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# Morphological and molecular characterization of interspecific hybrids of kenaf (*Hibiscus cannabinus* L.) and false roselle (*H. acetosella* Welw. ex Hiern)

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**Abstract**

**Interspecific hybrids of kenaf (*Hibiscus cannabinus*) and allotetraploid wild relative *H. acetosella* were produced in order to introgress desirable traits from wild to cultivated species. Hybrid seed was obtained using either species as pollen donor.The hybrid plants were of intermediate type, with similar morphological features. Molecular characterization using SSR and ISSR markers exhibited high diversity among hybrid plants and could clearly distinguish hybrids from parents. Both ISSR and SSR markers were efficient for hybrid identification and species discrimination.Mean resolving power of the ISSR primers was higher (0.44) compared to SSR markers (0.34), whereas SSR markers exhibited higher allele polymorphism. Cluster analysis and principal component analysis revealed that hybrids formed distinct groups and exhibited high genetic diversity.**

**Key words:** Interspecific hybridization, kenaf, *H. acetosella*, SSR, ISSR

## Introduction

Kenaf (*Hibiscus cannabinus* L.), commonly known as mesta or Deccan hemp in India is an important bast fibre crop having versatile applications. In India, the fibre from kenaf is primarily mixed with bast fibre obtained from jute for making bags, sacks, twines, ropes, cordages and carpets [1]. In the past decades, alternate use of kenaf fibres has increased considerably in automobile interior construction and fibre composite products. Kenaf biomass is consideredas a potential alternative for wood in the paper pulp industry in China, USA and European countries. Biomass from kenaf is also considered a potential source of biofuel and a good substitute for grain ethanol [2].

Although India is producing kenaf for past 200 years, the adoption of this crop has been very limited compared to other fibre crops like cotton and jute. Kenaf is grown in rainfed dry areas of Madhya Pradesh, Andhra Pradesh and parts of Tamil Nadu. Predominant mesta species grown in India is roselle (*Hibiscus sabdariffa*), a distant relative of kenaf. Since the fibres from jute, kenaf and roselle are mixed and are sold as raw jute, separate statistics for area and production of bast fibre from kenaf (*H. cannabinus*) are rarely available. Indian mesta production has been dwindling around 0.2 million tonnes during the past decades, of which about 20% comes from kenaf. The major reasons for lower adoption of kenaf compared to roselle in India may be attributed to lower productivity of kenaf under higher soil and atmospheric moisture characteristic of subtropical environment and higher susceptibility to diseases and pests.

The wild and weedy relatives of crop species carry valuable traits that can help in improving the productivity of cultivated crops. Wide hybridization has been exploited in crop improvement of several food and non-food crops including rice, wheat, potato, tomato, cotton, pulses etc. However, little progress has been achieved in transferring traits from other *Hibiscus* species to kenaf in India. False roselle or African rosemallow (*Hibiscus acetosella* Welw. ex Hiern), a semi-domesticated relative of kenaf is grown as ornamental foliage and for vegetable purpose in European and African countries [3]. It is a semi perennial herb, well-adapted to subtropical environment and resembles kenaf in appearance. It is highly resistant to root knot nematodes, which is a major pest of kenaf and also exhibits good resistance to yellow vein mosaic virus [4]. Previous attempts of interspecific hybridization between *H. cannabinus* and *H. acetosella* have been made for genomeic relationship studies, which indicate that *H.*

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*acetosella* (AABB, 2n = 4x = 72) and *H. cannabinus* (AA, 2n = 2x = 36) share common genome [5]. In the present study, we have attempted hybridization between the two species in order to transfer useful genes from wild to high yielding cultivated kenaf genotypes. Since molecular markers can efficiently detect hybrid plants from self-plants at very early stage of development, genome repeat region specific inter simple sequence repeat (ISSR) and simple sequence repeat (SSR) markers were used to confirm hybridity of the regenerated plants. These markers were also used to investigate the genomic relationship of the species.

