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Original scientific article

RAPD PROFILING IN DETECTING GENETIC VARIATION IN *Glaucium* (Papaveraceae) SPECIES: EDIBLE AND MEDICINAL PLANT

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Glaucium is a genus belonging to Papaveraceae subfam. Chelidonoideae Ernest that contains about 23 species. The distribution of *Glaucium* species relatively widely covers western Asia and the Mediterranean region and is decreased from central Asia to the European countries. As a country, Iran harbors relatively more species of the genus *Glaucium* (10 species) and hence, this country is considered as the hot spot of the genus. No detailed Random Amplified Polymorphic DNA (RAPD) studies were conducted to study *Glaucium* genetic diversity. Therefore, we collected and analyzed three species from 6 provinces of Iran regions. Overall, 60 plant specimens were collected. Our aims were 1) to assess genetic diversity among *Glaucium* species 2) is there a correlation between species genetic and geographical distance? 3) Genetic structure of populations and taxa. We showed significant differences in quantitative morphological characters in plant species. *G. flavum* var. *serpieri* depicted unbiased expected heterozygosity (UHe) in the range of 0.188. The Mantel test showed correlation (r = 0.66, p=0.0001) between genetic and geographical distances. We reported high

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genetic diversity, which clearly shows the *Glaucium* species can adapt to changing environments since high genetic diversity is linked to species adaptability. Present results highlighted the utility of RAPD markers and morphometry methods to investigate genetic diversity in *Glaucium* species.

Key words: Gene Flow, Random Amplified Polymorphic DNA (RAPD), *Glaucium* Species, Isolation, Morphometry.

INTRODUCTION

Glaucium is a genus with roughly 23 species in the Papaveraceae subfamily Chelidonoideae Ernest (KADEREIT, 1993; 1994). According to FEDDE (1909), there are 20 species, ten variants, and one subvariety. MORY (1979) split the genus *Glaucium* into two sections based on fruit dehiscence, morphological and anatomical properties of leaves, stems, seeds, and pollen grains: *G.* sect. *Acropetal* Mory (four species with acropetal dehiscence) and *G.* sect. *Glaucium* (four species with Glaucium dehiscence) (eight species with basipetal dehiscence). The genus is confined to Europe's Atlantic beaches, the Canary Islands, and Mongolia's Altai (MORY, 1979), and it thrives in both dry and open habitats (KADEREIT, 1993; MOBAYEN, 1985). The genus has a chromosomal base number of x=6, pinnate leaves, multicellularity, and terminally uniseriate trichomes, according to KADEREIT (1993). Seeds, on the other hand, are devoid of arils.

Seed and trichome micromorphology is beneficial for taxonomic classification and delimitation at all taxonomic levels and in many plant families in several studies (BARTHLOTT, 1981; SALMAKI *et al.,* 2009; SATIL *et al.,* 2011; SALIMI MOGHADAM *et al.,* 2015; TAVAKKOLI and ASSADI, 2016; ARABI *et al.,* 2017). In Iran, GRAN and SHARIFNIA (2008) looked at the seed ornamentations of 14 *Glaucium* species. Light microscopy (LM) and scanning electron microscopy (SEM) was used to investigate the seeds and trichomes of 15 *Glaucium* species found in Iran (TAVAKKOLI and ASSADI, 2019). *G. oxylobum* and *G. elegans* seeds are semicircular to reniform, whereas *G. oxylobum* and *G. elegans* seeds are reniform and elongated reniform, respectively. Verrucate–rugulate (the most common), verrucate–granulate, verrucate–perforate, verrucate–lineolate, rugulate–granulate, rugulate, and ocellate are the sculpturing varieties of the testa surface. According to their findings, seed and ovary trichome micromorphological traits give helpful and influential details for discriminating species and taxa within species and a diagnostic key to the taxonomy. *Glaucium* taxa's morphological, palynological, and phylogenetical traits were investigated by TAVAKOLI and ASSAD (2019). According to their study, some of these traits, such as micromorphology and the development of clades in phylogenetic trees based on matK and ITS3-6 DNA sequence data, vary across species. The Turkish genus *Glaucium* was separated into two subsections based on DNA research and morphological evidence: Glabrousae and Pubescentae (stem trichomes).

