Ginseng Conservation Program in Russian Primorye: Genetic Structure of Natural and Cultivated Populations

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Abstract

"The Regional complex long-term program of restoration (reintroduction) of Primoryes ginseng population up to 2005" elaborated by Primorye governor administration, Regional Committee of Natural Resources and Russian Academy of Sciences operates in Russian Primorye. The Institute of Biology and Soil Science (IBSS) provides the scientific implementation of this program including the genetic analysis of extant ginseng populations, plant reproduction and offspring identification. According to our investigations, the genetic resource of *P. ginseng* in Primorye is represented by three populations of wild-growing ginseng and a few private plantations. The results obtained by RAPD allowed concluding that this resourceis dispersed among the wild and cultivated ginseng sub-populations in such a way that each of sub-populations studied has to be represented in living plant collection as a stock material to maintain species genetic variability. The allozyme analyses also showed that the small sub-populations of natural ginseng are characterized by unique genetic diversity and, therefore, they all need to be represented in reintroduction centers. Additionally the allozyme analysis discovered that the Blue Mountain and Khasan populations possess the most genetic diversity. So, at least one more reproductive ginseng unit has to be created besides two already existing reintroduction centers representing the Sikhote-Alin and the Blue Mountain populations.

Introduction

In the first half of XX century a wild-growing *Panax ginseng* C.A. Meyer occupied the large forestlands in China, Korea and Russian Primorye, but now its area is drastically reduced. Only three localities, the Southern Sikhote-Alin, Blue Mountains in Spassk-Kirovsk region and Khasan-Nadezhdinsk-Ussuriysk region in Southern Primorye, represent now genetic resources

of ginseng [1-3]. Additionally the ginseng is cultivated in Primorye by few farmers and their plantations can also serve as a source of genetic variability because many cultivated lines of ginseng have originated from wild populations. Large areas in Korea and China are occupied by plantations of cultivated ginseng that was domesticated indefinitely many years ago.

The collaborators of Institute of Biology and Soil Science of Russian Academy of Sciences (IBSS) have studied the biology and ecology of wild-growing ginseng for more than fifty years. This study was used as a general platform to formulate the protection measures operated up to 1991:

- harvesting quotes of wild-growing ginseng were reduced;
- limit for minimal weight of harvested roots was established;
- the industrial farm "Ginseng" was organized with ginseng growing area of 30 hectares;
- favorable conditions for private ginseng farmers jointed in professional union were created.

After former Soviet Union disintegrated, the existed system of ginseng conservation was also destroyed. The politics of open frontier and liberalization of outer economic activity stimulated illegal ginseng market from whence the predatory harvest of wild-growing ginseng had acquired a character of mass business causing the degradation of ginseng natural populations. Besides, felling of coniferous forests, fires, and progressive river shallowing produced destructive influence upon recruitment of this valuable plant. To change the situation and to work out measures for protection of ginseng resources from exhaustion, the Primorye regional administration, Regional Committee of Natural Resources and Russian Academy of Sciences (IBSS) elaborated "Regional complex long-term program of restoration (reintroduction) of Primorye's ginseng population up to 2005".

In its reintroduction part, the Program supposes genetic analysis of extant ginseng populations for reasoned choice of number and emplacement of centers of reintroduction, search for more intact habitats for reintroduction and detailed recommendations for creation of reintroduced populations. During Program execution, IBSS provides centers of reintroduction with scientific implementation for the selection and propagation of sampled material and for offspring identification.

This paper contains materials of the principal steps of genetic analysis of Primorye ginseng population used for choice of number and emplacement of centers of reintroduction.

Material and Methods

From 1994 to 1999, plants of wild-growing ginseng were collected from 8 localities represent-

