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Utility of two mitochondrial markers for identification of *Picea abies* refugial origin

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Abstract: *Picea abies* (L.) Karst is one of the most important coniferous species of Europe from both ecological and economical points of view. Traditional methods for the gene pool conservation and biodiversity maintenance in forest ecosystems have been practiced in many countries. For progress in this field using highly polymorphic genetic molecular markers is needed. Our goal was to demonstrate the utility of two polymorphic mitochondrial markers mt15-D02 and *nad1* b/c in identification native Norway spruce stands. This molecular markers were tested in 1401 individuals from 59 Polish Norway spruce populations. We detected three alleles, which are called 1, 2 and 3, for locus mt15-D02 and two alleles , which are called 1 and 2, for locus *nad1* b/c in our material. All five variants of alleles indicate the natural origin of *P. abies*. Result of this study shows that molecular marker mt15-D02 is easy to use and more informative in compare to marker *nad1* b/c.

Additional key words: Norway spruce, PCR-RFLP, mtDNA, molecular markers

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Introduction

Most of forest ecosystems have been more or less strongly influenced by human activity especially during the last two centuries. In consequence, genetic structure of forest tree populations have been an unintentionally or intentionally changed by forest managers. Since genetic diversity is the basic feature for adaptability to survive in spatially and temporally heterogeneous environmental conditions, it is important to be aware of possible genetic changes that are to be expected along with different management practices (Müller-Starck et al. 1992; Savolainen and Karkkainen 1992; Rajora 1999; Rajora and Mosseler 2001). The knowledge about the level of genetic variation and population differentiation is essential for several aspects. Reliable information on the distribution of genetic variation is prerequisite for proper management of forest genetic resources in short-time and long-time perspective (Konnert and Bergman 1995; Palme and Vendramin 2002; Finkeldey and Ziehe 2004). The recognition of the existing genetic diversity is also the preliminary phase in development of an effective strategy for conservation of forest tree species gene pools.

Picea abies is widely distributed forest tree species throughout the Europe. The natural range of Norway spruce is divided into three main domains: the Baltico-Nordic, the Hercyno-Carpathian and the Alpine (Bucci and Vendramin 2000). These distribution areas were formed during the postglacial recolonisation of spruce from its refuges (Latałowa and Van der Knaap 2006; Tollefsrud et al. 2008). Norway spruce, as one of the most economically important conifer species in Europe, has been planted intensely within and beyond its natural range since middle of the 19th century (Schmidt-Vogt 1977). During that time, there were any regulations concerning

the trade and use of the forest reproductive material. Thus, the existence of artificial stands established with seed of unknown origin is highly probable. At the same time, the general principle, underlying the whole concept of gene conservation programs, is identification of native, local stands that are commonly considered to be valuable genetic resources. From that reason it is desired to be able to identify the origin of populations that are to be included in the conservation programs.

Due to the recent developments in the molecular biology, a variety of different techniques to analyze genetic variation has emerged. Up to now, several polymorphic regions have already been identified in the mitochondrial genome of Norway spruce (Grivet et al. 1999; Sperisen et al. 1998, 2000; Bastien et al. 2003; Maghuly et al. 2008). The usefulness of these markers in revealing population genetic differentiation in geographically distinct regions has been confirmed (Grivet et al. 1999; Vendramin et al. 2000; Sperisen et al. 1998, 2000, 2001; Gugerli et al. 2001; Collignon et al. 2002; Jaramillo-Correa et al. 2003; Bastein et al. 2003; Maghuly et al. 2007, 2008). The usefulness of markers generated from mitochondrial DNA (mtDNA) is mainly due the particular features of the mitochondrial genome itself. Plant mtDNA is extremely variable in size and gene arrangement but it has very low rate of gene sequence evolution (Wolfe et al. 1987; Palmer, 1992). It suggests much lower rate of point mutation than in chloroplast DNA (Palmer, 1992). Mitochondrial DNA is strictly maternally inherited in Pinaceae and thus is dispread only by the movement of seeds (Neale et al. 1989; Wagner 1992; Grivet et al. 1999). This particular transmission of mtDNA and lack of recombination enable to describe pattern of the genetics structure that has been shaped by various factors such as past demographic events associated with re-colonization from glacial refugia (Hewitt 1999, 2000, 2001; Mitton et al. 2000). Furthermore, the effective population size for the mtDNA is expected to be a half of that for the nuclear genes in outcrossing species (Comes and Kadereit 1998).