Taxonomy systematics research has already discovered *Glaucium* species. To the finest of our proficiency, no RAPD data on genetic variety research has been uncovered in Iran. A total of 60 models were examined. Our objectives were to 1) assess the genetic diversity of *Glaucium* species and identify genetic diversity. 2) Is there a link between the distance a species travels from its home and its species? 3) Population and taxon genetic structure 4) Is it possible for *Glaucium* species to swap genes?

MATERIAL AND METHODS

Plant materials

Three *Glaucium* species were discovered in Iran's various locations (Table 1). Morphological and molecular approaches were used to investigate these species. Sixty plant models (10-30 each plant species) were studied for morphometry. The number of models used in the random amplified polymorphic DNA study technique was restricted to 60. *G. grandiflorum* subsp. *refractum* (Náblek) Mory, *G. corniculatum* var. *flaviflorum* DC., and *G. flavum* var. *serpieri* (Heldr.) Halácsy were the species studied. All species were recognized based on prior references (TAVAKKOLI and ASSADI, 2016; ARABI *et al*., 2017).

Table 1. List of the investigated taxa including origin of voucher specimens

Taxa	Locality	Latitude	Longitude	Altitude(m)
G. grandiflorum subsp. *refractum* (Nábělek) Mory	Khorassan: Neyshabur, Rood village	37° 07' 48 "	49° 54' 04"	165
G. grandiflorum subsp. *refractum* (Nábělek) Mory	Esfahan: Delijan to Khomein, 20 km Khomein	38 ° 52' 93"	47 °25' 92"	1133
G. corniculatum var. *flaviflorum* DC.	Tehran: Ghum, Veshnaveh village	37° 07' 08"	49°54' 11"	159
G. corniculatum var. *flaviflorum* DC.	Gilan: Ghazvin road to Rasht, 960 m	38 ° 52' 93"	47 °25' 92"	1133
G. flavum var. *serpieri* (Heldr.) Halácsy	Tehran: 4 km N. W. Karaj, mountains of Baraghan	38°52' 93"	47 °25' 92"	1139
G. flavum var. *serpieri* (Heldr.) Halácsy	Golestan: Golestan national park, below Alme	35 °50' 36"	51° 24' 28"	2383

Morphometry

Twenty-nine morphological features were investigated (9 qualitative, 20 quantitative). Five to ten plant specimens were randomly chosen and assessed, or morphological studies were performed (Appendix). Before ordination, the data were converted (Mean=0, variance=1). Plants were categorized and ordinated using Euclidean distance (PODANI, 2000).

Random Amplified Polymorphic DNA

DNA was extracted from fresh leaves. The leaves had been dried before being used. The DNA extraction method was carried out exactly as before (ESFANDANI-BOZCHALOYI *et al*., 2019). The DNA purity was determined using an agarose gel. The DNA was amplified using RAPD primers (Operon technology, Alameda, Canada).

Table 2. RAPD primers and other parameters.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
OPD-02	5′-GGACCCAACC-3′	15	11	88.00%	0.33	6.86	15.88	5.45
OPD-03	5′-GTCGCCGTCA-3′	10	8	84.99%	0.47	7.51	18.43	6.85
OPD-05	5′ -TGAGCGGACA-3′	17	15	93.84%	0.55	2.66	10.33	2.67
OPD-08	5′-GTGTGCCCCA-3′	12	11	94.91%	0.42	4.21	17.50	3.65
OPD-11	5′-AGCGCCATTG-3′	11	9	95.74%	0.57	3.66	19.57	4.37
Mean		12	11	92.77%	0.52	5.9	17.55	4.5
Total		65	55					

Note: TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CBDP primers

Data analyses

We utilized Ward techniques and the Unweighted pair group approach to get the arithmetic mean (UPGMA). Multidimensional scaling and principal coordinate investigation were also used as ordering methods (PODANI, 2000). Investigation of variance was used to identify the morphological differences between species and populations (ANOVA).

