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Genetic Variation in *Hippophae rhamnoides* ssp. *sinensis* (Elaeagnaceae) Revealed by RAPD Markers

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Hippophae rhamnoides ssp. *sinensis is endemic to China, and it is a dioecious, outcrossing plant. Although many studies have been undertaken mainly on its agricultural, nutritional, medical, and ornamental value, little is known about its population genetics. This study uses random amplified polymorphic DNA to investigate the genetic diversity and population genetic structure of 13 natural populations of the subspecies sinensis. Fifteen primers amplified 107 reproducible bands, with 95 (88.79%) being polymorphic. The gene diversity within population was 0.168, considerably lower than that of tree species and most perennial, outcrossing species, but higher than that of annual or short-lived, selfing species. The* Gst *value showed that 18.3% of the total genetic variation resided among populations, a little lower than that of outcrossing species. The present results are quite similar to those previously reported in another subspecies, H ssp. . rhamnoidesrhamnoides. The low genetic differentiation among populations in ssp. sinensis may be attributed to the long-distance dispersal of seeds facilitated by birds, in addition to its characteristics of outcrossing, wind pollination, and widespread distribution. No association between genetic distance and geographical distribution was found. The population relationships revealed by the UPGMA dendrogram parallel this result, in that genetic distance did not increase with geographic separation. This pattern of population differentiation may imply the adaptation of ssp. s populations to the local environment, given that its habitats vary greatly across its distribution.*

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KEY WORDS: *Hippophae rhamnoides* ssp. *sinensis*; RAPD; genetic diversity; population differentiation.

INTRODUCTION

The genus *Hippophae* L., consisting of seven species and eight subspecies (Bartish *et al*., 2002), is a dioecious, wind-pollinated woody plant that reproduces asexually with root suckers and sexually with bird-dispersed seeds. All species in this genus are diploid ($2n = 24$) and are restricted to the Qinghai-Tibetan Plateau and adjacent areas, except *H. rhamnoides* L., which is distributed widely but fragmentally in Asia and Europe (Bartish *et al*., 2002; Lian *et al*., 2000; Rousi, 1971). In *H. rhamnoides*, several subspecies with different geographical distributions have been recognized (Lian *et al*., 2000; Rousi, 1971). *H. rhamnoides* ssp. *sinensis*, a primitive taxon of sea buckthorn (Lian *et al*., 2000; Yao and Tigerstedt, 1993), is endemic to China, growing mainly on sandy soils by riverbanks or along riverbeds, mountain slopes, and valleys. It is found on the eastern edges of the distribution of *H. rhamnoides*, ranging from Qinghai Province in the west to Hebei Province in the east, from Sichuan Province in the south to Inner Mongolia and Hebei Province in the north, with an altitude from 400 to 3900 m (Lian *et al*., 2000).

Hippophae rhamnoides ssp. *sinensis* is an important resource plant in China and exists as a pioneer plant with significant value for water and soil conservation. High diversity has been detected in this subspecies, especially for its fruits, leaves, and chemical components (Lian *et al*., 2000; Rousi, 1971; Zhao *et al*., 1991). Lian *et al*. (2000) recognized different infraspecific types in *H. rhamnoides* ssp. *sinensis*, based mainly on fruit characteristics, and indicated that much of the morphologically recognized variation occurred at 102°–104°E, 35°–36°N, with the altitude ranging from 2400 to 3400 m. During the last decades, many studies have been undertaken on sea buckthorn, concentrating mainly on its agricultural, nutritional, medical, and ornamental value (Eliseev *et al*., 1989; Singh *et al*., 1997; Tian, 1985; Yao and Tigerstedt, 1994). Molecular markers such as allozyme, RFLP, and RAPD have also been used to reveal genetic diversity and relationships in *Hippophae* species (Bartish *et al*., 1999, 2000, 2002; Sun *et al*., 2002; Yao and Tigerstedt, 1993). Although considerable morphological variation has been detected in *H. rhamnoides* ssp. *sinensis*, little is known about its genetic diversity and population genetic structure (see Lian *et al*., 2000).

