GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Biogeographic pattern of genetic diversity detected by RAPD and ISSR analysis in *Gypsophila* (Caryophyllaceae) species from Turkey

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Genet. Mol. Res. 14 (3): 8829-8838 (2015)
Received November 17, 2014
Accepted March 18, 2015
Published August 3, 2015
DOI http://dx.doi.org/10.4238/2015.August.3.6

ABSTRACT. *Gypsophila* L. is the 3rd-largest genus of Caryophyllaceae in Turkey, a country that includes 60 taxa belonging to 56 species of the 126 recognized in the genus. A total of 35 taxa are endemic to Turkey, with an approximately 60% endemism ratio. In this study, the genetic diversity of 14 *Gypsophila* species from Turkey was analyzed using random amplification of polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers. Sixteen RAPD and 6 ISSR primers produced 132 polymorphic bands. RAPD, ISSR, and RAPD + ISSR primers for the 14 species showed 92.7, 93.8, and 92.9% polymorphism, respectively. Our results indicate that RAPD and ISSR markers are reliable and effective for assessing the genetic diversity of *Gypsophila* species. *Gypsophila* species studied were separated into 2 clusters. Our analysis shows that the clusters correlated with geographic and phytogeographic regions.

Key words: *Gypsophila*; Random amplification of polymorphic DNA; Genetic diversity; ISSR

INTRODUCTION

The genus *Gypsophila* L. is mainly an Eurasian taxon that is present in the northern temperate region of the Old World from the Atlantic to the Pacific, and is mainly found between the latitudes of 30° and 60°. *Gypsophila* is a typical steppe element, and more than a half of the species in this region have a very limited geographic distribution. The *Gypsophila* genus originated in Caucasus, the Transcaucasian region, and the Eastern part of Turkey (Barkoudah, 1962). A total of 126 species in this genus can be found worldwide (Koyuncu et al., 2008; Korkmaz and Özçelik, 2011a). *Gypsophila* L. is the 3rd-largest genus of the Caryophyllaceae family in Turkey. Sixty taxa belonging to 56 species grow naturally in Turkey. Thirty-five of these are endemic to Turkey, with an endemism ratio of approximately 60% (Korkmaz and Özçelik, 2011a). The members of the genus are commonly known as "Coven" throughout the world ("Çöven" in Turkey), and they are very economically valuable. The plant roots are used for different purposes because of their rich saponin content. The extract produced from their roots can be used as a fire extinguisher, gold polisher, cleaner, and softener of delicate fabrics, as well as give crispness to halva. Turkey is known as the center of diversity of *Gypsophila* species, and they are indicators of gypsum areas. These species have a large geographical distribution from 100 to 2800 m altitude in Turkey (Korkmaz and Özçelik, 2011b; Korkmaz et al., 2012). Previous studies (Greenberg and Donoghue, 2011; Martínez-Nieto et al., 2013; Pirani et al., 2014) showed that most *Gypsophila* species form a clade, and the genus appears to be nonmonophyletic. This genus is very interesting from a biogeographic perspective. Although the most *Gypsophila* species are members of the Irano-Turanian elements, the distribution of the species include temperate Eurasia, the Mediterranean, and the Irano-Turanian regions. Thus, they are ideal models for studying both the effects of habitat fragmentation and selection based on genetic diversity.

DNA-based markers have gained attention because they are unaffected by the environment, detectable at all stages of development, and ubiquitous in number, covering the entire genome. These markers provide a large amount of information; they are highly polymorphic and analytically simple. Therefore, they have played an increasingly important role in the identification and measurement of genetic diversity of different plants (Ercisli et al., 2007). Random amplification of polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSRs) have been used to identify genetic variations between plant species because their applications does not require prior information regarding the target sequence in the genome (Khanuja et al., 1998) and require very little starting DNA template (Zietkiewicz et al., 1994). Thus, this technology has been widely used to investigate the genetic diversity and genetic structure of plant species (Singh et al., 2007; Liu et al., 2008; Ding et al., 2009; Ercisli et al., 2009). There have been no reports examining the genetic diversity of *Gypsophila* species. Thus, in the present study, we evaluated the genetic variability among the *Gypsophila* species growing in different phytogeographic regions in Turkey.