RESULTS

Morphometry

Plant species with significant ANOVA results showed variations in quantitative morphological features (P0.01). According to the substantial component study, the findings might explain 60% of the variance. The first PCA component was responsible for 40% of the overall conflict. The length and breadth of the calyx and the form of the leaf were found to have a positive relationship (>0.7) with the size and color of the corolla. Floral features, including the corolla apex, seed length, and the number of segment stem leaves, were detailed in the second and third components. The principal coordinate analysis (PCoA) and the unweighted pair group method with arithmetic mean (UPGMA) plots revealed symmetrical findings (Figure 1). Plant specimens from various species were often isolated due to morphological variations. *Glaucium* species were split into two groups based on morphological characteristics according to the UPGMA tree (Figure 1). The first category included populations of *G. flavum* var. *serpieri*. On the other hand, the second group was divided into two sub-groups. The first subgroup was generated by *G. corniculatum* var. *flaviflorum*. The second subgroup was *G. grandiflorum* subsp. *refractum.*

Glaucium individuals were divided into these categories and sub-groups based on physical distinctions. The use of physical features in identifying and dividing species into discrete groups was also validated by our PCoA findings (Figure not included). The same results were reported in the UPGMA tree (Figure 1).

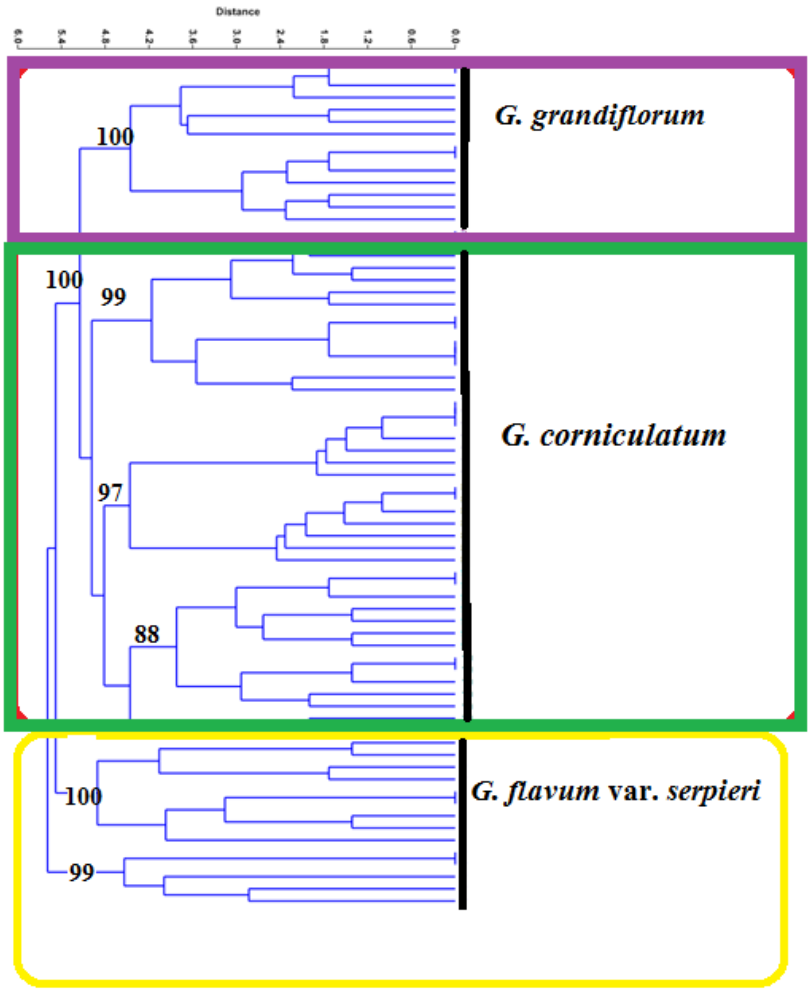

Figure 1. UPGMA clusters of morphological characters revealing species delimitation in *Glaucium* species

Species Identification and Genetic Diversity

Plant DNA (*Glaucium* species) may be amplified using the primers OPC-04, OPB-01, OPA-05, and OPD-11 (Figure not included). A total of 55 polymorphic bands were synthesized and amplified. The products were amplified to a range of 100 to 3000 bp. In OPD-05, we detected the most polymorphic bands. The polymorphic bands in OPD-03 were the smallest. Each primer included an average of 11 polymorphic bands. The polymorphic information content (PIC) ranged between 0.33 (OPD-02) to 0.57 (OPD-03) (OPD- 011). The average polymorphism information content of primers was 0.52.