The RAPD technique has several advantages over isozyme and other DNA markers, including speed, low cost, and the use of small amounts of

plant material. It has therefore been widely used for estimating genetic diversity and relatedness in plant populations (Ge *et al*., 1999; Heum *et al*., 1994; Huff *et al*., 1993). Bartish *et al*. (1999) conducted a RAPD analysis on the genetic variation of *H. rhamnoides* ssp. *rhamnoides*, which is distributed in the northwestern edge of the species distribution. Although 11 populations of *H. rhamnoides* ssp. *sinensis* were included in the study of Yao and Tigerstedt (1993), only six allozyme loci and use of seed sources rather than natural populations provided limited information on the population genetics of this subspecies. In the present study, we used RAPD markers to investigate 13 natural populations from throughout the geographical area of *H. rhamnoides* ssp. *sinensis*. Our specific goals were to (1) determine the extent and pattern of genetic variation within and between natural populations of the subspecies; (2) detect the relationship between genetic distances and geographical distance; and (3) compare the levels of genetic variation with those previously found for the same subspecies (Yao and Tigerstedt, 1993) and for *H. rhamnoides* ssp. *rhamnoides* (Bartish *et al*., 1999). Such information would contribute to a better understanding of the population genetics of this subspecies and facilitate its conservation and utilization as an important plant resource.

MATERIALS AND METHODS

Population Sampling

We sampled 13 natural populations representing the entire distribution area of *H. rhamnoides* ssp. *sinensis*. The 13 populations were divided into three groups according to their geographic regions. Group 1 consists of populations S4, S6, S7, S24, and S28, distributed in the Qinghai-Tibetan Plateau and the adjacent areas. Group 2 consists of populations S8 and S9, in the Liupan Mountains in the central part of the distribution. The remaining populations (S11, S13, S15, S16, S17, and S20) were included in Group 3, representing the populations in the Taihang Mountains in the eastern part of the distribution area. Populations S15 and S16 were collected from Wutaishan in Shanxi but at different altitudes. The locations and sample sizes of these populations are shown in Table I and Fig. 1.

About 20 individuals, including both male and female plants, were randomly sampled from each population at an interval of at least 5 m to prevent collecting ramets from a single individual, except for populations S4, S6, and S7, where samples were collected at 3-m intervals because of their small population sizes. Fresh leaves were harvested individually and dried with silica gel in the field.

Table I. Populations of *H. rhamnoides* ssp. *sinensis* Sampled in This Study

Pop. No.	Sample size	Location	Altitude (m)
S4	13	Chengduo, Qinghai	3900
S6	13	Luhuo, Sichuan	3400
S7	15	Between Songpan and Ruo-er Gai, Sichuan	3620
S8	19	Liupanshan, Ningxia	2360
S9	20	Heshui, Gansu	1380–1410
S11	20	Zhongyang, Shanxi	1300
S13	19	Between Anze and Qinyuan, Shanxi	1280
S15	18	Wutaishan, Shanxi	860
S16	20	Wutaishan, Shanxi	1810
S17	17	Weixian, Hebei	1180
S20	20	Qingshui, Inner Mongolia	1350
S24	20	Hezuo, Gansu	3150
S28	18	Qilianshan, Qinghai	2900

DNA Extraction and PCR Amplification

DNA extraction followed Ge *et al.* (1999) with minor modification according to the present material. Dried leaves were ground to a fine powder in a 1.5-mL Eppendorf tube, and then mixed with 750 μL of preheated 2 × CTAB extraction buffer containing 0.5% mercaptoethanol. The homogenate was incubated at 65°C for 30 min prior to adding 750 μL of chloroform:isoamyl alcohol (24:1, v/v). After mixing by inversion for 5 min the mixture was centrifuged at 10,000 *g* for 5 min at room temperature, and the supernatant was mixed with 2/3 volume ice-cold isopropanol. The DNA was recovered by centrifugation, washed with 500 μL of 70% ethanol and preserved in 100 μL of 1 × TE buffer. PCR amplification used the same system as Qian *et al.* (2001). Fifteen arbitrary RAPD primers that could amplify reproducible and clear DNA bands were selected from 136 primers (obtained from Sangon). DNA amplification was performed in a Rapidcycler 1818 and 1605 (Idaho Tech). Bands obtained from the two Rapidcyclers had been compared and were confirmed to be identical. Amplification products were resolved by electrophoresis on 1.5% agarose gel stained with ethidium bromide in 0.5 × TBE buffer and were imaged on Bio-Rad imaging devices (Gel Doc 2000 Gel Documentation System) supported by Quantity One (version 4.2). Molecular weights were estimated using a 100–3000 bp DNA ladder.

