Genetics and Molecular Research

Karyotype studies on *Lycoris radiata* populations from China

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ABSTRACT. *Lycoris radiata* is an important medicinal and ornamental plant of China. In the present study, somatic chromosome counts and karyotype analyses, which are important aspects of plant phylogeny and evolution, were performed in 466 individuals from 25 *L. radiata* populations by root tip squash method. Chromosome counts revealed that 10 populations were diploid (2n = 2x = 22) and 15 were triploid (2n = 3x = 33). Except for one diploid population containing some triploid plants, the remaining 24 populations showed a single cytotype. Karyotype analysis showed that the karyotypes of *L. radiata* varied in different populations and even within the same population. However, based on the Stebbins' system, the karyotype of all the populations could be classified in 4A classes. The cluster analysis and ordination methods demonstrated that the *L. radiata* populations grouped in two major clusters. Previous research has shown that the triploid strain of *L. radiata* is a genetically identical species. However, the cluster analysis revealed that the triploid strains clustered in two groups instead of one, which indicates that these strains may not be identical species, genetically. This study is expected to improve the understanding

of the genetic diversity in *L. radiata* and provide a basis for future studies on species differentiation, speciation, and taxonomy.

Key words: *Lycoris radiata*; Chromosome number; Karyotype; Ploidy

INTRODUCTION

Lycoris L. is one of the most important genera of Amaryllis family, most famous for perennial, bulbous plants. This genus consists of approximately 20 species, about 15 of which are endemic to China. It is distributed only in the warm temperate and subtropical zones of East Asia, from southwestern China to southern Korea and Japan, with a few species extending to northern Indo-China and Nepal (Hsu et al., 1994). Since 1928, *Lycoris* L., with a few, large chromosomes, has been a subject of several cytological and cytogenetic studies (Nishiyama, 1928). The chromosome numbers observed in this genus are: 2n = 12, 13, 14, 15, 16, 17, 18, 19, 21, 22, 24, 25, 27, 30, 32, 33, and 44 corresponding to the diploid, abnormal diploid, triploid, tetraploid, and aneuploid levels, with the basic chromosome number, x, being 6, 7, 8, and 11 (Shi et al., 2006).

Lycoris radiata Herb., an important member of the genus *Lycoris*, is widely distributed in China and is well known as the 'Red Spider Lily'. The plant prefers shady and moist areas and can be propagated from small bulbs. These bulbs have been used in traditional Chinese medicine for a long time, due to the presence of two main medicinal components, the lycorine and galantamine, in the bulbs. Furthermore, *L. radiata* is of great interest to horticulturists because of its scarlet flowers in autumn and green leaves throughout winter.

A perennial medicinal and ornamental herb, naturally occurring *L. radiata* includes diploid, triploid, and tetraploid species (Zhou et al., 2007). Triploid species are completely sterile, having a genomic constitution of 2n = 3x = 33 (Nishiyama, 1928), whereas diploids are fertile, with a genomic constitution of 2n = 2x = 22 (Shao et al., 1994). Based on data from our field investigations in China, the completely sterile species are very common and wide spread, but the fertile diploid species are isolated in East China. However, tetraploid species have a narrow distribution and, to the best of our knowledge, are only found in the Huoshan county of Anhui Province (Zhou et al., 2007).

Chromosome number, size, and karyotype are considered good taxonomic characters. Moreover, cytological data are essential in studies focusing on diversification (Stebbins, 1971). It was also reported that the chromosome numbers and karyotypes of some species vary in different populations and even within the same population (Zhou et al., 2007; Fiorin et al., 2013). Several cytological studies have been performed on *L. radiata* (Nishiyama, 1928; Inariyama, 1932; Kurita, 1987; Sun et al., 1998; Zhou et al., 2007). However, several arguments and problems related to the cytology of *L. radiata* still exist. In addition, such studies have been missing in the large number of investigations on this species in China (Mookerjea, 1955; Shao et al., 1994; Qin et al., 2004; Zhou et al., 2004).

In the present study, the main objective was to investigate the ploidy levels and chromosome numbers in *L. radiata* from 25 different regions and 13 provinces of China. To the best of our knowledge, this is the first study on the ploidy and karyotype patterns in *L. radiata*. The results obtained are expected to provide a basis for future studies on differentiation, speciation, taxonomy, and diversification of *L. radiata*.

