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Full Length Article

Development of Genomic SSR Markers and Analysis of Genetic Diversity of 40 Haploid Isolates of *Ustilago maydis* in China

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Abstract

Ustilago maydis (DC) Corda is an important pathogen of maize (*Zea mays* L.) in China. The poorly defined genetic diversity of this pathogen hinders efforts to better predict and complement resistance of maize in the breeding programs. In this study, we developed 2196 SSR markers in *U. maydis*, of which 122 were successfully experimentally validated and used to analyze genetic diversity and population structure of the 40 *U. maydis* isolates. In total, these markers generated 488 alleles with an average of 4.0 alleles per marker. The PIC values ranged from 0.094 to 0.811 with an average of 0.479. The averaged genetic similarity was 0.462 among all isolates. Cluster analysis and population structure analysis both classified the 40 isolates into six different groups. Group classification showed no clear relationship with mating type locus '*a*' or the geographical origin of the studied isolates. This study demonstrates a unique genetic diversity among *U. maydis* haplotypes in corn production areas in China.

Keywords: Genetic diversity; Mating type; Simple sequence repeats (SSR); *Ustilago maydis*

Introduction

The basidiomycete *Ustilago maydis* (DC) Corda is a ubiquitous causative agent of smut disease on maize (*Zea mays*), one of the world's most important cereal crops (FAO 2012, http://www.fao.org). This disease is especially important in China where it is estimated to be responsible for 5%~10% yield losses in annual maize production (Ding *et al*., 2008). In sexually reproducing fungi like *U. maydis*, sexual compatibility is controlled by mating type genes, which function to prevent self-fertilization and ensure the genetic diversity of the population (Wahl *et al*., 2010). In *U. maydis*, mating is accompanied with a dramatic change of biology and is controlled by two independent mating type loci, termed '*a*' and '*b*'. The recognition reaction of different products of these loci further leads to the formation of a dikaryon that requires a maize host for further propagation (Kronstad and Staben, 1997). Hence, infection by this smut fungus triggers gall formation in maize vegetation and reproductive organs, which can result in stunted plant growth and reduced yield, leading to economic losses (Lübberstedt *et al*., 1998; Baumgarten *et al*., 2007; Ding *et al*., 2008). However, in some places, such as Mexico, the resulting gall's of smut on the maize ear are sold as an expensive delicacy (Valverde *et al*., 1995; Juarez-Montiel *et al*., 2011).

U. maydis has recently been used as model system for investigating the molecular interactions between biotrophic fungal pathogens and plant hosts (Martinez-Espinoza *et al*., 2002; Kamper *et al*., 2006). While recent research of *U. maydis* has explored sex determination and morphogenesis, molecular biology, and genomics (Brefort *et al*., 2009) little has been done to assess genetic diversity of *U. maydis* strains, especially in China. Although mating type loci are believed to be very important for genetic diversity, Zambino *et al*. (1997) surprisingly found very low levels of differentiation in *U. maydis* populations with respect to mating type locus *b* variation. However, mating type locus *a*, and genome-wide markers have not been investigated for the ability to differentiate genetic diversity in *U. maydis*.

Microsatellites or simple sequence repeat (SSR) genetic markers are polymerase chain reaction (PCR)-based, frequently co-dominant, hyper-variable, multi-allelic, abundant and well distributed throughout all prokaryotic and eukaryotic genomes and would likely be useful to differentiate diversity in *U. maydis* (Powell *et al*., 1996; Zane *et al*., 2002; Kalia *et al*., 2010). SSRs have been successfully used for genetic diversity studies in many fungi (Scott and Chakraborty, 2008; Vogelgsang *et al*., 2009; Ren *et al*., 2012). To assess SSR flanking regions, sequences have to first be known for primer design. Recently developed genomics resources including the genomic sequence of *U. maydis* (Kamper *et al*., 2006) allow thousands of genome wide SSR markers to be developed now.

We hypothesized that SSR markers will be useful to better characterize the diversity in *U. maydis* and will correspond to mating type and geographic origins of isolates. Here, we developed a set of SSR markers in *U. maydis*: (1) to analyze genotype and genetic diversity, (2) to assess genetic relationship among isolates collected

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from the main production region for corn in China, and (3) evaluate the correspondence to mating type and geographic origin of isolates.