MATERIAL AND METHODS

Plant materials

The leaf samples of 14 *Gypsophila* species from Turkey were analyzed in this study. The species examined are *G. germanicopolitana* Hub.-Mor., *G. glomerata* Pall. ex Adams, *G. heteropoda* Freyn & Sint. subsp *minutiflora* Barkoudah, *G. hispida* Boiss., *G. lepidioides*

Boiss., *G. leucochlaena* Hub.-Mor., *G. muralis* L., *G. oblanceolata* Barkoudah, *G. perfoliata* L. var. *perfoliata, G. pilosa* Hudson, *G. pilulifera* Boiss. & Heldr., *G. pinifolia* Boiss. & Hausskn, *G. tuberculosa* Hub.-Mor., *G. venusta* Fenzl. The analyzed plant samples, including 10 representatives of the Irano-Turanian elements, 2 of the Euro-Siberian, and 1 of the Mediterranean Endemism, phytogeographic regions, and numbers and localities of the *Gypsophila* taxa analyzed are shown in Table 1.

Table 1. Taxonomic information of *Gypsophila* species studied.

Taxon name	Phyto-geographic region	Endemism	Record No.	Locality
G. germanicopolitana	Ir.-Tur.	+	Korkmaz:1689	Eskişehir
G. glomerata	Eu.-Sib.	-	Korkmaz:1971	Edirne
G. heteropoda subsp *minutiflora*	Ir.-Tur.	+	Korkmaz:1822	Sivas
G. hispida	Ir.-Tur.	-	Kandemir:7037	Erzincan
G. lepidioides	Ir.-Tur.	+	Korkmaz:1937	Erzincan
G. leucochlaena	Ir.-Tur.	+	Korkmaz:1967	Kütahya
G. muralis	Eu.-Sib.	-	Korkmaz:1454	Tekirdağ
G. oblanceolata	Ir.-Tur.	+	Korkmaz:1881	Niğde
G. perfoliata var. *perfoliata*	-	-	Korkmaz:1640	Sivas
G. pilosa	Ir.-Tur.	-	Korkmaz:1653	Sivas
G. pilulifera	Medit.	+	Korkmaz:1831	Kütahya
G. pinifolia	Ir.-Tur.	+	Korkmaz:1952	Malatya
G. tuberculosa	Ir.-Tur.	+	Korkmaz:1928	Erzincan
G. venusta	Ir.-Tur.	-	Korkmaz:1855	Konya

All plant materials were collected from different parts of Turkey (Figure 1). Plant samples were collected from different localities representing the species. After herbarium studies, scientific names of the plant samples were identified according to Davis (1967), Davis et al. (1988), Güner et al. (2000), and Güner (2012). In addition to determining the taxon names, their phytogeographic regions and the endemic taxa were also determined. Most taxa were Irano-Turanian elements. The number of the endemic taxa was 8 (57%). Herbarium samples from each taxa were deposited in the Erzincan University Herbarium.

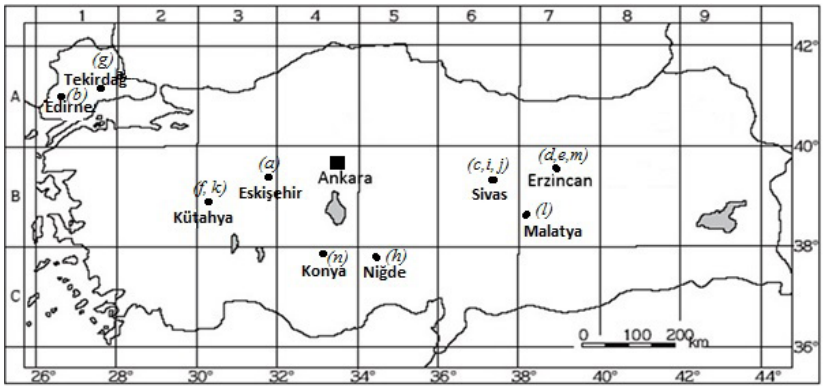

Figure 1. Geographic distribution of studied *Gypsophila* species.