**Materials and methods**

Leading *H. cannabinus* varieties HC 583 and MT150 were selected for interspecific hybridization with *H. acetosella* accessions WHIN 20 and WHIN 22. Pure genetic stocks were obtained from National Active Germplasm Site (NAGS) of Jute and Allied Fibres, Central Research Institute for Jute and Allied Fibres, Kolkata, India. The materials were grown in the rainy season of 2009 in 2 m x 2 m plots. Optimal management practices were adopted to develop healthy and robust plants. At the flowering stage, petals and anthers were removed from mature bud (which would flower next day) by forceps for emasculation. Pollination was performed next day around 9:30 AM by dusting stigma of HC583 with *H. acetosella* pollen. Reciprocal crosses were also made. Each pollinated flower bud was further covered with cotton ball to prevent unwanted pollination and tagged properly. The hybrid seeds were first germinated in small plastic pots with a mixture of sand and soil (1:1) and then young seedlings were transferred to field after one week. Morphological features of the hybrid in comparison with parental characters were noted.

Total genomic DNA was extracted from young germinated seedlings (5 days old) from each accession and immature leaves of hybrids by using CTAB method [6] with minor modifications. Extracted DNA was treated with RNAse A (5 µl/ml, 30 min., 37$^{o}$C) after precipitation and purification with isopropanol and ethanol. The concentration of extracted DNA was examined using a spectrophotometer (Eppendorf, Germany). A total of 10 ISSR and SSR markers each were used for hybrid confirmation and genome analysis. PCR amplifications were carried out in a thermal cycler (Biorad, USA) in a total volume of 25 µl (50 ng of template DNA, 0.2 µM of each primer pair, 1XTaq Buffer A, 2.5 mM $MgCl_2$, 0.2 mMdNTPs, and 1.25U Taq DNA polymerase). PCR conditions were set as preheating for 5 mins at 95$^{o}$C (initial denaturation); 35 cycles (denaturation for 45 s at 95$^{o}$C, primer annealing for 1 min at annealing temperature of specific primer pair and extension for 1 min at 72$^{o}$C) followed by a final extension at 72$^{o}$C for 10 min. The PCR products were separated by electrophoresis in a 10% denaturing polyacrylamide gel.In case of ISSR markers PCR mixture contained (100 ng of template DNA, 1µM of each primer, 2.5 mM $MgCl_2$, 0.2 mMdNTPs and 1.25U Taq DNA polymerase). Amplification reaction was carried out using following protocol – one initial cycle (95$^{o}$C for 4 min, 1 min at initial annealing temperature, extension at 72$^{o}$C for 2 min) followed by 43 cycles each (30 s at 95$^{o}$C, 1 min at primer annealing temperature and at 72$^{o}$C for 1 min); followed by a final extension at 72$^{o}$C for 10 minutes.PCR products were then separated in a 1.6% agarose gel in 1x TBE buffer by electrophoresis at 80 volts for 1.5-2 h.

Well resolved fragments were scored as present (1) or absent (0) for each marker locus and set in a binary matrix. Polymorphism information content (PIC) values for SSR primers were calculated for each marker according to Anderson *et al*. [7]. SPI (ISSR primer index) was calculated by summing up the PIC values of all the loci amplified by the same primer following Rajwade *et al*. [8]. Resolving power ($R_p$) values for ISSR and SSR markers were calculated following Prevost and Wilkinson [9]. Mean resolving power $R_p$ was calculated by dividing $R_p$ by number of total bands of each marker. Jaccard's coefficient of similarity was measured and a dendogram based on similarity coefficients were generated through NTSYS-PC by using unweighted pair group method with arithmetical averages (UPGMA) according to SAHN method [10]. Principal component analysis (PCA) was carried out using the 'Eigen' module of NTSYS-PC.

**Results and discussion**

The results of the interspecific hybridization attempted are presented in Table 1. Interspecific hybridization between *H. cannabinus* as female and *H. acetosella* as male resulted in setting of 12 seeds (20.69% per flower). Reciprocal cross resulted in 17.78% seed set per flower. However, considering the number of ovaries present in each flower, the efficiencyof interspecific hybridization was 1.15% for *H. Cannabinus*and 1.27% for *H. acetosella*. A total of 12 interspecific hybrids could be obtained from the harvested seeds. Menzel and Wilson [11] suggested crossing to be more successful taking *H. cannabinus* as female, although in the present study we did not observe significant difference between reciprocal crosses.