MI values ranged from 2.67 (OPD-05) to 6.85 (OPD-03), with an average of 4.5 for each primer. Deals of the effective multiplex ratio (EMR) are essential for distinguishing genotypes. EMR readings ranged from 10.33 (OPD-05) to 19.57 (OPD-11) in our research. The average EMR value per primer was 17.55. (Table 2). All three *Glaucium* species' genetic characteristics have been computed and are shown (Table 3). Unbiased anticipated heterozygozygosity (UHe) in *G. flavum* var. *serpieri* was in the range of 0.188. In *G. grandiflorum*, Shannon information was high (0.359). The lowest value, 0.22, was found in *G. flavum* var. *serpieri*. In *G. flavum* var. *serpieri* and *G. grandiflorum*, the observed number of alleles (Na) varied from 1.16 to 1.5. For *G. flavum* var. *serpieri* and *G. corniculatum* var. *flaviflorum*, the adequate number of alleles (Ne) was in the range of 1.078-1.44. In the *Glaucium* species, gene flow (Nm) was relatively low (0.456).

Table 3. Genetic diversity variables of Glaucium species

Taxon	N	Na	Ne	I	He	UHe	%P
G. grandiflorum subsp. *refractum* (Nábělek) Mory	20.000	1.500	1.311	0.359	0.367	0.387	60.00%
G. corniculatum var. *flaviflorum* DC.	30.000	1.500	1.441	0.330	0.233	0.233	50.00%
G. flavum var. *serpieri* (Heldr.) Halácsy	10.000	1.167	1.078	0.022	0.220	0.188	16.67%

(N = number of samples, Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism in populations)

The AMOVA test (Analysis of Molecular Variance) was used to find genetic variations between *Glaucium* species (P = 0.001). According to AMOVA, between-species genetic diversity accounts for 62 percent of all genetic variation. Within the species, there was a lot less variability (17 percent). (Please note that the table is not included.) Genetic similarity and dissimilarity, as measured by Genetic statistics (GST), had D est values of (0.678, p = 0.001) and (0.289, p = 0.001), respectively, indicating substantial differences.

Since the findings of the neighbor-joining tree and the PCoA plot of *Glaucium* populations based on RAPD data were comparable, only the PCoA plot of *Glaucium* populations based on RAPD data is given evaluated. The PCoA plot demonstrated that the three species are highly distinct based on genetic differences. *G. flavum* var. *serpieri* models were positioned widely apart in the UPGMA and PCoA plots. *G. corniculatum* var. *flaviflorum* was detected near G. grandiflorum but not near *G. flavum* var. *serpieri*. In both studies, *G. grandiflorum* was shown to be more closely related to *G. corniculatum* var. *flaviflorum*. The Kimura 2p distance was used to determine the genetic distance between the three species, which was 2.77.

In the *Glaucium* species, gene flow (Nm) was deficient (0.456). *Glaucium* members' genetic identity and phylogenetic distance are investigated (Table 4). Genetically, *G. corniculatum* var. *flaviflorum* and *G. flavum* var. *serpieri* were quite similar (0.924). Due to limited genetic resemblance, *G. grandiflorum* subsp. *refractum* and *G. flavum* var. *serpieri* were shown to be distinct (0.553). The Mantel test revealed a link between genetic and geographical distances ($r = 0.66$, $p=0.0001$).

In the STRUCTURE investigation, Evanno's test revealed $k = 3$. This gene cluster is shown in the UPGMA image above. *G. corniculatum* var. *flaviflorum* and *G. grandiflorum* subsp. *refractum* (differently colored) and *G. flavum* var. *serpieri* show genetic differences in a STRUCTURE plot (Fig. 2) based on $k = 3$.