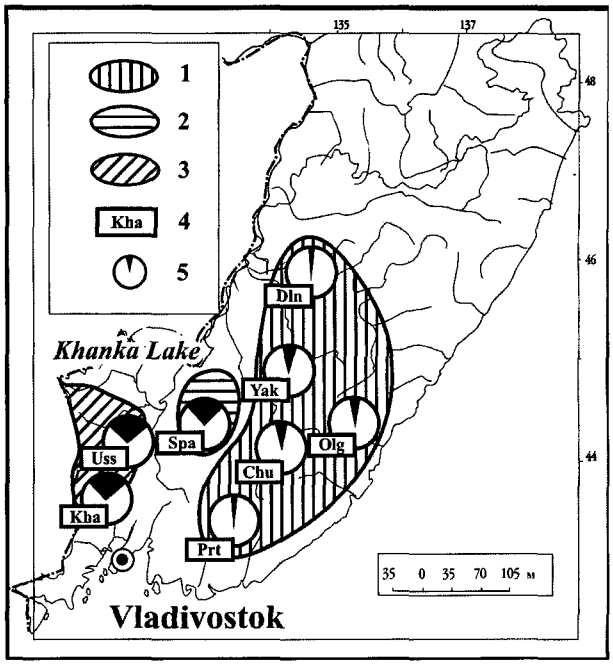

Fig. 1. Natural populations of *P. ginseng* in the Russian Primorye (1- the Sikhote-Alin population, 2 the Blue Mountain population, 3 the Khasan population), location of sampled sub-populations (4), and distribution of a rare allele of *Pgm-2* locus among sub-populations studied (5). Abbreviation: Kha Khassan sub-population, Uss Ussuriysk sub-population, Spa - Spassk sub-population, Ch - Chuguevka sub-population, Prt Partizansk sub-population, Olg Olga sub-population, Yak Yakovlevka sub-population, Dln Dalnerechensk sub-population.

ing three natural populations in the Russian Primorye. Namely, two samplings from the Khasan population (Khasan and Ussuriysk sub-populations, Kha and Uss, for short), five samplings from the Sikhote-Alin population (Chuguevka, Partizansk, Olga, Yakovlevka and Dalnerechensk sub-populations, Chu, Prt, Olg, Yak and Dln, for short) and two samplings from the Blue Mountains population (Spa sub-population, Spassk part of the Blue Mountain population) were covered with sampling (Fig. 1). Cultivated ginseng plants were sampled from private plantation in Dalnegorsk region. The cultivated plants were divided up into two groups of plants similar constitutionally as well as in the form and color of leaves to produce groups of two morphotypes. A total of 192 *P. ginseng* plants from natural populations and 23 cultivated plants were investigated.

RAPD [4] and allozyme methods were used as described earlier [5,6] to evaluate various genetic characteristics of ginseng populations comprehensively, because both methods have some advantages [7]. Fresh leaves of ginseng were sampled and stored in liquid nitrogen until

enzyme extraction. DNA was isolated from lyophilized powdered leaves. A total of 75 and 192 *P. ginseng* plants were investigated by RAPD and allozyme analysis respectively.

Allele frequencies and basic genetic parameters of the populations were computed using POPGENE [8] and TFPGA program [9]. Proportion of polymorphic loci ($P_{0,95}$), number of alleles observed per locus (A), effective number of alleles per locus (A_e), the expected (H_e) and the observed heterozygosity (H_o) were estimated, as well as a test on differentiation of populations was applied [10]. Because RAPDs are inherited as dominants, their allele frequencies and expected heterozygosity were calculated by indirect method [11,12]. Calculating Wright's F-statistics [13] was applied to the allozyme data to investigate the genetic structure of the natural ginseng populations. Genetic distance/identity measures were calculated for allozyme data as described by Nei [14] while for RAPD data pairwise similarity coefficients were computed using formula: $S= 2Nab/(Na+Nb)$ [15].

Dendrograms were constructed with UPGMA cluster analysis using NTSYS-pc [16] for RAPD data and TFPGA [9] for allozyme data.

Results

Evaluation of P. ginseng genome polymorphism

It is reasonable to believe that the most genetic variability of ginseng is connected with natural populations that are very thin, yet, until now occupied large area. Cultivated plant populations originate usually from a limited number of plants, and they are also exposed to selection press so only a part of species gene pool is involved in cultivated populations [17,18]. However, some cultivated populations, especially in the case of *P. ginseng*, can represent a very important part of species genetic variations, which do not occur already in the extant natural populations because of extinction of most wild (sub)populations many years ago.

Thus, investigation of genome variability of both natural and cultivated ginseng populations was needed to evaluate all existing genetic variants for conservation them in reintroduction centers and to determine the worth of sub-populations for conservation purposes. For this study, we used RAPD analysis [4] that operates with both coding and repeatable sites of DNA. Moreover, it was shown by some researchers [7], that RAPD markers could recover the essential DNA variations while genetic variability estimated by allozyme method was extremely low.

