also evident in individuals from four different populations of *M. simplicifolia* (Fig. 1). The absence of a scorable polymorphism between populations of a given species was a pointer to genetic homogeneity within these species at the population level. It is important to mention that all these RAPD primers amplified DNA bands of high, as well as low, intensity in both species. However, within a species at the inter-population level the intensity of both types of band were similar. Additionally, many RAPD primers could not generate 100% polymorphism and the position of many band were similar between the individuals of the two species of *Meconopsis*; marked differences were nonetheless observed in the intensity of specific bands (Fig. 1 c).

Although RAPD primers detect regions that are not otherwise scorable by RFLP probes (Welsh and McLelland 1990; Williams et al. 1990), the information content of an individual RAPD marker is very low and, therefore, it is essential to use many of these markers in the assessment of the genetic polymorphism of a species (Williams et al. 1990). Additionally, in a number of plant species, a comparison of isozyme analysis/RFLP and RAPD markers has been carried out and these studies have indicated that RAPDs have provided a level of resolution equivalent to RFLPs for the determination of genetic relationships among genotypes and breeding lines (Hallden et al. 1993; dos Santos 1994; Heun et al. 1994; Thormann et al. 1994; Vierling et al. 1994; Bhat et al. 1995). A few representative patterns of RAPD-based polymorphism is shown in Fig. 2. It is evident that RAPD primers can discriminate between the two species of *Meconopsis* investigated and can, therefore, serve as useful probes for genetic typing.

The present findings are in broad agreement with our earlier data (Sulaiman 1992; Sulaiman and Babu 1995; Sulaiman and Hasnain 1995; Sulaiman et al. 1995) on the low genetic polymorphism of *Meconopsis* species, observed via isozyme and DNA fingerprinting analyses, which may be threatening the survival of this endan-

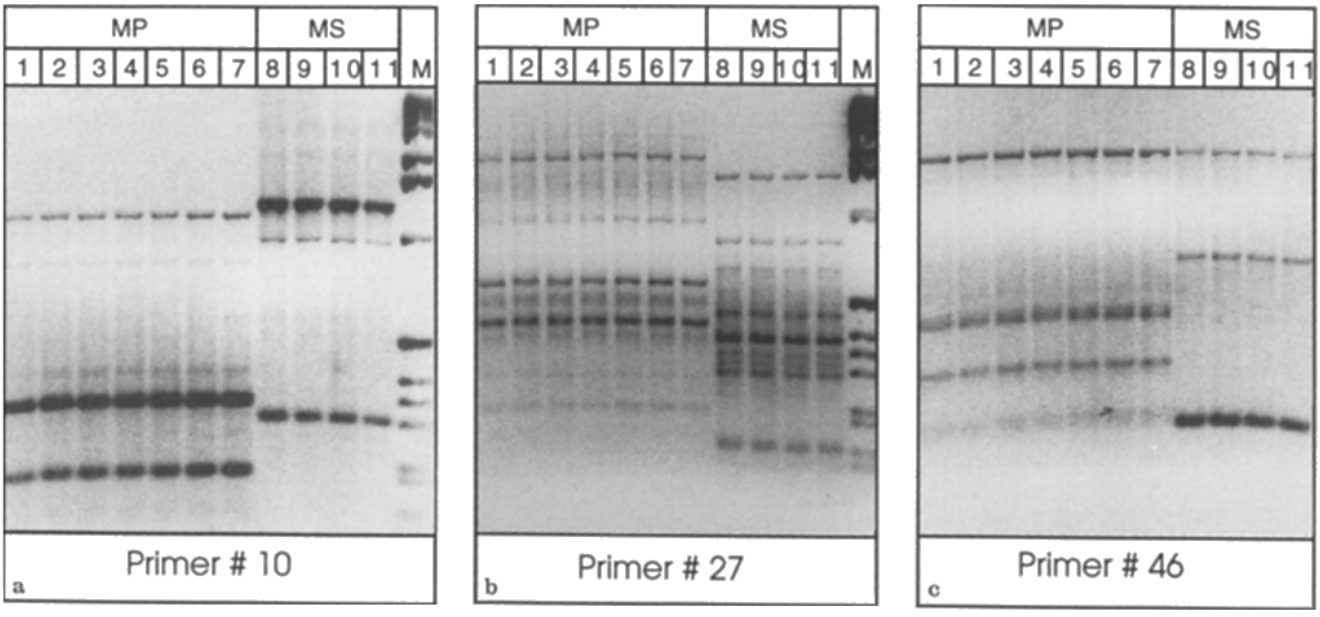
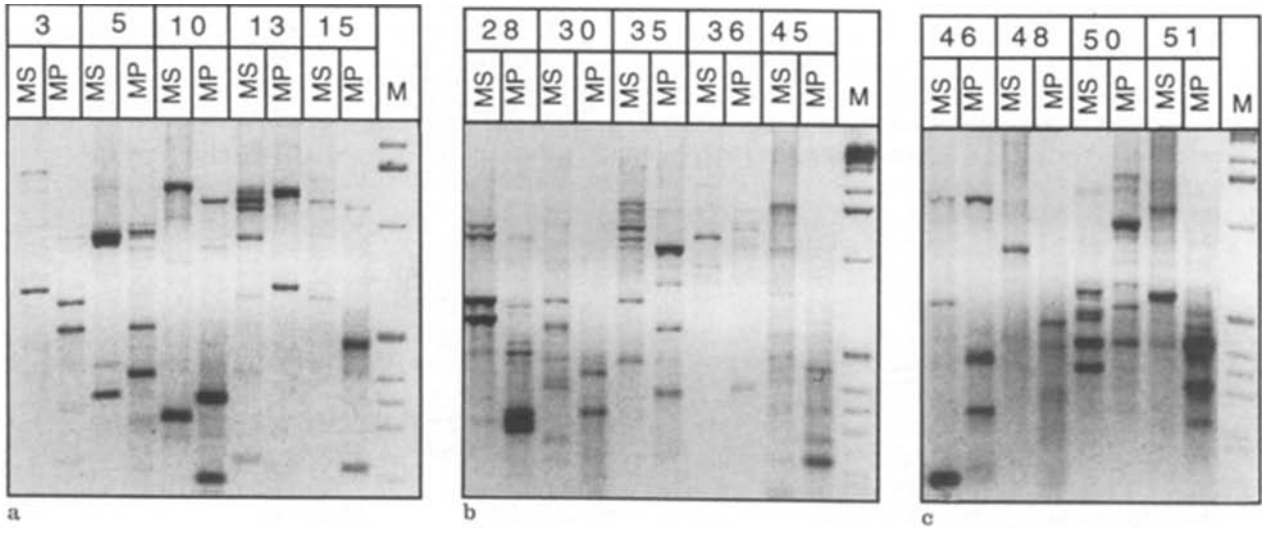