In the present study, we tested utility of two mtDNA markers *nad1* b/c and mt15-D02 in *Picea abies*. The mitochondrial marker, the intron b/c, of subunit 1 of NADH dehydrogenase (*nad1* b/c) was previously described by Sperisen (1998, 2000). This intron contains six mutations allowing to separate trees of Russian refugium from those of central and southeastern refugium (Sperisen et al. 1998, 2000). Polymorphism of the mitochondrial region at the locus mt15-D02 exhibited a 142 bp insertion/deletion mutation and was describe by Maghuly et al. (2008). Our aim was to choose the most informative marker that allows for Norway spruce individuals derived from different refugia to be distinguished. In this way we could testify the origin of Norway spruce stands and gain the information relevant for the conservation of Norway spruce genetic resources.

Materials and methods

Plant material and DNA extraction

For molecular analyzes we use 1401 trees from 59 Polish populations originating in the two domains within the natural distribution area of *Picea abies*: 17 populations from the Baltico-Nordic, 21 populations from the Hercyno-Carpathian. Additionally, 21 populations were sampled in the "spruceless zone" area (Fig. 1). Total DNA was extracted from needle tissue according to the procedures described by Dumolin et al. (1995). DNA concentration and purity were assessed with using DNA-Calculator (Eppendorf).

Mitochondrial markers

The second intron of the mitochondrial gene *nad1* b/c was amplified using a set of primers described by Demesure et al. (1995). A PCR-RFLP method was used to assess the polymorphism pattern of the maternally inheritanted mitochondrial *nad1* b/c intron (Grivet et al. 1999). The PCR mixture contained 1x PCR, pH 8.6 (NOVAZYM), $MgCl_2$ mM, 0.2 mM dNTPs, 0.8 μM of each primer, 10 ng/μl BSA, 1.25 units of AllegroTaq polymerase (NOVAZYM) and 30–50 ng of template DNA in a total volume of 25 μl. Amplification was carried out in a DNA thermal cycler (MJ Research PTC200) with the following temperature profile: an initial denaturation step of 94°C for 3 min, than 30 cycles of 94°C for at 1 min, 57°C for at 1 min, 72°C for at 2 min, and final extension at 72°C for 10 min. The results of amplifications were tested on 1.5% agarose gel. The PCR products were studied with restriction enzyme *Rsa*I (Fermentas). Thirteen microliters of PCR products were digested with 3 U restriction enzyme in total volume of 20 μl for 1.5 h in 37°C. Restriction DNA fragments were electrophoresed on 2% agarose gels containing ethidium bromide. Results were visualized under UV light and documented with a BioCaptMw documentary system (Vilber Lourmat).

The mitochondrial region at the locus mt15-D02 was amplified using a set of primers described by Maghuly et al. (2008). The PCR mixture contained 1x PCR buffer, pH 8.3 (NOVAZYM), 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 μM of each primer, 10 ng/μl BSA, 0.9 units of VivaTaq polymerase (NOVAZYM) and 30–50 ng template DNA in a total volume of 15 μl. Amplification was carried out in a DNA thermal cycler (MJ Research PTC200) with the following temperature profile: an initial denaturation step of 95°C for 15 min, than 35 cycles of 1 min for at 95°C, 1 min at 54°C, 2 min at 72°C , and final extension at 72°C for