MATERIAL AND METHODS

Plant material

Bulbs from 466 accessions representing 25 wild populations of *L. radiata* were collected for use in the study. As this species is extensively propagated clonally, the collected bulbs were from plants spaced 10-30 m apart, a standard interval considering the population size. Each surveyed population represented approximately 8-20 mature individuals. This sampling strategy covered almost the complete geographical range of *L. radiata* in China. All the bulbs were maintained in water until their root tips were harvested for the cytological studies. The names of the populations and details of the accessions, along with the geographic coordinates of the place of their collection, are presented in Table1.Voucher specimens were deposited in the herbarium of the Nanjing Forestry University, Nanjing, China.

Table 1. Geographical data of the collection sites for different *Lycoris radiata* populations.

Population	Location	Longitude and Latitude	Altitude (m)	Collector and Vouchers
L1	Chizhou city, Anhui Prov.	117°35'E, 30°19'N	158	Y.X. Liu & Y.Q. Zhi; 140301
L2	Xiuning county, Anhui Prov.	118°11'E, 29°39'N	137	Y.X. Liu & Y.Q. Zhi; 140302
L3	Yixian county, Anhui Prov.	117°48'E, 30°04'N	234	Y.X. Liu & Y.Q. Zhi; 140303
L4	Huainan city, Anhui Prov.	116°47'E, 32°37'N	118	Y.X. Liu & Y.Q. Zhi; 140304
L5	Maanshan city, Anhui Prov.	118°27'E, 31°39'N	115	Y.X. Liu & Y.Q. Zhi; 140305
L6	Chuzhou city, Anhui Prov.	118°16'E, 32°16'N	122	Y.X. Liu & Y.Q. Zhi; 140306
L7	Tongling city, Anhui Prov.	118°18'E, 32°28'N	27	Y.Q. Zhi; 140307
L8	Nanjing city, Jiangsu Prov.	118°37'E, 32°07'N	248	Y.X. Liu & Y.Q. Zhi; 140308
L9	Lianyungan city, Jiangsu Prov.	119°24'E, 34°42'N	42	Y.X. Liu & Q.R. Zhou; 140309
L10	Jinyun county, Zhejiang Prov.	120°18'E, 28°34'N	330	Y.X. Liu; 140310
L11	Linan city, Zhejiang Prov.	119°25'E, 30°20'N	1065	Y.X. Liu; 140311
L12	Guixi city, Jiangxi Prov.	117°15'E, 28°16'N	41	Y.X. Liu; 140312
L13	Qianshan county, Jiangxi Prov.	117°47'E, 27°40'N	282	Y.X. Liu; 140313
L14	Ganzhou city, Jiangxi Prov.	114°54'E, 25°55'N	144	Z.Y. Liao & J.L. Dai; 140314
L15	Longnan county, Jiangxi Prov.	114°33'E, 24°37'N	390	M. He; 140315
L16	Jianou city, Fujian Prov.	118°08'E, 27°02'N	281	Y.X. Liu; 140316
L17	Yanxin county, Hubei Prov.	115°02'E, 29°42'N	121	Y.X. Liu; 140317
L18	Xinning county, Hunan Prov.	110°51'E, 26°26'N	301	Y.X. Liu; 140318
L19	Qingyuan, Guangdong Prov.	112°46'E, 23°44'N	418	Y.X. Liu; 140319
L20	Duan county, Guangxi Prov.	108°02'E, 24°06'N	203	Y.X. Liu; 140320
L21	Guilin city, Guanxi Prov.	110°15'E, 25°12'N	196	Y.X. Liu; 140321
L22	Xian city, Shanxi Prov.	108°57'E, 34°12'N	432	Y.X. Liu & C.Liu; 140322
L23	Nanjiang county, Sichuan Prov.	106°41'E, 32°29'N	1002	Y.X. Liu; 140323
L24	Xishui county, Guizhou Prov.	106°11'E, 28°19'N	1181	Y.X. Liu; 140324
L25	Jingdong county, Yunnan Prov.	100°42'E, 24°36'N	1484	Y.X. Liu; 140325

Chromosome preparation

For cytogenetic analysis, the somatic chromosomes in the meristematic cells of the roots tips were used. Firstly, approximately 1-2 cm long root tips were collected and treated with 0.002 M 8-hydroxyquinoline for 6 h at 4°C. The tips were then fixed in 3:1 absolute ethanol:glacial acetic acid at 4°C for a minimum of 24 h. After washing with tap water, the root tips were macerated in 1 M hydrochloric acid at 60°C for 6 min, and then stained with phenol-fuchsin for 12 h. Finally, the well stained root tips were toned and tapped in 45% acetic acid and pressed using a microscope slide (Zhou et al., 2007; Liu et al., 2011). Photomicrographs of chromosomes in the mitotic metaphase were taken with a photomicroscope (Nikon Eclipse 50i, Japan), equipped with a photographic camera (Cool SNAP cf, Photometrics, USA); the images were stored in a computer and processed with Adobe Photoshop, using only those functions that could be evenly applied to the entire image.