DNA extraction

Genomic DNA was extracted from powdered plant materials using the method described by Li and Quiros (2001). Approximately 1 g tissue samples were ground to a fine powder in liquid nitrogen in 2-mL Eppendorf tubes. Next, 1 mL DNA extraction buffer (100 mMTris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 500 mMNaCl, 20% sodium dodecyl sulfate, and 10 mM 2-mercaptoethanol) was added and mixed well. The mixture was incubated at 65°C in a water bath for 45 min with intermittent shaking at 5-min intervals. The mixture was centrifuged at 12,000 *g* for 15 min at 4°C and the supernatant was transferred into a new 1.5-mL tube. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently. The DNA was precipitated adding 0.6 volume freezer-cold isopropanol and incubated at -20°C for 10 min. The upper phase was discarded after centrifugation for 10 min at 12,000 *g* and -4°C. The pellet was washed with 70% ethanol. The dried DNA was exposed to dry air before being dissolved in 100 µL TE buffer.

RAPD analysis

Samples were screened for RAPD variation using the standard 58-base primers supplied by Eurofins MWG Operon, LLC (Huntsville, AL, USA). The samples were screened for RAPD variations using the standard 10-base primers supplied by Eurofins MWG Operon. A 30-µL reaction cocktail was prepared as follows: 3 µL 10X polymerase chain reaction (PCR) buffer, 1.8 µL 10 mg/mL bovine serum albumin, 1.2 µL 10 mM dNTPs, 1.2 µL 25 mM $MgCl_2$, 3 µL 100 ng/µL DNA, 1.2 µL 5 µM primer, 0.4 µL 5 U/µL *Taq* DNA polymerase (D6677; Sigma, St. Louis, MO, USA), and 17.4 µL water.

ISSR analysis

Twenty-five ISSR primers were screened and 6 primers were selected for further analysis. PCRs were carried out using a single primer in individual reaction in a 25-µL volume containing 25 ng genomic DNA, 0.1 mM dNTPs, 2.5 mM $MgCI_2$, 1 U *Taq* DNA polymerase, 1X Taq buffer, and 0.5 µM primer. Amplification was performed using a thermal cycler programmed for an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 1 min at the specific annealing temperature, and 1 min at 72°C, with a final extension step for 7 min at 72°C.

Electrophoresis

Next, 27 µL PCR products were mixed with 3 µL 6X gel loading buffer and separated by 1.5% (w/v) agarose gel electrophoresis in 0.5X Tris-borate-EDTA buffer at 70 V for 150 min. The gel was stained in ethidium bromide solution containing 2 µL ethidium bromide and 100 mL 1X Tris-borate-EDTA buffer for 40 min and visualized under UV light in a Bio Doc Image Analysis System with Uvisoft analysis package (Cambridge, UK).

Data analysis

PCR products were scored as the presence (1) and absence (0) of a band for each species and the data were then analyzed. The data were used to calculate a Jaccard (1908) similarity index, from which an unweighted pair group method with arithmetic mean dendrogram

was constructed. Dendrograms were produced according to the unweighted pair group mean arithmetic method using the NTSYS-pc ver. 2.02 software (Rohlf, 1998). All experiments in this study were repeated twice. For every primer, 2 PCRs were performed.

RESULTS

RAPD analysis

Fifty-eight primers were screened for their ability to generate consistently amplified band patterns, as well as to assess polymorphisms in the species tested. Among these primers, only 16 had generated reproducible and polymorphic bands. The following primers were used: OPC02, B-20, OPD-16, OPBA-03, OPB-10, OPA-01, OPD-20, OPA-13, OPH-10, OPH-16, OPY-15, OPA-17, OPY-7, OPW-1, OPW-8, and OPW-18. A total of 110 bands were recorded and 102 polymorphisms were detected. A total of 92.7% of the bands were polymorphic. Amplicon sizes ranged from 250 to 3600. Primers OPY-7 presented the highest number of RAPD products (11). Primers OPB-10 presented the lowest number of RAPD products (3) (Table 2).

Table 2. Details of banding pattern revealed by RAPD primers.