We used the RAPD profiles generated by 5 primers - OPC-08, OPD-02, OPD-07, OPD-11,

Table 1. Primers using for studying of genetic diversity of natural and cultivated populations of *P. ginseng*

Primer	Nucleotide sequence (5'→3')	Number of fragments	Number of polymorphic fragments
OPA-20	GTTGCGATCC	14	5
OPB-12	CCTTGACGCA	13	2
OPC-08	TGGACCGGTG	8	1
OPC-15	GACGGATCAG	12	2
OPD-02	GGACCCAACC	18	0
OPD-07	TTGGCACGGG	19	3
OPD-11	AGCGCCATTG	9	1
OPD-13	GGGGTGACGA	12	0
OPD-20	ACCCGGTCAC	11	1
OPE-11	GAGTCTCAGG	11	2
OPF-05	CCGAATTCCC	11	0
In general:		138	17

Table 2. Indices of proportion of polymorphic loci (P) and number of alleles per locus (A) in studying samples of *P. ginseng* (based on 66 RAPD-loci revealed by 5 primers)

	Cultivated plants			Wild-growing plants			
Indices	Morphotype I (N=14)	Morphotype II (N=9)	Summarized sample (N=23)	Khasan (N=11)	Spassk (N=11)	Chuguevka (N=15)	Summarized sample (N=37)
P, %	3.6	3.6	7.6	3.0	9.1	7.6	10.6
A	1.05	1.05	1.08	1.03	1.09	1.08	1.11

OPD-13 (Table 1) to compare genetic variability of cultivated and wild-growing *P. ginseng*. In this experiments, genetic polymorphism for 37 wild-growing ginseng plants collected from Kha, Spa, and Chu sub-populations was 10.6 % and for 23 cultivated plants - 7.6 % (Table 2). The level of polymorphism for the individual sub-populations studied was found to be 3.0%, 9.1% and 7.6 %, accordingly.

Genetic polymorphism of "mixed" sample of plants from different populations can be used to characterize the genetic potential of species. Determined by RAPD in artificially mixed probe, it shows as a rule the higher value, than that of any groups involved in combined sample. Moreover, the more pronounced difference between individual groups combined in "mixed" probe

exists, the higher resulted polymorphism of combined probe can be expected, yet, this value is not a simple sum of polymorphism indices for individual probes since a part of polymorphic loci is/may be common for sub-probes. In our experiments (Table 2), each sub-group contributes significantly to joint polymorphism value, thus demonstrating high degree of dispersion of genetic potential of ginseng. Therefore, the RAPD data obtained impel to think that each of populations and even most of big sub-populations have to be present in living plant collections as a stock material.

We obtained an additional support for this supposition when analyzed another sample of 15 ginseng plants from the Spassk natural sub-population using primers OPB-12, OPC-08, OPC-15, OPD-02, OPD-07, OPD-11, OPD-13, OPD-20. These plants were transferred from their habitats to "forest farm" in 1995. RAPD analysis of this sample has showed higher indices of polymorphism (P = 14.63; A = 1.1258, Table 3), than it was in any of three natural sub-populations mentioned above (Table 2). We compared levels of genetic diversity of the sample from "forest farm" with those of the cultivated plants. Genetic differentiation between plants from "forest farm" and cultivated ginseng was revealed by the test on population differentiation ($\div^2 = 480.7248$, df = 318, p = 0,0000). In fact, the plants from "forest farm" showed higher indices of genetic diversity and lower coefficient of intragroup similarity than it was calculated for any of two cultivated plant groups (morphotype I and morphotype II), yet, in the "summarized" sample combining both

Table 3. Indices of genetic variability of *P. ginseng* (based on 102 RAPD loci revealed by 8 primers)