Materials and Methods

SSR Mining and Primer Design

Genomic sequences of *U. maydis* were downloaded from the Broad Institute (USA, http://www.broadinstitute.org/). SSR loci were screened using the MISA program (http://pgrc.ipk-gatersleben.de/misa/) with default parameters. Compound SSRs were defined as ≥ 2 SSRs interrupted by ≤100 bases (Sonah *et al*., 2011). Then 126 randomly selected primer pairs were then synthesized (Invitrogen, Shanghai, China).

U. maydis Haploid Isolates

U. maydis-affected galls in maize were collected in the summers of 2011 and 2012 mainly from East China, located between longitude 87.58°- 126.65° W to E, and latitudes 32.05°- 45.75° S to N representing the major region of corn production in China. At least eight haploid isolates were isolated from each gall using a monospore separation method. In our previous study (Zhang *et al*., 2013) two pairs of sequence-specific primers were used to detect two alleles (*mfa1* and *mfa2*) at locus *a*, a mating type locus that controls haploid cell fusion of two *U. maydis* strains. Only two strains with either *mfa1* or *mfa2* were kept for further DNA isolation and SSR analysis. SG200, a modified solopathogenic fungal strain derived from the *U. maydis* strain FB1 with *mfa1* locus (Doehlemann *et al*., 2008), was used as reference. Table 1 lists the isolates used in this study.

PCR Amplification and Product Detection

Genomic DNA was extracted from strains of *U. maydis* using the CTAB protocol as described by Weising *et al*. (1995). For PCR amplification, a ten µL reaction mixture was made containing 1 µL 1×PCR Buffer, 0.5 U Taq DNA polymerase, 4 nmol/L dNTPs, 10 pmol/L primer pairs and 20 ng gDNA. PCR amplification conditions were as follows: pre-denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension step of 72°C for 10 min. PCR products were resolved on a 8.0% (w/v) non-denatured polyacrylamide gel at a constant current 70 mA for 1h. Detection of SSR bands was visualized by the silver staining method.

Data Analysis

Allele size was estimated using a 50-bp ladder (Promega, Beijing). The number of alleles, major allele frequency, and polymorphisminformationcontent(PIC)valueswere calculated using Power Marker version 3.25 (Liu and Muse, 2005).

The genetic diversity assuming four geographic origin populations (Table 1) was analyzed using POPGENE (V1.32; Yeh *et al*., 1997). The polymorphic bands of each SSR marker were treated as binary characters for their presence (1) or absence (0) in the 40 haploid isolates and was analyzed using the NTSYS-pc program (V2.1, Rohlf, 2002). Genetic similarity between isolates was calculated by the Dice similarity coefficient based on the proportion of shared alleles (Dice, 1945; Nei and Li, 1979) with the SIMQUAL (similarity for qualitative data) subprogram. Cluster analysis was performed using the Un-weighted Pair Group Method with Arithmetic average (UPGMA, Yu *et al*., 2006) in the SAHN subprogram.

Population structure analysis was performed with STRUCTURE version 2.3.4 (Pritchard *et al*., 2000). The optimum number of populations (*K*) was selected by testing *K*=1 to *K*=12 using five independent runs of 10,000 burn-in period length at fixed iterations of 100,000 with a model allowing for admixture and correlated allele frequencies. After the optimal *K* (*K*=6 in this study) was determined according to Murray *et al*. (2009), an additional run of 5,000,000 burn-in and sampling iterations were used to infer population structure.

Results

Characterization of SSR Primers in *U. maydis* Genome

U. maydis genomic DNA totaling 19.68 Mbp belonging to 274 contigs (average of 71.8 Kb per contig) were investigated for SSRs. The contigs were assembled into 47 supercontigs, among which 35 contigs could be placed into the 23 linkage groups of *U. maydis*. We identified 2462 SSR loci on 240 contigs with the average of 9 SSRs per contig and 125 SSRs per million base pairs. Because of the existence of 249 compound formation SSRs, 2213 candidate SSR loci were used for further primer design. 2196 pairs of SSR primer were successfully designed with the average product size of 221 bp.