**Table 1.** Details on interspecific hybridization of *H. cannabinus* and *H. acetosella*

| S.No. | Cross combination | No. of female genotypes used | No. of male genotypes used | No. of flower crossed | No. of seeds obtained | Seed set per flower (%) | No. of seeds germinated | No. of seedlings survived |
|---|---|---|---|---|---|---|---|---|
| 1. | *H. cannabinus* x *H. acetosella* | 2 | 2 | 58 | 12 | 20.69 | 10 | 9 |
| 2. | *H. acetosella* x *H. cannabinus* | 2 | 2 | 45 | 8 | 17.78 | 5 | 3 |
| | Total | | | 103 | 20 | 19.42 | 15 | 12 |

The interspecific hybrid exhibited characters intermediate of the parental species. The interspecific hybrids exhibited reddish tints in leaf and red patches in the stem and calyx. Detailed characters of the parents and the interspecific hybrids are presented in Table 2. The flowers were light pink with rudimentary anthers. Flower was open, showy with anthers fixed to staminal column and superior stigma. Variation in leaf shape and size were noticed (Fig. 1) in the hybrids. Leaf shape in hybrids also varied with developmental stages.

A total of 32 alleles were amplified by the 10 ISSR primers (200 bp-920bp).The 10 SSR primers amplified 35 alleles with a size range of 80 bpto 850 bp. The ISSR primers BDB $(ACA)_5$, $DDC(CAC)_4CA$, $DHB(CGA)_5$, $DBD(AC)_7$, $BDB(CAC)_5$, $(CCG)_6$ and SSR primers MJM432, MJM467, MJM563, MJM591, MJM606, MJM618 and MJM634 could clearly distinguish the interspecific hybrids from the parents. These primers producing reproducible and clear polymorphism were selected for further analysis (Table 3). Both the SSR and ISSR data sets were combined to obtain the more informative and precise result depicting genetic relationship. Representative ISSR and SSR amplification patterns in four parents and 12 hybrids are presented in (Fig. 2). These markers are thus promising for hybrid characterization and further genetic analysis in segregating generations. The SSR markers exhibited higher allele polymorphism but lower mean resolving power compared to ISSR markers. Per cent polymorphism of ISSR markers varied from 50-67% with a mean of 60.17%. SSR markers revealed wide range of polymorphism (33-100%) with mean polymorphism of 72.43%. Mean PIC value for ISSR markers (0.48) was lower than that of SSR markers (0.54) (Table 3). This indicates high amount of marker polymorphism in the population. Resolving power of the ISSR markers (1.62) was lower compared to SSR markers (1.65). However, when $\overline{R_p}$ values were determined, ISSR markers (0.42) exhibited higher resolvability than SSR markers (0.33), indicating ISSR primers produced more informative bands. The genomic repeat specific markers like ISSR and SSR have also been found to be suitable for genetic diversity analysis, genome association studies and hybrid identification in many other crops due to their high polymorphism and resolving power [12].

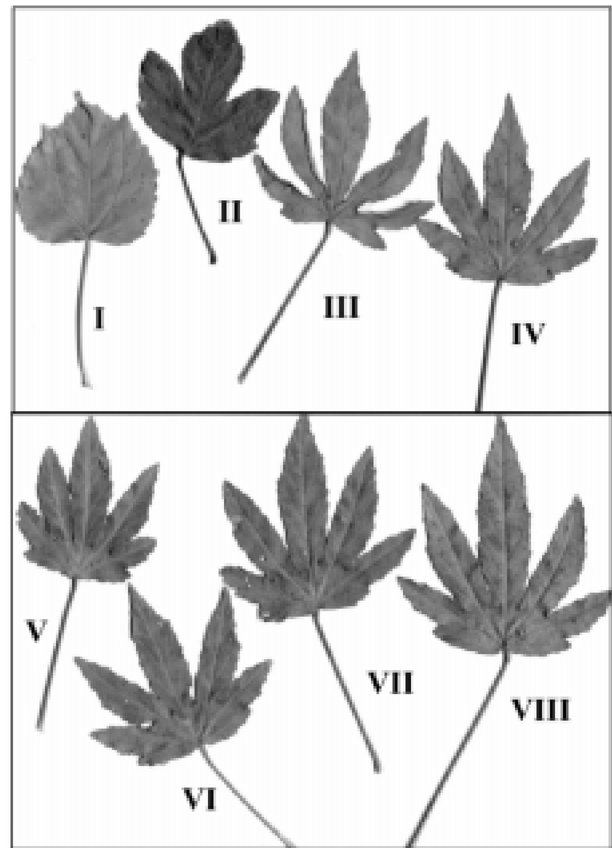


**Fig.1. Comparative leaf morphology of parents and interspecific hybrids. I –*H. cannabinus*, II –*H. acetosella*, III-VIII – interspecific hybrids.**