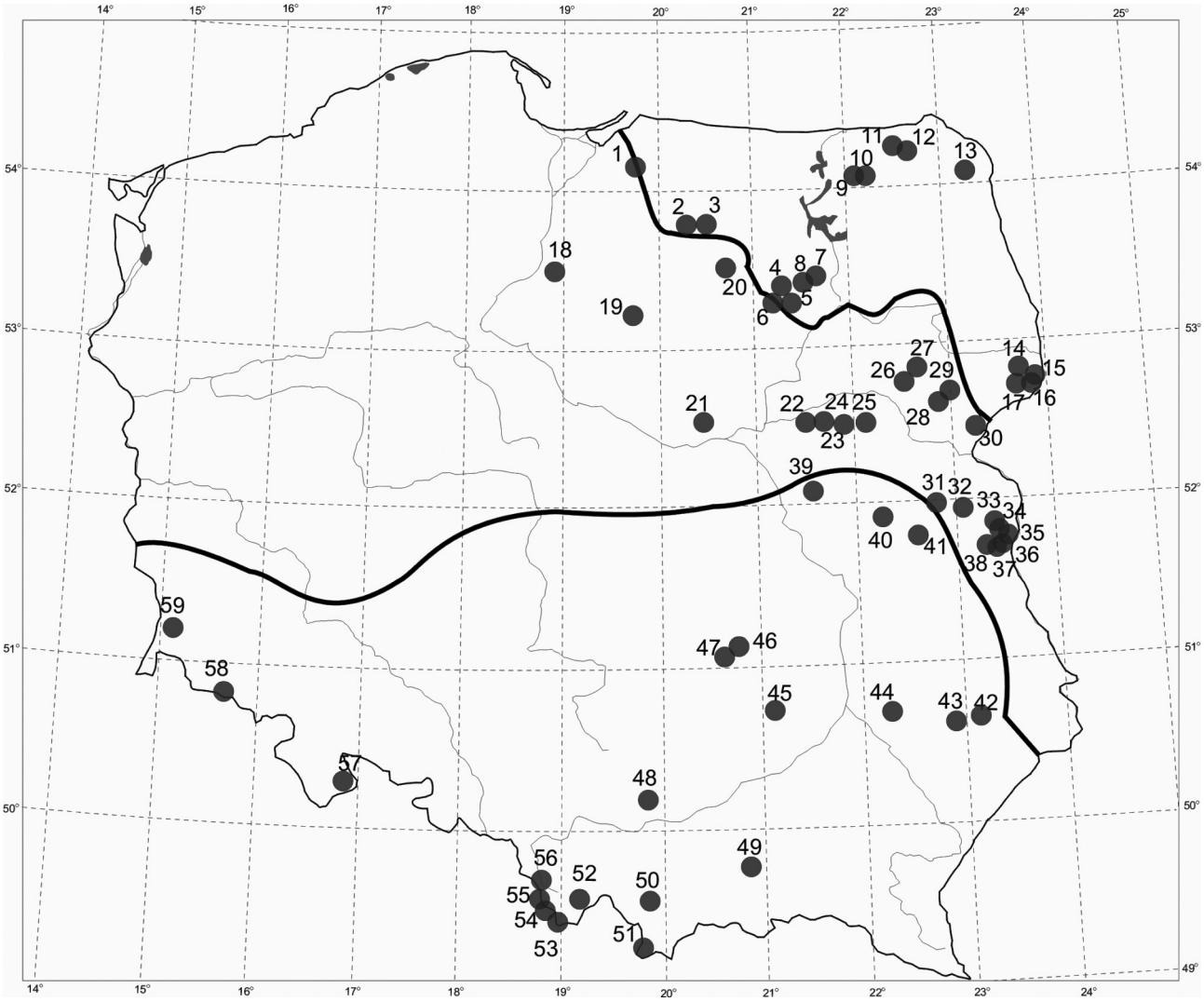

Fig. 1. Geographic distribution of *Picea abies* stands in Poland (exact data containing Table 1)

10 min. Ten microliters of the PCR products were electrophoresed on 2% agarose gel containing ethidium bromide. Results were visualized under UV light and documented with a BioCaptMw documentary system (Vilber Lourmat).

Results and Discussion

Two polymorphic mtDNA markers were employed to investigate the origin of *Picea abies* individuals from 59 different Polish populations. In our material five different mitotypes were scored for the two mtDNA molecular markers tested. Detailed information concerning alleles frequency in all investigated populations are presented in Table 1.