Fig. 1a–c Random amplified polymorphic DNA markers revealed intra-species genetic homogeneity in individuals from different geographically isolated populations of the two endangered Himalayan species, *M. paniculata* and *M. simplicifolia*, with respect to the primers tested. (10, panel **a**; 27, panel **b**; 46, panel **c**). The different lanes represent genomic DNA isolated from individuals from seven geographically isolated populations of *M. paniculata* (*lanes 1–7*) and four geographically isolated populations of *M. simplicifolia* (*8–11*). *Lane 12* represents a 1-kb ladder

gered genus in its natural endemic habitat. A number of isozyme markers revealed a low to zero genetic variation in these *Meconopsis* species both at intra- and inter-population levels (Sulaiman and Babu 1995). Absence of microsatellite-based polymorphism and low recombination in *Meconopsis* was evident using simple repeat $(GT)_n$ and O-chi probes (Sulaiman et al. 1995). An homologous repetitive sequence probe from partial genomic libraries of *M. paniculata* and *M. simplicifolia* also generated data pointing to the absence of genetic variation among the individuals belonging to different geographically isolated populations of *M. paniculata* and *M. simplicifolia* (Sulaiman and Hasnain 1995; Sulaiman et al. 1995).

A direct comparison of the genetic diversity data of *Meconopsis* species generated by the three markers [isozyme analysis of three enzyme systems – acid phosphatase, esterase, glutamate dehydrogenase (Sulaiman and Babu 1995); DNA fingerprinting using probes derived from *M. simplicifolia* and *M. paniculata* partial genomic libraries (Sulaiman and Hasnain 1995, Sulaiman et al. 1995]; and RAPDs (present work)] was made. Data analyses were carried out using Jaccard's coeffi-

Fig. 2a–c A representative RAPD profile generated by a 10-mer primer (Genosys, USA: 3, 5, 10, 13, 15, panel **a**; 28, 30, 35, 36, 45, panel **b**; 46, 48, 50, 51, panel **c**) showing inter-species genetic polymorphism between *M. simplicifolia* and *M. paniculata*. The different lanes represent *M. simplicifolia* and *M. paniculata* genomic DNA isolated from individuals belonging to MS2 and MP1 populations, respectively. *M* 1-kb ladder