Statistical analyses

The chromosome counts were determined using at least 20 well-spread metaphase cells for each population. Chromosome measurements were carried out in at least 5 well-spread metaphases for each population. The following parameters were calculated: the absolute (mm) and relative (%) length of each chromosome pair; the absolute size of the haploid complement (mm), the arm ratio (long arm/short arm) of each chromosome pair, centromeric index, and coefficient of variation in the chromosome size. The karyotype asymmetry index (AsK%) was calculated using the formula described by Arano and Saito (1980). The karyotype formulae (KF) were determined from the chromosome morphology based on the centromere position in accordance with the classification of Levan et al. (1964) and the karyotype classification was done as described by Stebbins (1971). To group the studied populations by similarity in their karyotypic features, we used the unweighted pair group method with arithmetic mean (UPGMA) clustering, together with ordination based on the principal coordinate analysis (PCoA).

RESULTS

A total of 466 individuals from 25 populations of *L. radiata* were examined. Chromosome counts revealed that 10 populations were diploid (2n = 2x = 22) and 15 were triploid (2n = 3x = 33). Except for one diploid population (L14) containing some triploid plants, the remaining 24 populations showed a single cytotype. The tetraploid individuals were not present in any the cytotypes observed. The pictures of the mitotic metaphases and karyograms of the populations are presented in Figure 1. The somatic chromosome numbers (2n), ploidy levels, haploid chromosome length (HCL), AsK%, symmetry classes of Stebbins, and KF are summarized in Table 2.

Figure 1. Mitotic metaphase chromosomes in the meristematic cells of root tips in *L. radiata* populations (L1-L25); L7, L12, L14, and L21 populations possessed two karyotypes.

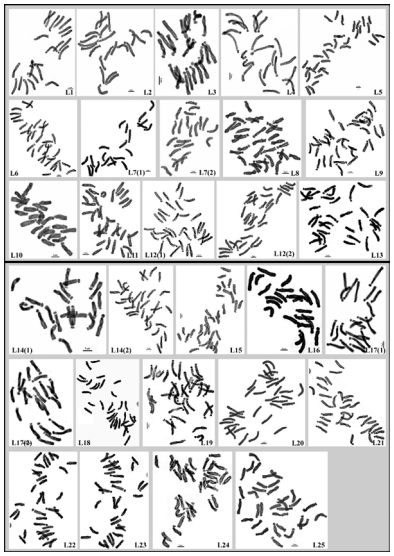

Figure 1. Mitotic metaphase chromosomes in the meristematic cells of root tips in *Lycoris radiata* populations (L1-L25); L7, L12, L14, and L21 populations possessed two karyotypes.

Table 2. Karyotypic features in 25 *Lycoris radiata* populations occurring in China.