Restriction analysis of *nad1*b/c using *Rsa*I shows 33 bp insertion/deletion mutation. The presence of this 33 bp sequence is attributable for Norway spruce originated from central and southeastern Europe, whereas absence of this sequence is specific for Norway spruce from northern Europe (Fig. 2). According to their refugial origin, variants were referred to as "southern" (allele 1) and "northern" (allele 2) haplotypes (Sperisen et al. 2000) (Fig. 2). The distribution of both haplotypes within the studied area shows a clear geographical pattern which refers to the structure of the natural range of the species. In the north-eastern part of Poland we noted the "northern"

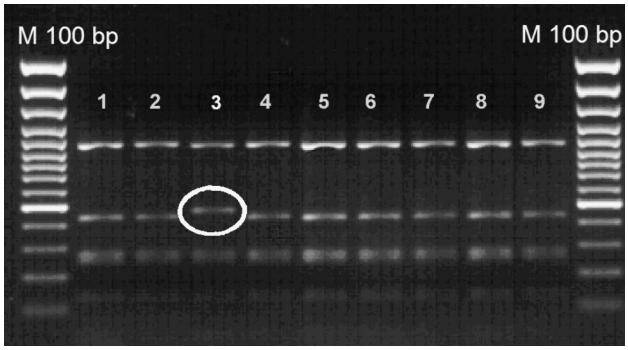

Fig. 2. Result of PCR-RFLP analysis of *nad1* b/c fragment. In lines 1,2, 4–9 are show individuals without 33 bp indel sequence and in line 3 individual containing 33 bp sequence is presented

Table 1. Characteristic of sites and frequency of detected alleles

Sites	Number of individuals	Locus mt15-D02			Locus nad1 b/c	
		1	2	3	1	2
		Frequency of alleles %				
Northern range						
1. Królewo	6	33.3	33.3	33.3	66.6	33.3
2. Nowe Ramuki	22			100.0		100.0
3. Rykowiec	22			100.0		100.0
4. Rozogi	5	20.0		80.0	20.0	80.0
5. Surowiec	41			100.0		100.0
6. Czarnia	25			100.0		100.0
7. Leman	32			100.0		100.0
8. Pisz	17			100.0		100.0
9. Diabla Góra	22			100.0		100.0
10. Borki	22			100.0		100.0
11. Zacisze	21			100.0		100.0
12. Maków	21		4.8	95.2	4.8	95.2
13. Maćkowa Góra	22			100.0		100.0
14. Długi Bród	42	73.8		26.2	73.8	26.2
15. Bank Genów Białowieża	48	60.4		39.6	60.4	39.6
16. Białowieski Park Narodowy	48	50.0		50.0	50.0	50.0
17. Góra Batorego	41	63.4		36.6	63.4	36.6
"Spruceless zone"						
18. Budy	5	100			100.0	
19. Lidzbark (WDN)	47	4.9	40.4	54.7	45.3	54.7
20. Nowy Las	6	16.7		83.3	16.7	83.3
21. Płońsk (WDN)	21	42.9	47.6	9.5	90.5	9.5
22. Jeżyska I	13	23.0	23.0	54.0	46.0	54.0
23. Jeżyska II	10	90.0	10.0		100.0	
24. Puszcza Kamieniecka	28	39.3	60.7		100.0	
25. Wrotnów	32	37.6	59.3	3.1	96.9	3.1
26. Olendy	31	37.5		58.0	37.5	58.0
27. Rutka	33		3.0	97.0	3.0	97.0
28. Dołubowo I	28	50.0		50.0	50.0	50.0
29. Dołubowo II	30	100.0			100.0	
30. Rezerwat „Sokóle"	19	47.4	52.6		100.0	
31. Rezerwat „Liski"	25	100.0			100.0	
32. Wisznice	32	96.9		3.1	96.9	3.1
33. Matiaszówka I	15	26.6		73.4	26.6	73.4
34. Matiaszówka II	31	51.6		48.4	51.6	48.4
35. Matiaszówka III	36	22.2		77.8	22.2	77.8
36. Adampol	29	79.3	13.8	6.9	93.1	6.9
37. Marynki	31	35.5	19.3	45.2	54.8	45.2
38. Zahajki	5		100.0		100.0	
Southern range						
39. Rezerwat „Jedlina"	27	63.0	26.0	11.0	89.0	11.0
40. Rezerwat „Jata"	29	76.0		24.0	76.0	24.0
41. Biała	26	77.0	23.0		100.0	
42. Roztoczański Park Narodowy	20	100.0			100.0	
43. Hedwiżyn	17	100.0			100.0	
44. Janów Lubelski	19	100.0			100.0	
45. Łagów	19	100.0			100.0	
46. Skarżysko-Kamienna	20	100.0			100.0	
47. Świnia Góra	22	100.0			100.0	
48. Miechów	21	4.8	95.2		100.0	
49. Gromnik	15	93.4	6.6		100.0	
50. Raba Wyżna	22	77.3	22.7		100.0	
51. Dolina Chochołowska	11	100.0			100.0	
52. Prusów	21	100.0			100.0	
53. Ujsoły	21	100.0			100.0	
54. Jaworzynka	21	81.0	19.0		100.0	
55. Bukowiec	22	100.0			100.0	
56. Dobka	20	100.0			100.0	
57. Nowa Morawa	21	100.0			100.0	
58. Kocił Szrenicki	22	100.0			100.0	
59. Węgliniec	21	100.0			100.0	