There were 653 SSRs with mono-nucleotide repeat motifs, 600 with di-nucleotide, and 861 with tri-nucleotide. Comparably, there were 348 SSRs with tetra, penta- and hexa- repeat motifs, suggesting SSRs with 1~3 repeat motif(s) dominate in the genome of *U. maydis* (Fig. 1). Linkage groups one to five contained the greatest number of SSRs, which corresponded to greater sequence lengths (r^2=0.89). An uneven distribution for different motif type was observed among different linkage groups with the exception of di- and tetra-nucleotide (data not shown). SSR density (number of SSRs per Mb) was lowest on linkage groups 1, 3, 6, 7 and 9.

Validation of SSR Markers

To validate the SSR primers we randomly selected 126 SSR markers for further analysis. Of 126 SSR markers only 4 JAAS0014, JAAS0017, JAAS0055 and JAAS0094 did not

Table 1: Description and geographic origins of isolates used in this study

Name	Collection date	Allele[a]	Province	City	Geographical group[b]	Longitude	Latitude	Cluster group[c]
UM01_JS	6/2011	*mfa2*	Jiangsu (JS)	Nanjing	S. China	118° 46'E	32° 03'N	I
UM02_JS	6/2011	*mfa1*	Jiangsu (JS)	Nanjing	S. China	118° 46'E	32° 03'N	I
UM03_JS	6/2011	*mfa2*	Jiangsu (JS)	Nanjing	S. China	118° 46'E	32° 03'N	I
UM04_HB	10/2011	*mfa1*	Hebei (HB)	Shijiazhuang	N.E. China	114° 30'E	38° 02'N	I
UM05_HB	10/2011	*mfa2*	Hebei (HB)	Shijiazhuang	N.E. China	114° 30'E	38° 02'N	I
UM06_BJ	10/2011	*mfa2*	Beijing (BJ)	Beijing	N.E. China	116° 23'E	39° 55'N	I
UM07_HLJ	8/2011	*mfa1*	Heilongjiang (HLJ)	Harbin	N.E. China	126° 39'E	45° 45'N	I
UM08_HLJ	8/2011	*mfa2*	Heilongjiang (HLJ)	Harbin	N.E. China	126° 39'E	45° 45'N	I
UM09_JS	7/2011	*mfa1*	Jiangsu (JS)	Yancheng	S. China	120° 07'E	33° 23'N	III
UM10_JS	7/2011	*mfa2*	Jiangsu (JS)	Yancheng	S. China	120° 07'E	33° 23'N	III
UM11_SD	9/2011	*mfa2*	Shandong (SD)	Jinan	Huang-Huai	116° 59'E	36° 40'N	III
UM12_SD	9/2011	*mfa1*	Shandong (SD)	Jinan	Huang-Huai	116° 59'E	36° 40'N	III
UM13_LN	10/2011	*mfa2*	Liaoning (LN)	Dandong	N.E. China	124° 23'E	40° 07'N	III
UM14_LN	10/2011	*mfa1*	Liaoning (LN)	Dandong	N.E. China	124° 23'E	40° 07'N	III
UM15_JS	9/2011	*mfa2*	Jiangsu (JS)	Xuzhou	Huang-Huai	117° 11'E	34° 16'N	II
UM16_JS	9/2011	*mfa1*	Jiangsu (JS)	Xuzhou	Huang-Huai	117° 11'E	34° 16'N	II
UM17_JS	10/2011	*mfa2*	Jiangsu (JS)	Lianyungang	Huang-Huai	119° 10'E	34° 36'N	II
UM18_JS	10/2011	*mfa1*	Jiangsu (JS)	Lianyungang	Huang-Huai	119° 10'E	34° 36'N	II
UM19_JS	10/2011	*mfa1*	Jiangsu (JS)	Huaian	Huang-Huai	119° 01'E	33° 35'N	II
UM20_JS	10/2011	*mfa2*	Jiangsu (JS)	Huaian	Huang-Huai	119° 01'E	33° 35'N	II
UM21_JS	6/2012	*mfa2*	Jiangsu (JS)	Nanjing	S. China	118° 46'E	32° 03'N	II
UM22_JS	6/2012	*mfa1*	Jiangsu (JS)	Nanjing	S. China	118° 46'E	32° 03'N	II
UM23_JS	7/2011	*mfa2*	Jiangsu (JS)	Taizhou	S. China	119° 54'E	32° 29'N	II
UM24_JS	7/2011	*mfa1*	Jiangsu (JS)	Taizhou	S. China	119° 54'E	32° 29'N	II
UM25_JS	10/2011	*mfa1*	Jiangsu (JS)	Suqian	Huang-Huai	118° 15'E	33° 56'N	V
UM26_JS	10/2011	*mfa2*	Jiangsu (JS)	Suqian	Huang-Huai	118° 15'E	33° 56'N	V
UM27_JS	10/2012	*mfa1*	Jiangsu (JS)	Suqian	Huang-Huai	118° 15'E	33° 56'N	V
UM28_JS	10/2012	*mfa2*	Jiangsu (JS)	Suqian	Huang-Huai	118° 15'E	33° 56'N	V
UM29_HN	10/2012	*mfa2*	Henan (HN)	Xunxian	Huang-Huai	114° 23'E	35° 35'N	V
UM30_HN	10/2012	*mfa1*	Henan (HN)	Xunxian	Huang-Huai	114° 23'E	35° 35'N	V
UM31_XJ	8/2011	*mfa1*	Xinjiang (XJ)	Urumqi	N.W. China	87° 35'E	43° 48'N	IV
UM32_XJ	8/2011	*mfa2*	Xinjiang (XJ)	Urumqi	N.W. China	87° 35'E	43° 48'N	IV
UM33_SX	10/2012	*mfa2*	Shanxi (SX)	Taiyuan	Huang-Huai	112° 28'E	37° 43'N	V
UM34_SX	10/2012	*mfa1*	Shanxi (SX)	Taiyuan	Huang-Huai	112° 28'E	37° 43'N	V
UM35_JS	10/2012	*mfa1*	Jiangsu (JS)	Nanjing	S. China	118° 46'E	32° 03'N	V
UM36_HN	10/2012	*mfa1*	Henan (HN)	Zhengzhou	Huang-Huai	113° 38'E	34° 45'N	IV
UM37_XJ	8/2012	*mfa1*	Xinjiang (XJ)	Urumqi	N.W. China	87° 35'E	43° 48'N	IV
UM38_XJ	8/2012	*mfa1*	Xinjiang (XJ)	Urumqi	N.W. China	87° 35'E	43° 48'N	IV
UM39_SD	7/2012	*mfa2*	Shandong (SD)	Jinan	Huang-Huai	116° 59'E	36° 40'N	IV
UM40_USA	9/2011	*mfa1*	USA					VI