**Table 2.** Comparative morphological features of parents and hybrids

| S.No. | Morphological features | *H. cannabinus* | *H. acetosella* | Interspecific hybrid |
|---|---|---|---|---|
| 1. | Growth habit | Tall, erect | Tall, semi-erect | Tall, semi-erect |
| 2. | Stem | Cylindrical, surface comparatively smoother, green with few reddish pigmentation | Cylindrical, surface rough, red to deep red colour | Cylindrical, surface smooth, green with few reddish pigmentations |
| 3. | Branching habit | No branching | Branched | Comparatively more branched than parents |
| 4. | Leaf | Green, palmate with almost 1-2 shallow lobes. Leaf marginshave small serrations. | Red, palmate with 2-4 distinct medium deep lobes. Leaf marginshave small serrations. | Green with reddish patches, palmate with distinct 5-6 deeper lobes than parents. Leaf margin serrated with reddish pointed edges |
| 5. | Flower type | Solitary, Complete | Solitary, complete | Solitary, complete |
| | Pollination behavior | Self-pollinated with high rate of cross-pollination | Self-pollinated with occasional cross-pollination | Self-pollination inhibited due to rudimentary anthers. |
| 6. | Calyx | Pergamentaceous, five lobed with green acuminate to subcaudate lobes with prominent nectary gland on each of five midribs. White wooly tomentum present. Small bristles are present on calyx. | Pergamentaceous, blackish red with 10 strong prominent veins, five bearing small nectary glands. Small hard bristles present on calyx. | Pergamentaceous, green with strong prominent nectary glands on each five midrib. Few small bristles present. |
| 7. | Corolla | Five large showy petals with lemon yellow colour | Corolla smaller, pale red with prominent veins running through corolla | Five smaller corolla with light pinkcolour |
| 8. | Androecium | Anthers bilobed, attached to light pink stamina column | Anthers bilobed, attached to deep pink stamina column | Anthers bilobed, attached to light pink stamina column |
| 9. | Gynoecium | Stigma five, pink to light brown colour. Style long, slender, light pink | Stigma light to dark brown, style shorter, deep pink | Stigma five, pink to light brown colour. Style long, slender, light pink |

Jaccard's pair wise similarity coefficient (J) values between hybrids and *Hibiscus cannabinus* ranged from 0.27 to 0.47 with an average of 0.38 showing low genetic association. Interspecific hybrids exhibited higher genetic similarity with the other parent *Hibiscus acetosella* (J = 0.37 to J = 0.59) with an average similarity coefficient of 0.51. Genetic association between *Hibiscus cannabinus* and *Hibiscus acetosella* was low (J = 0.33). The hybrids exhibited high genetic diversity with Jaccard's similarity coefficients ranging from 0.43 to 0.82. Such wide range demonstrates high level of variability present among hybrid plants. Genetic association between hybrids were higher among the hybrid plants than the parents, which was evident from the closer association of the 11 out of 12 hybrid plants developed in the present study. The dendrogram revealed high within-species genetic association for the two species *H. cannabinus* and *H. acetosella* (Fig. 3). At a coefficient value of 0.6, the hybrids formed two distinct groups, suggesting that the distribution of the genomic segments during hybridization of diploid kenaf and tetraploid *H. acetosella* may not be random. Suchnon-random variation among hybridplants has also been observed in interspecific crosses of *Jatropha* species [13]. The result from principal component analysis supported cluster analysis and effectively distinguished the interspecific hybrids from the parental lines (Fig. 4). A total of 16 PCA coordinates were obtained, of which the first four PCA coordinates explained 76.05% of the total variation of the data set. High genetic diversity among the hybrids also suggests