RAPD Analysis

Amplified DNA fragments were scored by presence (1) or absence (0) for each DNA sample, which formed a matrix of the RAPD phenotypes. The bands smaller than 300 bp or larger than 2000 bp as well as the faint bands were not used in the analysis because they were not stable. Bands of

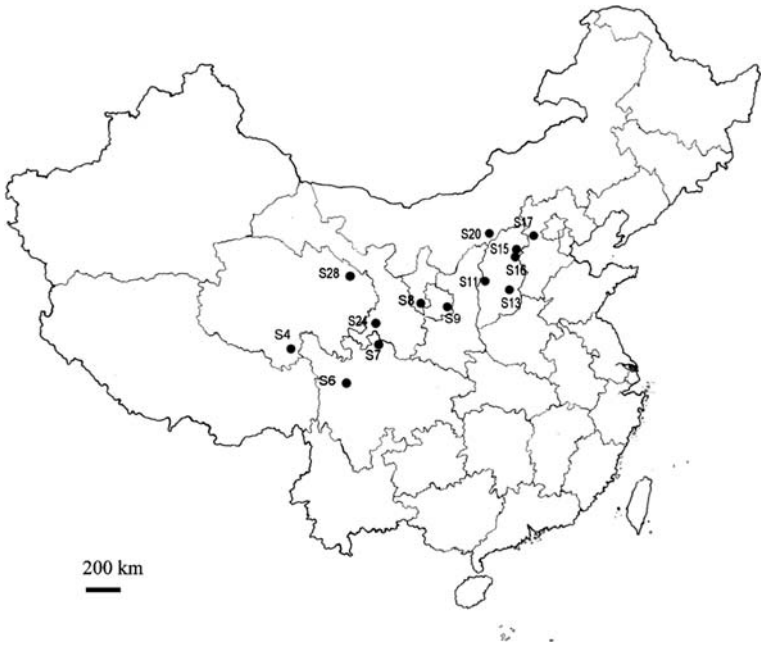

Fig. 1. Localities of 13 populations of *Hippophae rhamnoides* ssp. *sinensis* sampled in this study.

identical size amplified with the same primer, regardless of intensity, were considered to be homologous. The matrix was analyzed using the computer program PopGene (Yeh *et al.*, 1997) with the following genetic parameters calculated: the percentage of polymorphic bands (PPB), Nei's gene diversity (*h*), Shannon's diversity index (*I*), population gene diversity (*Ht*), subpopulation gene diversity (*Hs*), and subpopulation differentiation (*Gst*). These parameters were all calculated at population, group, and subspecies levels.

In addition, Nei's unbiased genetic distance matrix (Nei, 1978) was used to cluster populations by PopGene. A matrix of Nei's genetic distance was used to cluster the populations by the unweighted pair group method with arithmetic averaging (UPGMA) using SAHN in NTSYS.

RESULTS

Genetic Diversity

In total, 107 bands were generated from 232 individuals from the 13 populations using 15 primers. The number of bands amplified by each pair

of primers varied from 5 to 10, with an average of 7.1 bands per primer. Of the total 107 bands, 95 (88.79%) were polymorphic, with 6.3 polymorphic bands per primer on average. The size of the amplified fragments ranged from 300 to 2000 bp, but most were from 300 to 1300 bp. Examples of the polymorphism detected with primers S429 and S506 are shown in Fig. 2.

Four parameters measuring genetic diversity for each population and each group are shown in Table II. These genetic parameters varied considerably across populations. The PPB value within populations varied from 44.86% (population S13) to 66.36% (population S16), and the effective number of alleles, Nei's gene diversity, and Shannon's index showed that population S6 maintained the highest diversity (n_e = 1.374, h = 0.223, I = 0.335). The four parameters all indicated that population S13 exhibited the lowest levels of variability (PPB = 44.86%, h = 0.125, I = 0.197). The total means of the four parameters at the population level were PPB = 55.81%, n_e = 1.274, h = 0.168, and I = 0.259.