Primer	Sequence (5'-3')	Length of amplified bands	No. of bands	No. of polymorphic bands	P (%)
OPC02	GTGAGGCGTC	300-2100	7	6	85.7
B-20	GGACCCTTAC	500-2700	6	6	100.0
OPD16	AGGGCGTAAG	750-3200	5	5	100.0
OPBA-03	GTGCGAGAAC	250-3000	8	7	87.5
OPB-10	CTGCTGGGAC	400-1800	3	3	100.0
OPA-01	CAGGCCCTTC	300-3500	10	9	90.0
OPD20	ACCCGGTCAC	250-2800	6	6	100.0
OPA-13	CAGCACCCAC	400-3000	7	5	71.4
OPH-10	CCTACGTCAG	500-2600	8	7	87.5
OPH-16	TCTCAGCTGG	600-3400	4	4	100.0
OPY-15	AGTCGCCCTT	500-2800	9	8	88.8
OPA-17	GACCGCTTGT	300-2700	8	8	100.0
OPY-7	AGAGCCGTCA	750-2100	11	10	90.9
OPW-1	CTCAGTGTCC	500-2800	6	6	100.0
OPW-8	GACTGCCTCT	800-3600	4	4	100.0
OPW-18	TTCAGGGCAC	250-2750	8	8	100.0
Total		250-3600	110	102	92.7

A dendrogram was constructed according to the RAPD data from 14 *Gypsophila* species, which divided the species into 2 main clusters (Figure 2). The first cluster included *G. glomerata* and *G. muralis*. The second cluster, which included the largest number of species (12 species), was also divided into 2 sub-clusters: the first sub-cluster included *G. pinifolia*, *G. tuberculosa*, *G. lepidioides*, *G. pilosa*, *G. perfoliata*, *G. heteropoda*, *G. leucochlaena*, *G. hispida*, and *G. venusta*. The second sub-cluster included the species *G. oblanceolata*, *G. pilulifera*, and *G. germanicopolitana* (Figure 2). The greatest similarity was observed between the species *G. pinifolia* and *G. tuberculosa* (0.184), and the greatest dissimilarity was observed between the species *G. muralis* and *G. pinifolia* (0. 931).

ISSR analysis

We surveyed 14 *Gypsophila* species using 6 ISSR primers. A total of 32 bands were

amplified with 6 primers, and 30 bands were polymorphic. The percentage of polymorphic bands produced by each primer ranged from 80 to 100%. A total of 93.8% of bands were polymorphic. The primer UBC-827 showed the highest number of ISSR bands (8), while the UBC-818 and UBC-848 primers showed the lowest number of bands (4). In general, the size of amplified DNA fragments scores ranged from 250 to 2800 bp (Table 3).

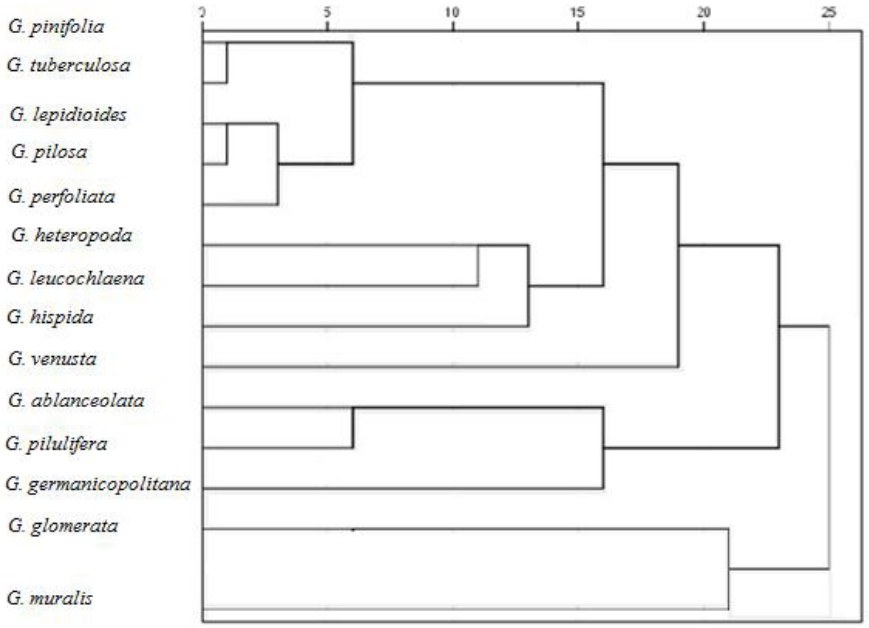

Figure 2. Dendrogram showing the clustering pattern among 14 samples of *Gypsophila* species as revealed by RAPD markers.