[a]Genetic mating type loci *a*

[b]Geographical regions and characterization of corn production. N.E. China represented for Northeast of China, Huang-Huai for Huang-Huai river area, S. China for South of China, and N.W. China for Northwest of China

[c]Group identified in cluster analysis as described in results

amplify any bands in the 40 isolates. 72.9% (89 of 122) SSR primers were polymorphic and amplified unique bands in the 40 isolates. Calculated product sizes were well matched with that as predicted. Examples of typical amplification pattern were shown in Fig. 2. Most markers generated reproducible, clear, distinct and polymorphic amplifications products, suggesting these SSRs were suitable for genetic diversity analysis.

PIC of SSR Markers

The polymorphic loci resulted in a total of 488 alleles for the 40 isolates. The number of alleles per locus ranged from two to 10, with an average of 4.0 alleles across the 122 loci. The marker JAAS0064, flanking the CAG repeated motif, produced 10 different loci in 40 isolates, the highest among the 122 SSR markers. The following top two were JAAS0023 and JAAS0025, which produced eight different alleles each.

The PIC values ranged from 0.094 to 0.811 with an average of 0.479 for all SSR markers. Of these markers, JAAS0025 with the repeated motif of TGCAAG showed the highest PIC value (0.811); JAAS0023 ranked second (PIC=0.807). Despite having the most alleles JAAS0064 had a lower PIC value (0.787). Among polymorphic markers JAAS0037 showed the highest value for major allele frequency (0.950) and the lowest value for PIC (0.094). Statistical analysis showed no significant difference between the different SSR repeat type and PIC value ($F_{(6, 121)}$=1.88, p=0.089).