A similar effect was seen on the plot next door. K-Means and STRUCTURE studies backed up the limited gene flow findings. There was no evidence of significant gene flow among *Glaucium* species that we could find. Because these populations were positioned near together, this matched the grouping we got via Neighbor-joining (Figure not included). According to the STRUCTURE plot based on the admixture model, these common alleles make up a miniscule proportion of the genomes in these populations. These data suggest a high degree of genetic stratification among *Glaucium* species.

Table 4. The Nei genetic similarity (Gs) estimates using RAPD markers

pop1	pop2	pop3	pop4	pop5	pop6	
1.000						pop1
0.666	1.000					pop2
0.560	0.924	1.000				pop3
0.850	0.730	0.827	1.000			pop4
0.774	0.797	0.744	0.794	1.000		pop5
0.553	0.770	0.788	0.755	0.646	1.000	pop6

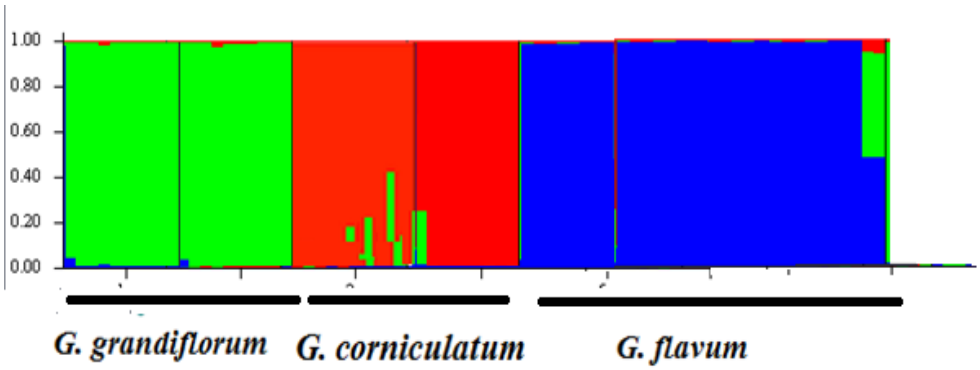

Fig. 2. STRUCTURE plot of RAPD data in *Glaucium* populations studied

The neighbor-joining plot also showed the same result. Limited gene flow results were supported by K-Means and STRUCTURE analyses too. We could not identify substantial gene flow among the *Glaucium* species . This result is in agreement with grouping we obtained with Neighbor- joining (Figure not included), as these populations were placed close to each other. As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise very limited part of the genomes in these populations and all these results are in agreement in showing high degree of genetic stratification within *Glaucium* species.

DISCUSSION

Glaucium is a taxonomic category having several morphological traits that make it challenging to identify and categorize *Glaucium* species (KADEREIT, 1993; SALARI *et al.*, 2013; 2020; JAHANI *et al.*, 2019; ESFANDANI-BOZCHALOYI, *et al.*, 2017, 2018a, 2018b, 2018c, 2018d). Other techniques to improve the central taxonomic methodology must be investigated because of the complexity (ERBANO *et al.*, 2015; MIAO *et al.*, 2018; JI *et al.*, 2020a, 2020b). Plant taxonomists may now use molecular techniques to explore plant groupings thanks to advances in molecular technology (MORY 1979; SHEN *et al.* 2020). Glaucium species' genetic diversity was examined using morphological and molecular methods. To determine genetic diversity and affinity in *Glaucium* species, we mainly employed RAPD markers. Similar patterns emerged from our grouping and ordination techniques. The application and utility of morphological traits in *Glaucium* species were successfully shown using morphometry data. The use of morphological features to distinguish *Glaucium* species was supported by PCoA plot findings. The researchers determined that morphological characteristics such as corolla color, leaf shape, leaf length, stamen location, leaf edge, and corolla length may be used to distinguish the *Glaucium* group. The morphological distinctions between *Glaucium* species were discovered. The divergence, we think, is due to disparities in quantitative and qualitative characteristics.

We describe the morphology and genetic diversity of three *Glaucium* species for the first time. This research aimed to identify diagnostic traits that may be used to distinguish various *Glaucium* species in Iran. As previously stated by KADEREIT (1993), morphological features are a good aid for species identification.