Sample	N	A	A_e	P_{95},%	$S_{ñð}$	H_e
P. ginseng, cultivated plants: morphotype I	14	1.0943	1.0583	12.20	0.9760 (0.0097)	0.0479 (0.0113)
P. ginseng, cultivated plants: morphotype II	9	1.0755	1.0548	9.76	0.9755 (0.0105)	0.0408 (0.0114)
"Summarized" sample of cultivated ginseng	23	1.1698	1.0936	21.14	0.9592 (0.0198)	0.0783 (0.0129)
P. ginseng, "forest farm" plants, originating from the Spassk natural population	15	1.1258	1.0799	14.63	0.9636 (0.0148)	0.0600 (0.0128)
All over *P. ginseng*	38	1.2830	1.1715	35.77	0.9236 (0.0406)	0.1348 (0.0142)

Note: N, sample size; A, number of alleles observed per locus; A_e, effective number of alleles observed per locus; P_{95},%, proportion of polymorphic loci, 95 % criteria; S, the mean coefficient of intragroup similarity; H_e, the mean expected heterozygosity over all loci. Standard deviations showed in parentheses.

groups of cultivated plants, polymorphism index was significantly higher (Table 3). Thus, for the "summarized" sample of cultivated ginseng, a proportion of polymorphic loci was 21.14 and 14.63 for "forest farm" plants while expected heterozygosity for these samples was 0.0783 and 0,0600 respectively. These results show that even the most polymorphic population does not keep all of genetic diversity of the species. Estimated value of genetic polymorphism was found to be 35,77% for the "summarized" probe constructed from wild and cultivated *P. ginseng* plants (Table 3), that is similar to the polymorphism data published for *P. quinquefolius* [19]. If so, an important conclusion for ginseng conservation is that plants originating from different natural and cultivated populations must be presented in plant collection of the reintroduction centers to maintain species genetic variability.

UPGMA cluster analysis clustered the mixture of wild-growing and cultivated plants in two groups (data are not presented). Cultivated plants of "wild" morphotype I combine in one cluster together with wild-growing plants whereas cultivated plants of "plantation" morphotype II form a separated cluster. So, at least a part of traits used in morphotype division have genetic nature. Similar conclusion was made by Bai et all in experiments with morphological traits of *P. quinquefolius* [19]. Both observations make it possible to use a visual selection as a preliminary action in completing the stock plant collection. Besides it may be considered that plants of the morphotype I are genetically more close to plants from natural habitats and in some cases might be used as stock material for reintroduction. This observation is important because permits to manage reintroduction program with lesser number of plants from taiga.

Our results showed that an average value of H_e for *P. ginseng* was 0.1348 (Table 3). This estimate of H_e is high enough to affirm that *P. ginseng* plantings in Primorye kept the principal part of its gene pool dispersed now across the natural populations and different private plantations. Nevertheless, neither cultivated moiety nor wild-growing one recollect all the ginseng species variability. Therefore, both sources of ginseng genetic diversity have to be present in stock collections of reintroduction centers to support species gene richness and heterozygosity.

Genetic structure of natural ginseng populations over the range

To develop the strategy of ginseng conservation, one must look into the genetic structure of the species along the existent habitats. First, some estimates of genetic variability, heterogeneity of populations and differentiation within and among populations are needed to evaluate the sites of concentrated genetic diversity and to conserve them. Second, the reintroduction strategy has to

involve restoring the pattern of allele frequencies characteristic of the populations to provide a stability of reintroduced populations. Finally, the stock material for reintroduction should comprise the most heterozygous individuals from the populations to decrease a level of inbreeding among progeny plants.

To resolve these problems an allozyme analysis was used because inheritance of allozymes as co-dominants allows to estimate allelic frequencies and heterozygosity directly and to use routine methods of gene diversity statistics. Our early studies [20] showed that *P. ginseng* possesses the low level of allozyme diversity. Nevertheless, three loci among 39 loci studied were selected as molecular markers to characterize ginseng populations. These loci, *Pgm-2*, *Pgm-3* and *Gpt-2*, were polymorphic and the mode of inheritance of these enzymes was verified by inheritance analysis.

Two sub-populations from the Khasan population (Kha and Uss), five sub-populations from the Sikhote-Alin population (Chu, Prt, Olg, Yak and Dln) and one Spa sub-population were sampled for this experiment (Fig. 1). All the observed alleles were found in each sub-population and differences in allele frequencies among the sub-populations varied from 0.12 for *Pgm-3* to 0.29-0.30 for *Pgm-2* and *Gpt-2*. Significant heterogeneity of sub-population allele frequencies was observed for *Pgm-2* locus only, while general chi-square test for heterogeneity of sub-populations over all loci showed differences in allele frequencies ($p<0.01$). Fig. 1 demonstrates the distribution of rare (black) and basic (white) alleles of *Pgm-2* locus among the sub-populations of ginseng. Rare allele frequency are around 0.3 in the sub-populations within the Khasan and the Blue Mountain populations (Kha, Uss, Spa) whereas it was much less in the sub-populations within the Sikhote-Alin population (Chu, Dln, Prt, Yak, Olg).