haplotype in clear majority (populations no 2, 3, 5, 6, 7, 8, 9, 10, 11 and 13; Table 1), although an admixture with the "southern" haplotype was also found in some populations (populations no 1, 4, 12, 14, 15, 16 and 17; Table 1). The average frequency of the northern haplotype within the northern range was about 80% and the southern one was 20%. Within the "spruceless zone" we noted intermixing of the "southern" and "northern" haplotypes (populations no 19, 20, 21, 22, 25, 26, 27, 28, 32, 33, 34, 35, 36 and 37; Table 1). The frequency of each haplotype in individual population varied considerably However, we found some regularity that is higher frequency of the southern haplotype in most of stands from the transitional area. In the southern Poland "southern" haplotype was reported from all except two populations (populations no 39 and 40; Table 1) that seems to fit with the hypothesis of southern spruce range formation based on palynological data (Obidowicz et al. 2004).

Amplification of mt15-D02 region in studied tree has revealed the presences of three mtDNA size variants (Fig. 3). Fragment 1249 bp and fragment 1107 bp were described previously by Maghuly et al. (2007) in Austrian Norway spruce populations. We detected third, additional fragment sized around 800 bp in northern part of Poland. All detected alleles were called according to their electrophoretic mobility: 1 (length 1249 bp), 2 (length 1107 bp) and 3 (length 800 bp) (Fig. 3).

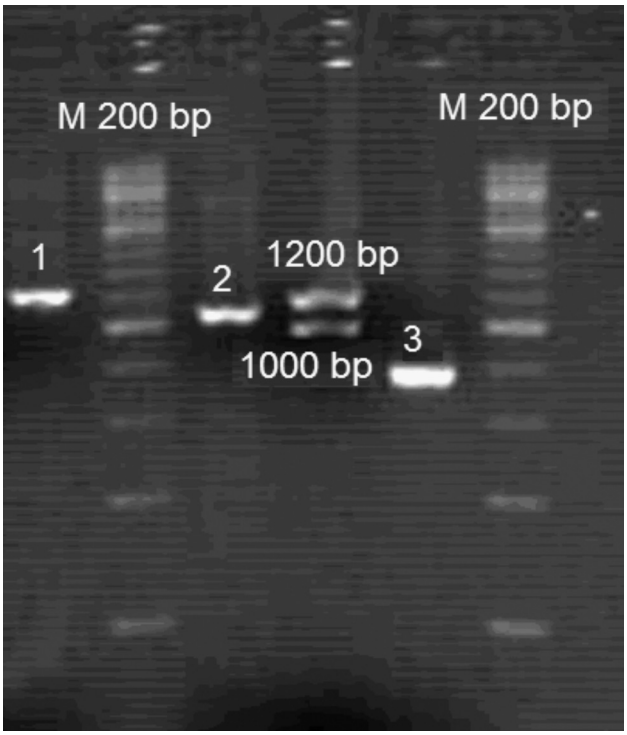

Fig. 3. Result of electrophoresis of mt15-D02 in 2% agarose gel: allele 1 (1249 bp), allele 2 (1107 bp) and allele 3 (800 bp)