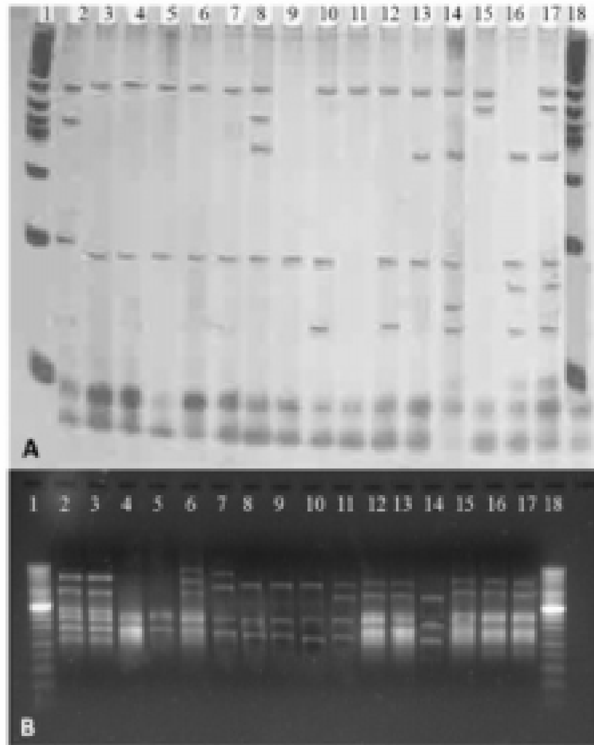

**Fig. 2. Representative ISSR and SSR amplification patterns of the parental lines and interspecific hybrids of *H. cannabinus* and *H. acetosella*.A-amplification pattern of SSR marker MJM 634, B-Amplification pattern of ISSR marker BDB(CAC)$_5$. Lane 1, 18-50 bp DNA ladder, Lane 2, 3 – *H. cannabinus*, Lane 16, 17 – *H. acetosella*, Lanes 4 – 15, interspecific hybrids H1-H12.**

better possibility of removing linkage drag in the segregating generations.

The present study examined prospect of wide hybridization in kenaf for transfer of useful genes from *H. acetosella* and confirmed the genetic identity of the interspecific hybrid. This is the first report of molecular