At the subspecies level, the PPB was 88.79%, n_e was 1.321, h was 0.204, and Shannon's index was 0.325. At the group level, Group 1 was the highest (PPB = 82.24%, n_e = 1.328, h = 0.206, I = 0.325) and Group 2 was the lowest (PPB = 71.03%, n_e = 1.286, h = 0.178, I = 0.281), with the group means of PPB = 77.26%, n_e = 1.310, h = 0.193, and I = 0.304.

Genetic Divergence among Populations

Population differentiation is shown in Table III, with 18.3% of genetic variation found among populations (*Gst* = 18.3) at the subspecies level. At the group level, the *Gst* values were 16.1%, 9.8%, and 15.2% for groups 1,

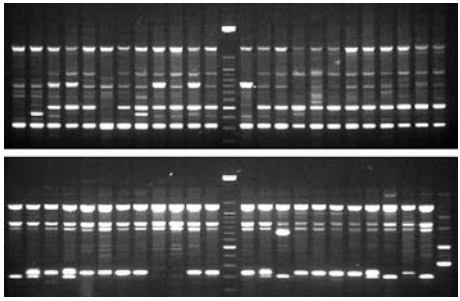

Fig. 2. RAPD amplification products generated from *Hippophae rhamnoides* ssp. *sinensis* genomic DNA: (top) obtained with primer S429; (bottom) obtained with primer S506.

Table II. Genetic Parameters for 13 Populations of *H. rhamnoides* ssp. *sinensis*

Population	PPB (%)	n_e	h	I
Group 1				
S4	57.94	1.292	0.178	0.275
S6	64.49	1.374	0.223	0.335
S7	50.47	1.264	0.160	0.244
S24	53.27	1.268	0.163	0.251
S28	57.01	1.253	0.158	0.248
Group 2				
S8	53.27	1.230	0.145	0.229
S9	58.88	1.290	0.174	0.269
Group 3				
S11	53.27	1.270	0.164	0.252
S13	44.86	1.192	0.125	0.197
S15	57.01	1.299	0.179	0.274
S16	66.36	1.324	0.198	0.307
S17	54.21	1.261	0.161	0.250
S20	57.94	1.266	0.163	0.255
Total mean	55.81 (5.4800)	1.274 (0.0425)	0.168 (0.0231)	0.259 (0.0330)
Subspecies	88.79	1.321	0.204	0.325
Group 1	82.24	1.328	0.206	0.325
Group 2	71.03	1.286	0.178	0.281
Group 3	78.50	1.315	0.195	0.307
Group mean	77.26 (5.7075)	1.310 (0.0213)	0.193 (0.0143)	0.304 (0.023)

Note: PPB, Percentage of polymorphic bands; n_e, Effective number of alleles; h, Nei's (1978) gene diversity; I, Shannon's information index.

2, and 3, respectively. It is obvious that genetic differentiation was significantly higher in groups 1 and 3 than in Group 2 (Table III).

The UPGMA phenogram based on Nei's unbiased genetic distance matrix is shown in Fig. 3. It is obvious that populations S6 and S13 are distinct, while the remaining populations form three clusters. The first cluster consists of populations S4, S24, and S8; the second includes populations S7 and S28; and the last one of populations S9, S11, S15, S16, S17, and S20. However, the populations from some groups occur in different clusters. To investigate a possible correlation between genetic relationships and geographic distances, we compared Nei's unbiased genetic distance matrix with a corresponding geographic distance matrix. The two matrices were not significantly correlated ($r = 0.38$, $P = 0.997$).

DISCUSSION

Genetic Diversity

Genetic diversity and population genetic structure of *H. rhamnoides* have been investigated recently using molecular markers such as allozyme and

Table III. Coefficient of Gene Differentiation

	Ht	*Hs*	*Gst*
Subspecies	0.206 (0.0281)	0.169 (0.0180)	0.183
Group 1	0.210 (0.0299)	0.176 (0.0206)	0.161
Group 2	0.177 (0.0315)	0.160 (0.0255)	0.098
Group 3	0.195 (0.0317)	0.165 (0.0215)	0.152

Note: Ht, population gene diversity; *Hs*, subpopulation gene diversity (standard errors in parentheses); *Gst*, coefficient of gene differentiation.