Table 3. Details of banding pattern revealed by ISSR primers.

Primer combination	Sequence (5'-3')	Length of amplified bands	No. of bands	No. of polymorphic bands	P (%)
UBC-808	AGAGAGAGAGAGAGAGC	300-900	5	4	80.0
UBC-811	GAGAGAGAGAGAGAGAC	250-1600	6	6	100.0
UBC-818	CACACACACACACACAG	500-1400	4	4	100.0
UBC-825	ACACACACACACACACT	250-1800	5	5	100.0
UBC-827	ACACACACACACACACG	300-2800	8	7	87.5
UBC-848	CACACACACACACACACG	300-1700	4	4	100.0
Total		250-2800	32	30	93.8

Cluster analysis revealed 2 major clusters similar to that detected in the RAPD analysis (Figure 3). *G. glomerata*, *G. muralis*, and *G. germanicopolitana* formed the 1st cluster, while the other cluster included 11 species. The other cluster was resolved into 2 sub-groups: the 1st subgroup included *G. venusta*, *G. pilulifera*, *G. oblanceolata*, *G. heteropoda*, *G. perfoliata*, *G. leucochlaena*, and *G. hispida*, and while the other included *G. tuberculosa*, *G. pilosa*, *G. lepidioides*, and *G. pinifolia.* The genetic distance based on ISSR data ranged from 0.145 (*G. muralis vs G. glomaretaglomerata*) to 0.907 (*G. muralis vs G. tuberculosa*).

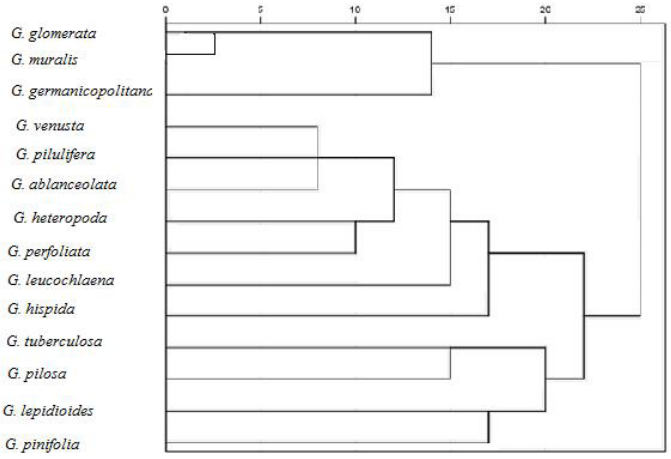

Figure 3. Dendrogram showing the clustering pattern among 14 samples of *Gypsophila* species as revealed by ISSR markers.

Combined RAPD and ISSR analysis

Two different types of markers were employed to assess genetic diversity among 14 *Gypsophila* species. The combined data generated from RAPD and ISSR marker analyses provided 142 bands, 132 bands of which (92.9%) were polymorphic, and a dendrogram was constructed (Figure 3). Fourteen *Gypsophila* species were separated into two 2 distinct clusters, each with containing two 2 species. The 1st cluster included *G. muralis* and *G. glomareta*. The 2nd cluster was further separated into 2 sub-clusters with 12 species. The 1st sub-cluster included *G. lepidioides*, *G. pinifolia*, *G. tuberculosa*, *G. perfoliata*, *G. heteropoda*, *G. pilosa*, *G. leucochlaena*, *G. oblanceolata*, *G. hispida*, and *G. venusta*, and while the 2nd sub-cluster included *G. pilulifera* and *G. germanicopolitana.* The combined analysis revealed that the similarities of the species ranged from 0.168 (*G. pinifolia* and *G. lepidioides*) to 0.892 (*G. pinifolia* and *G. lepidioides*).