Although various molecular marker techniques have been used to investigate Papaveraceae germplasm, previous studies have either compared the Papaveraceae to other families in the order Papaverales or investigated genetic relationships and diversity within and among populations and a small number of species in the same genus. The levels of study at the interspecific and intergeneric levels have attracted comparatively little attention.

Ten *Glaucium* taxa (TAVAKKOLI and ASSADI, 2019) were studied for morphological, palynological, and phylogenetic properties. Although the majority of the studied species' morphological traits matched those listed in Flora of Turkey (CULLEN, 1966), few of their characteristics were found to be unique. The data from MORY (1979) were compared to the findings of our measurements. The morphological and palynological features were the most comparable in this research. GRAN and SHARIFNIA (2008) established that *G. haussknechtii* and *G. grandiflorum* are similarly based on the investigation of 28 qualitative and 37 quantitative features in a micromacromorphological investigation of 18 *Glaucium* species. The *Glaucium* taxa were split into two groups based on stem hairs, according to TAVAKKOLI and ASSADI (2019). *G. grandiflorum* var. *grandiflorum, G. grandiflorum* var. *torquatum, G. grandiflorum* var. *torquatum, G. grandiflorum* var. *haussknechtii, G. grandiflorum* var. h*aussknechtii, G. grandiflorum* var. *haussknechtii, G. grandiflorum* var. *haussknecht* Phylogenetic research using matK and ITS3-6 DNA sequences revealed that the Glaucium species are divided into two major clades in ML trees, which corresponds to the hairiness of their stems, petal color, and seed testa outline. These two sub-clades had taxa that were likewise consistent with the ovary tubercle. Genetic diversity and population structure in *Glaucium* species identification were investigated using molecular markers (RAPD) and morphometry research. There were genetic differences among all of the species. According to the current findings, isolation and restricted gene flow are the vital deterministic factors that define the *Glaucium* population. We also discovered that the *Glaucium* species has a high amount of genetic diversity, suggesting that it can adapt to changing circumstances since high genetic diversity is associated with species adaptability.

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RAPD PROFIL U OTKRIVANJU GENETSKIH VARIJACIJA KOD VRSTA GLAUCIJUMA (PAPAVERACEAE): JESTIVA I LJEKOVITA BILJKA

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Izvod

Glaucium je rod koji pripada podfamu Papaveraceae. Chelidonoideae Ernest koji sadrži oko 23 vrste. Rasprostranjenost vrsta *Glaucium* relativno široko pokriva zapadnu Aziju i region Mediterana i smanjena je od centralne Azije do evropskih zemalja. Kao država, Iran ima relativno više vrsta iz roda *Glaucium* (10 vrsta) i stoga se ova zemlja smatra vrućom tačkom roda. Nisu sprovedene detaljne studije nasumično pojačane polimorfne DNK (RAPD) za proučavanje genetske raznolikosti *Glaucium*. Stoga smo prikupili i analizirali tri vrste iz 6 provincija iranskih regiona. Ukupno je prikupljeno 60 biljnih primeraka. Naši ciljevi su bili 1) da procenimo genetičku raznovrsnost među vrstama *Glaucium* 2) da li postoji korelacija između genetske i geografske udaljenosti vrsta? 3) Genetička struktura populacija i taksona. Pokazali smo značajne razlike u kvantitativnim morfološkim karakteristikama kod biljnih vrsta. *G. flavum* var. *serpieri* je prikazao nepristrasnu očekivanu heterozigotnost (UHe) u opsegu od 0,188. Mantelov test je pokazao korelaciju (r = 0,66, p=0,0001) između genetičke i geografske udaljenosti. Prijavili smo visoku genetsku raznovrsnost, što jasno pokazuje da se vrste *Glaucium* mogu prilagoditi promenljivim sredinama jer je visoka genetska raznolikost povezana sa prilagodljivošću vrsta. Sadašnji rezultati su istakli korisnost RAPD markera i metoda morfometrije za istraživanje genetske raznolikosti vrsta *Glaucium*.

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