Analysis of Genetic Diversity Revealed by SSR Markers

Estimated parameters of genetic diversity for four

Table 2: Genetic diversity within 4 geographic groups of *U. maydis*

Geographic group	N_A	N_E	I	PPB(%)
N.E. China	2.309±1.001[a]	1.927±0.804	0.636±0.439	76.4
Huang-Huai	3.26±1.085	2.273±0.945	0.868±0.380	98.4
S. China	2.789±0.977	2.132±0.793	0.793±0.366	93.5
N.W. China	1.756±0.657	1.578±0.552	0.437±0.363	63.4
average	2.528	1.978	0.683	82.9
All	3.984±1.431	2.474±1.038	0.983±0.391	

N_A = observed number of alleles; N_E = effective number of alleles; I = Shannon's information index; PPB(%) = percentage of polymorphic bands
[a] Mean ± standard error

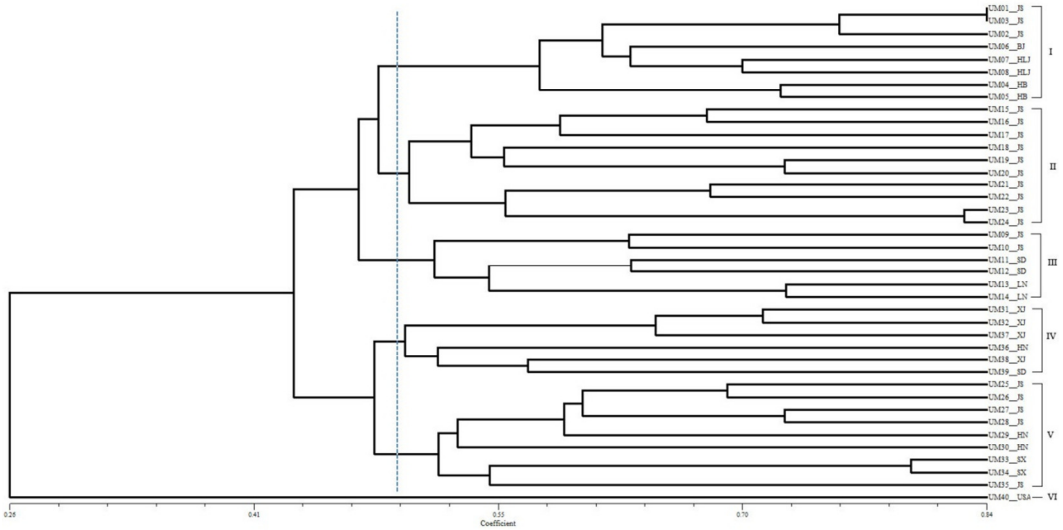

Fig. 3: Clustering tree
UPGMA cluster dendrogram showing the genetic relationships among the 40 strains based on 122 SSR loci. Each isolate was identified by number and collection region with the abbreviation of province (Table 1). The horizontal axis indicated the Dice similarity coefficient

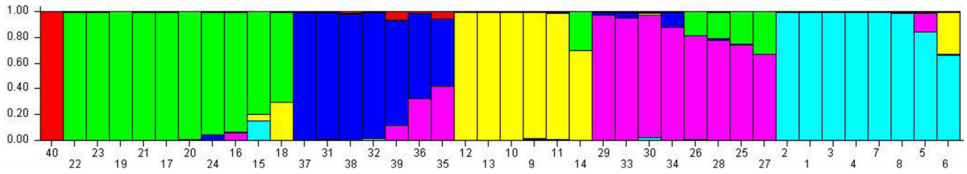

Fig. 4: Population structure analysis of 40 *U. maydis* isolates
Analysis was carried out using STRUCTURE software with K set at 6. Inferred ancestries of the 40 U. maydis isolates were based on 6 genetic populations. Each population is represented by a different color. Cluster I is in cyan, cluster II in green, cluster III in yellow, cluster IV in blue, cluster V in pink, and red represents Cluster VI for the USA

geographic populations in China are presented in Table 2. There was abundant genetic variation of *U. maydis* at both the population level and the isolate level. Shannon's information index (I) varied between 0.437 (N.W. China) and 0.868 (Huang-Huai) with an average of 0.683 at the population level. Population Huang-Huai had 98.4% of polymorphic bands (PPB). At the isolate level Shannon's information index (I) was 0.983 with standard error of 0.391.