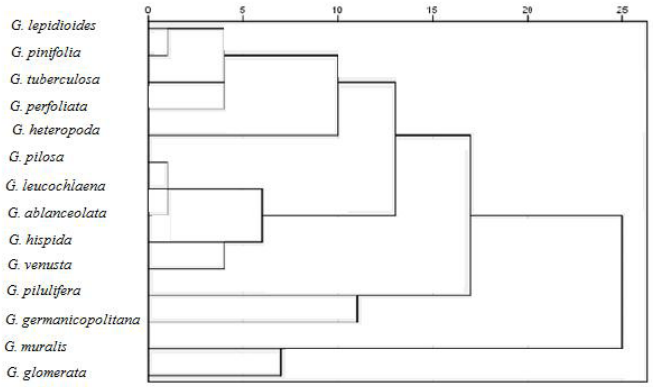

Figure 4. Dendrogram showing the clustering pattern among 14 samples of *Gypsophila* species as revealed by RAPD and ISSR markers.

DISCUSSION

RAPD and ISSR molecular markers have been used to study plant genetic diversity (Liu et al., 2008; Xinjuan et al., 2009; Ramírez et al., 2014). We used PCR-based DNA molecular markers of RAPD and ISSR to analyze the genetic diversity in 14 *Gypsophila* species. The level of polymorphism of ISSR (93.8%) in the *Gypsophila* species was higher than that detected in the RAPD analysis (92.7%). Similar results were reported for turmeric (Singh et al., 2012) and Turkish apricot (Yilmaz et al., 2012), with polymorphism levels of 95.4 and 88% for ISSR and 91.4 and 77% for RAPD, respectively. Other studies have shown that the ISSR marker is a more efficient marker system than the RAPD marker (Goulão and Oliveira, 2001; Fernández et al., 2002; Behera et al., 2008). In previous studies, ISSR markers were used to determine genetic relationships in the Caryophyllaceae family, such as in *Dianthus* (Fu et al., 2008) and *Silene vulgaris* (Egea-Gilabert et al., 2013). Ferrão et al. (2013) reported that the efficiency of a molecular marker indicated a balance between the level of polymorphism and its capacity to identify multiple polymorphisms. Therefore, the ISSR marker is a more efficient technique because of its capacity to reveal a relatively larger number of polymorphisms.

The efficiency of a molecular marker technique depends upon the number of polymorphisms, with greater polymorphism levels indicating greater genetic diversity. RAPD and ISSR molecular markers showed polymorphism levels of greater than 90%. These results indicate high genetic diversity among different species of *Gypsophila.*

Based on the combined data obtained from RAPD and ISSR marker analyses, 14 *Gypsophila* species were separated into 2 major clusters. The genetic distance of *G. glomerata* and *G. muralis* with other species correlated with their different phytogeographic regions, as they are Euro-Siberian elements. *G. pilulifera* is only Mediterranean element of the species studied. Dendrogram analysis based upon different marker systems revealed a relationship between the geographical and phytogeographic distribution of the species. The species originating from similar phytogeographic regions, such as *G. glomerata* and *G. muralis*, were clustered in the same group. Similar results were previously reported for *Curcuma* (Islam, 2004), *Vicia amoena* (Liu et al., 2013), *Lilium* (Lihua et al., 2014), and *Dioscorea* (Ramírez et al., 2014) in studies that described the similarity between genetic relationships and geographic distribution using RAPD and ISSR marker analyses.

Two taxa (*G. glomerata* and *G. muralis*) were Euro-Siberian elements, and only 1 species (*G. pilulifera*) was a Mediterranean element. The region of *G. perfoliata* var. *perfoliata* is unknown because of its wide geographic distribution. Species originating from the same phytogeographic regions were clustered in the same group (*G. glomerata* and *G. muralis*). This may have been observed because of the wide range of ecological and climatic conditions. In this study, we found that RAPD and ISSR markers were powerful tools for fingerprinting and examining the genetic diversity of *Gypsophila* species and that ISSR markers were more informative than RAPD markers.

ACKNOWLEDGMENTS

Some plant samples of this study were collected during the project studies numbered as TUBITAK HD 105T299, TUBITAK 105T147, and EUBAP-2011-10.01.05. We are very thankful for the support provided by the institutions. We also thank our colleague Dr. Ali

Kandemir for collecting plant samples of *G. hispida*.

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