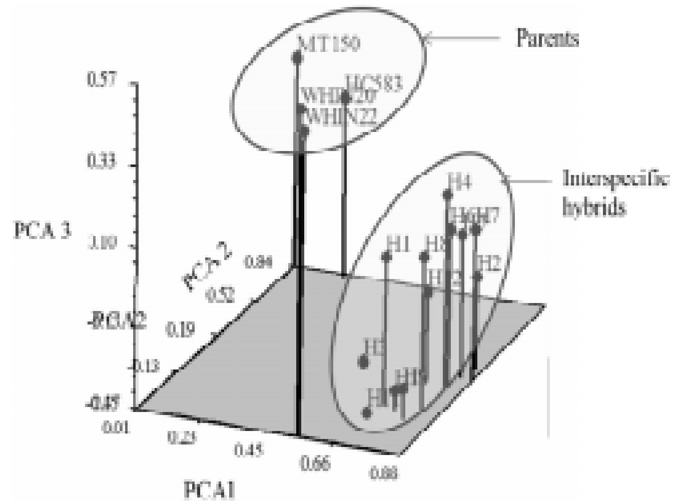


**Fig. 4. The 3-dimensional PCA plot using ISSR and SSR markers.**

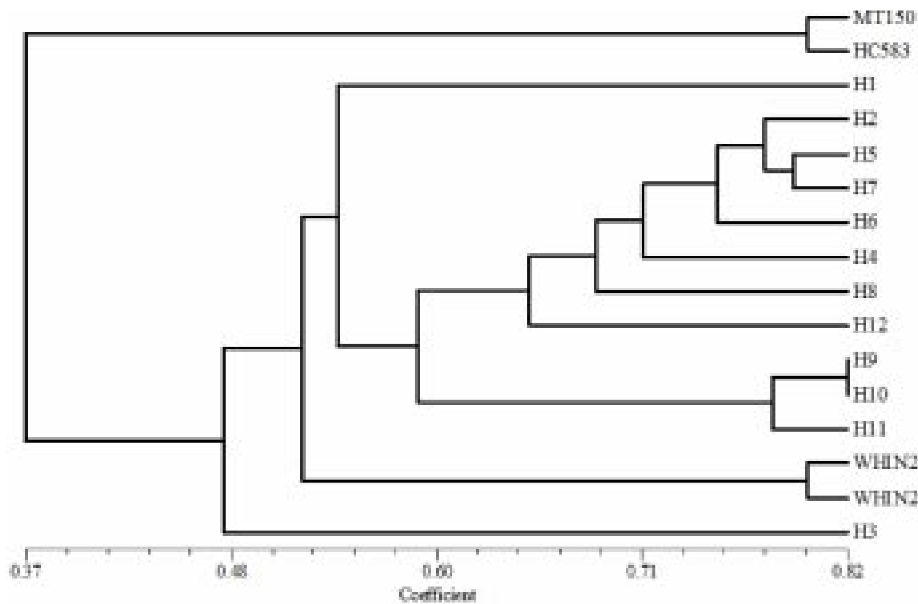


**Fig. 3. Dendrogram generated by UPGMA based on 6 ISSR and 7 SSR markers. HC 583 and MT 150 (*H. cannabinus*) and WHIN 20 and WHIN 22 (*H. acetosella*) are parental lines. H1-H12 represents 12 intersepcific hybrid plants generated in the study.**

**Table 3.** Nucleotide sequences of ISSR primers and information on band polymorphism on 4 parents and 12 interspecific hybrids

| Sl No | Primer designation | Primer sequence | PP | PIC | $R_p$ | Mean $\overline{R_p}$ |
|---|---|---|---|---|---|---|
| 1. | SISSR1 | BDB $(ACA)_5$ | 50 | 0.54 | 1.11 | 0.28 |
| 2. | SISSR2 | DDC $(CAC)_4$CA | 67 | 0.39 | 1.39 | 0.46 |
| 3. | SISSR3 | DHB $(CGA)_5$ | 60 | 0.49 | 2.11 | 0.42 |
| 4. | SISSR5 | DBD $(AC)_7$ | 67 | 0.60 | 1.62 | 0.54 |
| 5. | SISSR6 | BDB $(CAC)_5$ | 67 | 0.63 | 2.86 | 0.48 |
| 6. | UISSR 2 | $(CCG)_6$ | 50 | 0.22 | 0.63 | 0.31 |
| 7. | MJM 618 | F-CGTTATCAAGCAAATCCAACC<br>R-CATCTGGTGACTGCTTCGTCT | 87 | 0.71 | 3.61 | 0.41 |
| 8. | MJM 634 | F-GGAGAATATAAGGCCGCGTAG<br>R-CAGCGGTGTAAGGCTCTCTC | 77 | 0.81 | 3.00 | 0.33 |
| 9. | MJM 563 | F-CTTGGTTGTGGTGGTTGAACT<br>R-AAACCCACCATAGTTGTGTGC | 80 | 0.74 | 1.74 | 0.35 |
| 10. | MJM 606 | F-GGTACTGGTGCATGCTGATTT<br>R-TTCTGTGGAACCTGAGCATCT | 80 | 0.68 | 1.25 | 0.25 |
| 11. | MJM432 | F-CAAGCTTCTGCAGGTATGCTC<br>R-GGACTGAGATGGCAACAGTCT | 100 | 0.23 | 0.32 | 0.32 |
| 12. | MJM467 | F-CATGAATTGAGTGAGCATCCA<br>R-ATCTTCAAGCCCAAATATGCC | 50 | 0.29 | 0.62 | 0.31 |
| 13. | MJM591 | F-TGTCACTTGCCTATGATCGTG<br>R-AAACAACACCATGAACAGCAT | 33 | 0.29 | 0.99 | 0.33 |

B = (C, G, T), D = (A, G, T), H = (A, C, T); PP = percent polymorphism, PIC = polymorphism information content, $R_p$ mean resolving power, Mean $\overline{R_p}$ = mean resolving power.

characterization of interspecific hybrids of *H. cannabinus* and *H. acetosella*. The utility of ISSR and SSR markers in interspecific hybrid characterization, species relation studies and further marker assisted screening in segregating generations is also confirmed by high amplification, marker polymorphism and resolving power.

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