Cluster Analysis of 40 *U. maydis* Strains

A similarity coefficient matrix was calculated and then used for cluster analysis. The similarity coefficient of overall strains averaged 0.462. Strains UM01 and UM03 both from Jiangsu Province showed the greatest similarity with each other (similarity coefficient =0.845). Isolate UM19 from Jiangsu province and the USA strain UM40 were the most distant (similarity coefficient of 0.193).

UPGMA clustering result showed the 40 isolates could be classified into six major groups (Fig. 3). Isolates from UM01 to UM08 formed group I. Isolates from UM15 to UM24 (all from Jiangsu) together formed group II. Group III included isolates from UM09 to UM14. Group IV contained six members from UM31 to UM39 excluding

UM33 to UM35. Group V consisted of nine members (isolates from UM27 to UM30, UM33, UM34 and UM35). Strain UM40 (Group VI) from the USA did not cluster with any Chinese isolates and is an outgroup for this study. Most clusters were further supported by STRUCTURE analysis (Fig. 4) when the number of populations K=6. Clustering and STRUCTURE results both suggest that mating type locus *a* does not correlate to SSR genetic diversity (p=0.845 by χ^2 test) and we did not observe isolates with *mfa1* or *mfa2* clustered in the same group.

Discussion

In our study 2,462 SSRs were identified in *U. maydis*, which was less than the 6,854 SSRs suggested by a previous study across seven filamentous fungi that included *U. maydis* (Li *et al*. 2009). This was likely a result of different calculation methods and did not reduce the usefulness of this study because more than could be experimentally validated were still detected. Among those tested 96.7% of designed SSR markers this study developed were successful and 72.9% of SSR markers generated unique and useful bands. Both Li *et al.* (2009) and this study found that SSRs were unevenly distributed among chromosomes and linkage groups.

Genetic diversity high at the population and isolate level indicates a relatively high level of genetic exchange within and among the different sample populations. However, isolates collected from the same city were genetically similar to each other and often clustered together. Members of Group I all came from geographic group S. China and N.E. China, while Group II contained most of isolates from Jiangsu (Huang-Huai and S. China). Members of Group V came from Huang-Huai with only one exception (isolate UM35). However, overall cluster groups were not statistically correlated with geographic origin. For example, isolates from Jiangsu were scatted into most groups (excluding group IV), while Group II contained isolates all from Jiangsu.

The importance in recognition of different alleles at mating type loci *a* was expected to differentiate isolates. However, from the cluster analysis (Fig. 4), the isolates with the same allele at locus *a* did not group together, meaning that mating type loci *a* is not directly correlated with genetic diversity in these isolates. There are a number of potential causes for this unexpected result. First, it might be partly due to high recombination rate in the *U. maydis* genome (Kamper *et al*., 2006). This would lead to low linkage disequilibrium between locus *a* and other loci and low genetic correlations. Second, it could be due to the high mutation rates of SSRs in this species. High mutation rates are useful for providing separation between genotypes but could provide too much divergence. Similarly, if the mutation rates are high enough and SSRs do not follow a stepwise mutation model, these loci can back mutate resulting in homeoplasy in allele lengths. It would therefore be interesting to use SNP markers, which have a much lower mutation rate to see if the same patterns were observed.

Different combinations of two strains of *U. maydis* might have different virulence to the host (J Shi, personal communication). But until now, little has been known about the relationship between virulence and genetic diversity. Though recently both genetic and molecular tools have facilitated to identify virulence-related genes more information about the genetics and evolution of virulence at the population level needs to be further elucidated in *U. maydis* (Chacko and Gold, 2012; Rodriguez-Kessler *et al*., 2012; Heimel *et al*., 2013; Karakkat *et al*., 2013; Mueller *et al*., 2013).

Conclusion

In conclusion, this paper reports the development of 122 highly polymorphic SSR markers useful for characterizing *U. maydis*. The applications of these markers encompass strain identification, analysis of genetic diversity, and population structure studies. Genetic relationship between Chinese strains is not correlated with geography.

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