RAPD (Bartish *et al*., 1999, 2000; Yao and Tigerstedt, 1993). Based on 6 isozyme loci, Yao and Tigerstedt (1993) studied the genetic diversity of 11 populations of *H. rhamnoides* ssp. *sinensis* and found low levels of genetic diversity at the population level, with an *h* value of 0.117. In their RAPD study on 10 natural populations of ssp. *rhamnoides* distributed in Europe, Bartish *et al*. (1999) found that 89.7% and 55.2% of the scorable markers were polymorphic at the subspecies and population levels, respectively, and that the within-population gene diversity (*h*) was 0.159. In the present study on *H. rhamnoides* ssp. *sinensis*, 88.78% and 55.81% polymorphic bands were detected at subspecies and population levels, respectively. Nei's gene diversity varied from 0.125 to 0.223 in different populations, with an average

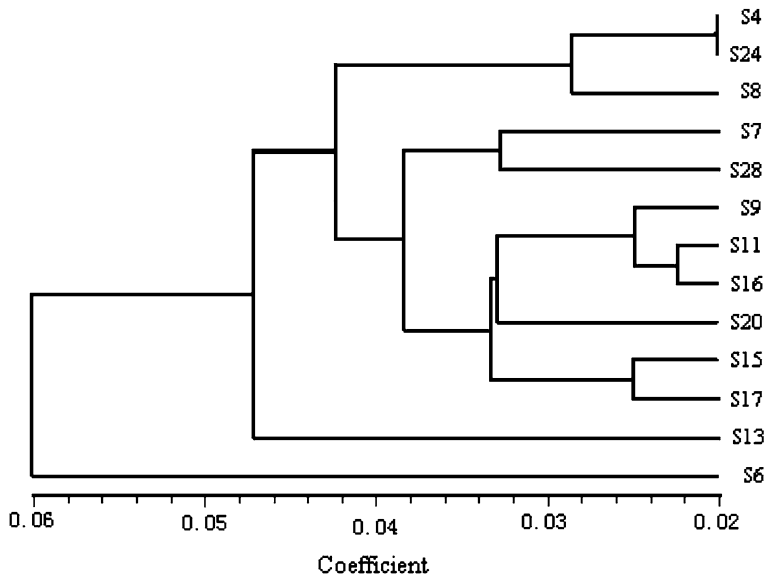

Fig. 3. Dendrogram of genetic distances of 13 populations of *H. rhamnoides* ssp. *sinensis* based on Nei's (1978) unbiased genetic distance coefficients.

of 0.168 (Table II). It is expected that there is higher variation in the present study than that revealed by Yao and Tigerstedt (1993) because RAPD often detects much higher genetic diversity than allozyme data (Liu and Furnier, 1993; Wong and Sun, 1999). It is interesting to note that the within-population genetic diversity of ssp. *sinensis* was comparable to ssp. *rhamnoides* (Bartish *et al*., 1999), although the ssp. *sinensis* populations sampled in this study have a wider distribution (Fig. 1).

By reviewing the published RAPD data, Bartish *et al*. (1999) divided previous studies into three groups: (1) the group that comprises taxa that are outcrossing, wind-pollinated, woody, and long-lived species, which are generally supposed to harbor comparatively high levels of within-population variability; (2) the group that comprises taxa that are outcrossing, perennial, and mainly herbaceous and insect-pollinated species; (3) the group that comprises taxa that are annual or short-lived perennial and mainly selfing species and generally harbor comparatively little within-population diversity. On this basis, Bartish *et al*. (1999) concluded that the breeding system appears to be critical for explaining variation in within-population genetic diversity, and considered that their estimates for *H. rhamnoides* ssp. *rhamnoides*, as a woody, moderately long-lived, obligately outcrossing and wind-pollinated species, might be regarded as somewhat lower than expected.