The expected heterozygosity over all loci (H_e) ranged from 0.2705 for the Prt sub-population to 0.3690 for the Uss sub-population, the observed heterozygosity (H_o) ranged from 0.2051 for the Olg sub-population to 0.3243 for the Spa sub-population. The biggest estimates of heterozygosity were shown in all sub-populations for *Gpt-2* locus, whereas heterozygosity at *Pgm-2* and *Pgm-3* loci for the sub-populations of Spa, Kha, and Uss differed from those of the Sikhote-Alin population (Chu, Dln, Prt, Yak, Olg). Yet, higher estimations of heterozygosity at *Pgm-2* locus were found in the Blue Mountain and the Khasan populations (Spa, Kha, Uss), however, these indices at *Pgm-3* locus were higher in the Sikhote-Alin population (Chu, Dln, Prt, Yak, Olg). In general, mostly high mean values of expected heterozygosity were found in the sub-populations of the Blue Mountain and the Khasan populations (Fig. 2).

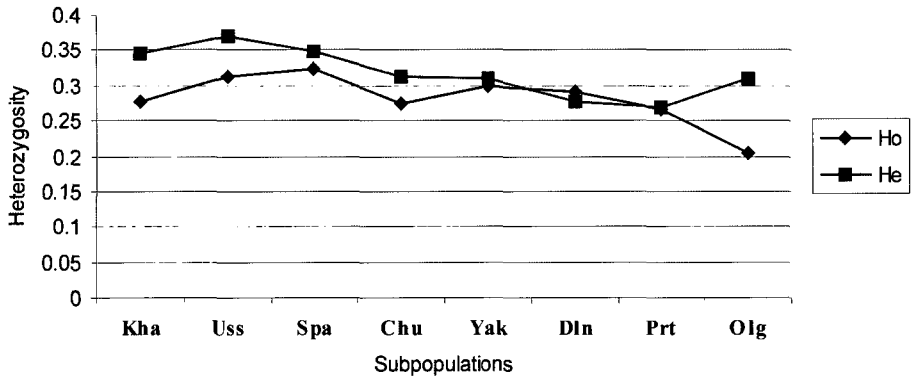

Fig. 2. Heterozgosity observed (H_o) and expected (H_e) in the sub-populations of *P. ginseng* based on the allele frequencies of three polymorphic allozyme loci (abbreviations as in Fig. 1).

Table 4. F-statistics analysis of the sub-populations of *P. ginseng*

Locus	F_{IT}	F_{IS}	F_{SP}	F_{ST}
Pgm-2	0,5987	0,2011	0,4976	-0,5152
Pgm-3	0,2148	0,0808	0,1458	-0,0340
Gpt-2	0,0775	0,0664	0,0119	-0,0321
Over all loci	0,2826	0,0981	0,2046	-0,1896

F_{IT}, inbreeding coefficient of individual respecting the whole species. F_{IS}; inbreeding coefficient of individual respecting a sub-population. F_{SP}; indicator of differentiation of sub-populations within populations. F_{ST}; the indicator of differentiation of populations.

We estimated the genetic diversity of *P. ginseng* sub-populations using Wright's F-statistics (Table 4). The analysis shows that F_{is} for loci ranged from 0.0664 to 0.2011 with mean value of 0,0981 over all loci. This mean estimation suggests that about 10 % deficit of heterozygotes corresponds to a mean sub-population of *P. ginseng*. Mean value of F_{it} was 0.2826 showing 28 % deficit of heterozygotes in the species as a whole. Indicators of differentiation at sub-population level (F_{sp}) and at population level (F_{st}) were 0.2046 and -0.1896, respectively. This value of F_{sp} suggests that about 20 % of genetic diversity of *P. ginseng* were found between sub-populations, whereas the rest diversity was concentrated within sub-populations. Negative estimates of F_{st} suggests that genetic diversity of the species is rather concentrated within the sub-populations than within the populations.