Similarly to *nad1* b/c marker, polymorphism in mt15-D02 also shows specific geographical distribution and clearly define the boundaries of the natural range of the Norway spruce in Poland. These results confirm that the natural range of the Norway spruce in Poland was formed at least, by the two different refugial areas, as has previously been claimed based on palynological (Latałowa and Van der Knaap 2006) and on genetic data (Lewandowski and Burczyk 2002; Tollefsrud et al. 2008). The presence of allele 3 in populations from the north-eastern part of Poland referrers to the Russian glacial refugium that has shaped the whole present boreal part of Norway spruce range in Europe (populations no 2, 3, 5, 6, 7, 8, 9, 10, 11 and 13; Table 1). Its average frequency within the northern range was 80%. In the southern Poland we noted allele 1 in clearly majority and its average frequency was 89,2% (populations no 42, 43, 44, 45, 46, 47, 51, 52, 53 55, 56, 57, 58 and 59; Table 1). It probably referrers to Carphatian refugium. It should be stressed, that the frequency of this allele decreases along with the increasing latitude. Its rare occurrence in the northern populations strongly suggests their non-local origin. As for allele 2, it is probably not native to Poland. It may be indicative for other than Carphatian southern refugium because its occurrence in Poland is not abundant and irregular. It is reported mainly from stands from "spruceless zone" (population no 19, 21, 22, 23, 24, 25, 27, 30, 36 and 37; Table1) as well as from two stands located within the north-eastern and the southern parts of Poland (populations no 1 and 12; Table1) but its frequency is not high. The average frequency of allele 2 in "spruceless zone" was 15,7%. However, allel 2 is commonly found within the Alpinie region (Maghuly et al. 2007). Based on this knowledge and our results, we have assumed that the occurrence of the allele 2 in Polish Norway spruce stands may suggests their non-native origin. To confirm this definitively, the distribution of this allele in other regions of Europe should be analyzed.

We compared all five mitochondrial alleles. Practical, technical and diagnostic features of *nad1* b/c and mt15-D02 were considered. We have observed constant pattern in co-occurrence of some alleles. The presence of allele 1 in *nad1* b/c was correlated with the presence of allele 1 or 2 in mt15-D02, while allele 2 in *nad1* b/c was always observed with allele 3 in mt15-D02 (Table 2). Geographic distribution of detected alleles and the patterns of their co-occurrence enabled us to make following conclusions:

1. Allele **1** of mt15-D02 as well as allele **1** *nad1* b/c define southern range of *Picea abies* originated in one of the southern refugium. In the case of Norway spruce from southern Poland it is probable Carpathian refugium;

Table 2. Co-occurrence of the alleles of two analyzed *loci* *nad1* b/c and mt15-D02

Locus mtDNA		Origin
Nad1 b/c	*mt15*-D02	
1 (483 bp)	1 (1249 bp)	southern
1 (483 bp)	2 (1117 bp)	southern
2 (450 bp)	3 (800 bp)	northern

2. Allele **2** of mt15-D02 defines southern range of *Picea abies* originated in another southern refugium apart from the Carphatian refugium. Its origin probably is due to use of foreign seed material to afforestations in the19th and the beginning of 20th century;

3. Allele **3** of mt15-D02 as well as allele **2** *nad1* b/c define the northern range of *Picea abies* originated from Russian refugium.

Investigation of genetic variation of mitochondrial marker *mt15-D02*, affords more information about phylogeography linkages of Norway spruce in Poland than marker *nad1* b/c. Furthermore, marker mt15-D02 is easy to use mainly because it is based only on a single PCR reaction and does not require more molecular approaches. It is also less time- and money-consuming in comparison with *nad1* b/c marker. We are convinced, that this marker may be successfully employ in verification of the origin of Norway spruce stands.

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