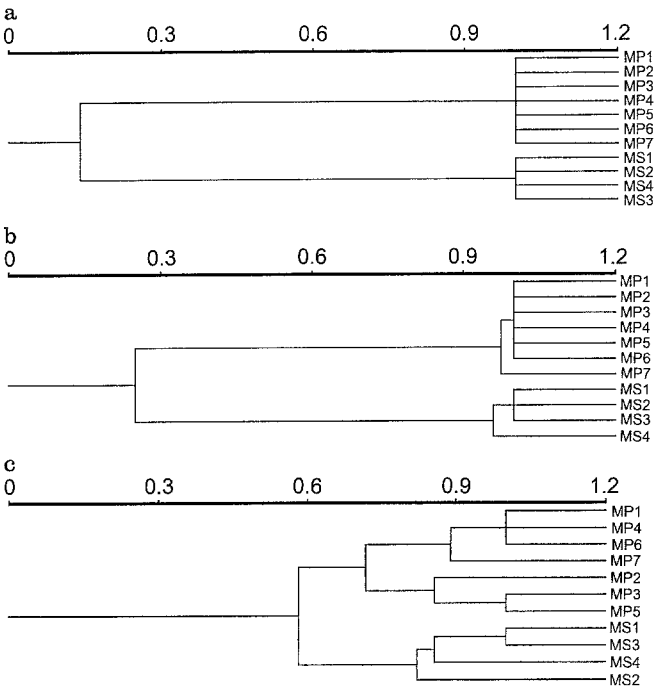


Fig. 3a–c Dendrograms of *M. simplicifolia* and *M. paniculata* populations based on cluster analysis (NTSYS-pc, Rholf 1992) from isozyme data (panel **a**), DNA fingerprinting data (panel **b**), and RAPD data (panel **c**)

cients of similarity (Rholf 1992). These three markers provided independent estimates of genetic relationships by cluster analyses, which are expressed as dendrograms in Fig. 3. For all three marker systems, the dendrograms were essentially similar; the populations of each species grouped in a distinct cluster. The relative positions of populations of a species were also similar in each cluster. However, for isozyme-based cluster analysis, populations of *M. simplicifolia* and *M. paniculata* grouped at relatively lower positions, which is a reflection of the inherent resolution problem with isozyme analysis (Wesing et al. 1991), and is further evident from the detection of very low polymorphism even at the interspecies level (Sulaiman and Babu 1995). A comparison of the similarity matrices obtained for each marker system by the Mantel test was carried out. This statistical analysis is a test for matrix correspondence which takes two matrices, plots one against the other (Mantel 1967), and gives the product-moment correlation (r) and the value of a test criterion (Z) to compare the degree of relationship between two matrices. Rholf (1992) has suggested that the degree of fit can be interpreted as follows: $0.9 \leq r$, very good fit; $0.8 \leq r < 0.9$, good fit; $0.7 \leq r < 0.8$, poor fit; $r < 0.7$, very poor fit. In order to estimate the similarity between dendrograms, a new set of matrices based on the co-phenetic values were constructed and compared. The correlation between matrices of co-phenetic values from the dendrograms based on RAPD vs DNA fingerprinting was very high ($r = 0.9995$). However, comparisons of the dendrograms based on isozyme vs DNA fingerprinting and isozyme vs RAPD resulted in relatively lower correlations ($r = 0.8472$ and $r = 0.8476$, respectively). It is important to recall that the Mantel analysis for the comparison of dendrograms based on the three marker systems employed demonstrated similar genetic relationships at the population level for the two *Meconopsis* species investigated.

While our results document the potential of RAPD markers in the genetic analysis of this endangered Himalayan species of potential horticultural and medicinal value at the intra- and inter- population level, they suggest intra-species genetic homogeneity in geographically isolated populations of *M. paniculata* and *M. simplicifolia*. Low genetic variation is frequently observed in plant species due to their reproductive strategies such as selfing and vegetative propagation (Waller et al. 1978), which is further reduced in small isolated populations (Schwaegerle and Schaal 1979). Similar absence of genetic variation has also been reported in the endangered plant species *Eucalyptus recurva* (Crisp 1988) and *Pedicularis forbishae* (Waller et al. 1987). This may also be one of the reasons for the absence of genetic variation in *Meconopsis*. Experiments are underway to evaluate genetic diversity amongst various other genera of the family Papaveraceae.

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