The additional support for this assumption may be our observations on genetic diversity of gin-

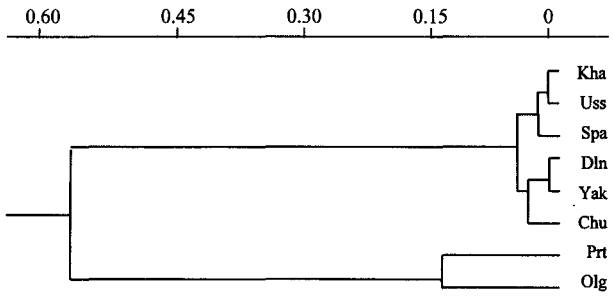

Fig. 3. Dendrogram of genetic relationship among sub-populations of *P. ginseng* based on the allele frequencies of three polymorphic allozyme loci (abbreviations as in Fig. 1).

seng plants from Ussuriysk and Dalnerechensk regions obtained by 5 primers (OPC-05, OPC-14, OPC-15, OPD-11, OPD-13). These sub-populations, as shown above (Fig. 1), are geographically distant from each other. Possibly the geographic factor caused significant differences in values of polymorphism (15.22% and 4.35%, respectively) and in another genetic variability parameters for these sub-populations.

The dendrogram in Fig. 3 represents genetic relationships of the sub-populations studied. The sub-populations are clustered in three groups. The first group involves three sub-populations from the Blue Mountain and the Khasan populations and characterized by minimal intra-sub-population genetic distances (Spa, Kha, Uss). Three sub-populations from the Sikhote-Alin population (Dln, Yak and Chu) are clustered in the second group, which is close to the first group. Prt and the Olg sub-populations differ both from each other and from the rest ginseng sub-populations, they create a separate group. These data most probably reflect the evolutionary history of the species. Indeed, lack of variation in the sub-populations from the Sikhote-Alin population (Fig. 1, 2) could be attributed to the founder effect probably originated from deficiency of refuges for ginseng in the central Sikhote-Alin during the last period of climate cooling.

The results have several conservation implications. Inasmuch as our data suggest that the Blue Mountain and Khasan populations of *P. ginseng* demonstrate the most genetic diversity, these two populations deserve to be protected in the first instance. However, the other small sub-populations of ginseng are characterized by unique genetic diversity. So, at least a few plants from small sub-populations of the greatest variability have to be represented as a stock material in reintroduction centers. Sample sizes from such sub-populations have to correspond to there own sizes in order to maintain stability of future reintroduced population. Gene diversity dispersed among populations and sub-populations, makes, on the one hand, a problem of conservation of

ginseng gene pool more complicated. On the other hand, this situation promotes saturation of stock material with heterozygous parent plants.

Genetic analysis of extant populations and sub-populations indicates that genetic variability in recent ginseng area drops generally towards the North-East direction. Having no more natural populations to analyze, we can suppose only that the center of genetic richness and, possibly, a center of ginseng origin was situated in South-West from extant natural populations, may be in the place of high industrial activity in modern China.

Practical results

Sikhote-Alin population was the first population covered by conservation program activity because its size and density are biggest among extant natural populations. The living plant collection of this population was completed with plants collected from southern regions of Sikhote-Alin. To multiply this stock-material, the reproductive ginseng nursery was built in settlement "Shumniy", and documents for the protected area "Natural park Ararat" are in preparation. Yet, during genetic study on populations, we have revealed that the most genetic variability of ginseng is concentrated in the other two populations Khasan and Blue Mountain ones. So a need to create some additional reintroduction centers appeared.

In 2000, thanks to the financial support of World Wildlife Foundation and ROLL Foundation, the Blue Mountain reintroduction center was created in the settlement Vasilkovka, Spassk region. More than 250 wild-growing plants were collected from the range of Blue Mountain to represent the Spa sub-population in collection of living plant, and only 183 from "indigenous plants" were chosen to serve as a stock material for reintroduction.

At present the Khasan population is not included yet in reintroduction program. That means that the more genetically reach population is not covered by reintroduction activity. It is of great importance to fill up this gap, the more so, as this population could be used for the reintroduction in neighboring countries - China and Korea.

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