Bartish *et al*. (1999) attributed the lower values of spp. *rhamnoides* to the fragmented distribution and isolation between island populations. It seems, however, that this factor could not explain the similar level of variability within ssp. *sinensis* populations because this subspecies has a relatively wider and less isolated distribution in China. One alternative explanation may be related to the successional stage. Subspecies *sinensis* is a pioneer plant and mainly occupies areas of early successional stages. As pointed out by Hamrick and Godt (1990), early successional species usually tend to have relatively lower levels of variability within populations. In addition, ssp. *sinensis* exhibits a high degree of vegetative reproduction through root suckers, which makes the individuals an agglomerate structure (Lian *et al*., 2000). Investigations indicated that each individual of sea buckthorn can produce 10 to several hundred new plants through root suckers (Lian *et al*., 2000). Therefore, the high level of clonal reproduction may also be responsible for the relatively lower genetic diversity within populations of sea buckthorn species, as evidenced in other plants (Godt and Hamrick, 1998; Tsyusko *et al*., 2005).

It is worthwhile mentioning that genetic diversity within populations of ssp. *sinensis* detected in the present study is remarkably similar to the morphological studies on fruit and vegetative traits of the subspecies, i.e. the RAPD-based genetic variation within population correlated well with that

detected by morphological observations. For example, populations S16 and S13 show high and low morphological diversity within population, respectively, especially for shape, color, and size of fruits. The RAPD-based values reveal similar trends (Table II). In addition, recent morphological studies indicate that the highest infraspecific variation of ssp. *sinensis* is found in populations from 2400 m to 3400 m in altitude (Lian *et al.*, 2000). As shown in Table II, populations of Group 1 that occur on the Qinghai-Tibetan Plateau ranging from 2360 m to 3900 m in altitude maintain the highest genetic diversity. These phenomena, however, need to be tested further in future investigations.

Population Genetic Structure

Bussell (1999) summarized the RAPD data of 35 species and found that on average 19.3% of total genetic diversity resides among populations for 29 outbreeding species, and 62.5% of total diversity resides among populations for 6 inbreeding species. Therefore, it is expected that the majority of diversity should be found within populations in both ssp. *sinensis* (81.7% based on Gst value) detected by the present study and ssp. *rhamnoides* (84.9% based on AMOVA analysis) revealed by Bartish *et al.* (1999) because *H. rhamnoides* is an outcrossing and wind-pollinated species. These values are lower than the average of other outbreeding species (Bussell, 1999).

In addition to the mating system, as pointed out by many authors, gene flow among populations has a significant influence on the distribution of genetic variation (Hamrick and Godt, 1990). Although recent observations indicate that the pollen of ssp. *sinensis* usually disperses no more than 20 m, with 60–90% falling within 12 m (Tian *et al.*, 1993; Wang *et al.*, 1989), the incidence of seed dispersal facilitated by birds and small animals was very high in ssp. *sinensis* (see Lian *et al.*, 2000 for details). Therefore, the limited genetic differentiation among populations in ssp. *sinensis* may be attributed to its relatively long-distance seed dispersal.

In many plant species there exists significant correlation between geographic and genetic distances among populations, which can be explained by the isolation-by-distance hypothesis (e.g., Comes and Abbott, 2000; Dawson *et al.*, 1995; Raybould *et al.*, 1996). In their study of 10 populations of *H. rhamnoides* ssp. *rhamnoides*, Bartish *et al.* (1999) found no correlation between genetic and geographic distances among populations based on two RAPD datasets ($r = 0.11$, $P = 0.763$; $r = 0.04$, $P = 0.641$). Similarly, our RAPD data were unable to detect significant correlation between the genetic and geographical distances for 13 ssp. *sinensis* populations ($r = 0.38$, $P = 0.997$). The same can be said of the UPGMA phenogram based on Nei's unbiased genetic distances, where populations within each group did

not cluster together before forming a cluster with any population of other groups (Fig. 3). For example, population S6 of Group 1 and population S13 of Group 3 were the most genetically differentiated from others, but the geographically more distant populations, such as S4 and S24 as well as S11 and S16, appeared to be very closely related genetically. This pattern of population differentiation may imply the adaptation of ssp. *sinensis* populations to the local environment, given that the habitats of ssp. *sinensis* vary greatly across its distribution (Lian *et al*., 2000). Alternatively, as Bartish *et al*. (1999) suggested, it is possible that large-scale geographical and ecotypic differentiation is not reflected in RAPD profiles. Further studies of reproductive biology, ecology, and population genetics utilizing other molecular techniques are currently under way and should yield valuable information for the conservation and utilization of this economically important species.

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