Population	2n	PL	HCL	L/S	DRL	CI	CV	AsK%	SC	KF
L1	22	2x	155.47	1.53	3.94	10.49	11.44	89.56	4A	4st+18t (2SAT)
L2	22	2x	154.38	1.70	4.93	11.05	14.33	88.99	4A	4st+18t
L3	22	2x	150.71	1.30	2.38	11.79	9.28	88.16	4A	4st+18t
L4	22	2x	156.39	1.77	5.29	10.71	17.70	89.27	4A	2st+20t
L5	33	3x	114.06	1.39	2.91	12.07	10.88	87.98	4A	9st+24t
L6	33	3x	129.41	1.44	3.33	11.80	10.87	88.22	4A	9st+24t
L7	22	2x	134.32	1.32	2.54	13.32	9.35	86.72	4A	10st+12t, 12st+10t
L8	33	3x	121.25	1.44	3.25	12.16	10.44	87.90	4A	9st+24t
L9	33	3x	124.66	1.29	2.36	11.61	9.83	88.40	4A	15st+18t
L10	22	2x	116.86	1.31	2.43	13.44	9.25	86.60	4A	10st+12t
L11	33	3x	115.06	1.59	4.35	12.53	13.06	87.49	4A	9st+24t
L12	33	3x	123.37	1.58	4.30	10.87	13.64	89.12	4A	6st+27t, 33t
L13	33	3x	131.44	1.50	3.77	10.43	11.23	89.57	4A	6st+27t
L14	22,33	2x,3x	116.52	1.41	3.10	11.70	10.65	88.32	4A	10st+12t, 33t
L15	33	3x	113.88	1.68	4.81	14.54	15.90	88.97	4A	6st+27t
L16	22	2x	140.63	1.63	4.52	11.37	13.82	88.69	4A	8st+14t
L17	22	2x	158.02	1.71	4.92	11.29	15.60	88.82	4A	8st+14t, 10st+12t
L18	33	3x	112.73	1.51	3.81	11.58	12.98	88.41	4A	6st+27t
L19	33	3x	149.11	1.71	5.00	10.05	15.31	90.01	4A	33t
L20	33	3x	145.21	1.51	3.78	10.36	11.37	89.62	4A	33t
L21	33	3x	134.36	1.60	4.39	10.77	13.26	89.31	4A	6st+27t
L22	33	3x	148.66	1.71	4.76	12.06	15.72	87.98	4A	15st+18t
L23	33	3x	114.54	1.30	2.36	12.34	10	87.66	4A	12st+21t
L24	33	3x	115.99	1.40	2.99	11.92	10	88.09	4A	9st+24t
L25	33	3x	121.02	1.39	2.82	10.82	11	89.17	4A	3st+30t

2n = somatic chromosome number; PL = ploidy levels; HCL = haploid chromosome length; L/S = longest/shortest chromosome ratio; DRL = difference of relative length range; CI = centromeric index; CV = coefficient of variation; AsK% = karyotype asymmetry index; SC = symmetry classes; KF = karyotype formula; subterminal-centromeric(st), terminal-centromeric (t).

Among all the populations of *L. radiata*, HCL was highest (158.02 mm) in the Yanxin population (L17), which was diploid with 2n = 22, whereas it was lowest (112.73 mm) in the Xinning population (L18), which was triploid with 2n = 33. Most of the triploid populations had relatively higher HCL in comparison to the triploid populations. Among the populations studied, L4 possessed the highest CV (17.70) indicating the highest variation among its chromosomes compared to the other *L. radiata* populations, whereas L10 possessed the lowest value (9.25). These populations also varied in their KF (Table 2). The chromosomes were mostly telocentric and subterminal, except in two populations (L19 and L20) that possessed only telocentric chromosomes. Compared to the other populations, L1 had one pair of satellite chromosomes. However, some populations possessed the same KF. For example, the KF of L1, L2, and L3 populations were 4st+18t; those of L5, L6, L8, L11, and L24 were 9st+24t, of L13, L15, L18, and L21 were 6st+27t; of L9, L22 were 15st+18t; L19, and of L20 were 33t. However, different cytotypes were found in the same population, such as L7, L12, L14, and L17. The AsK % varied from 86.60 (in the diploid population, L10) to 90.01 (in the triploid population, L19).

Analysis of the chromosome constitution in the 25 *L. radiata* populations revealed that the arm ratios of the 25 pairs of chromosomes, both in the diploid and triploids, exceeded 2.0, indicating a very high intrachromosomal asymmetry. However, the ratio of longest to the shortest chromosome was <2. Both the above-mentioned characteristics classified the karyotype of all the diploid and triploid populations as 4A (Stebbins, 1971; Table 2).

The UPGMA clustering of the species based on the karyotype data produced two major clusters (Figure 2). The first major cluster I comprised of two sub-clusters: one composed of

eight triploid populations (L12, L19, L25, L20, L21, L15, L18, and L13) together with three diploid populations (L1, L2, and L3) and the other composed only of the diploid population L4. The second cluster II also comprised of two sub-clusters: one composed of two diploid populations (L7 and L10) and the other composed of eight triploid populations (L9, L22, L24, L5, L23, L6, L8, and L11) together with three diploid populations (L14, L16, L17), all in close proximity to each other.

The PCoA analysis, performed to visualize the displacement of the populations, revealed that the first three principle coordinates based on Eigen values accounted for 99.96% of the total variations among all the populations studied. The first (PCoA 1), second (PCoA2), and third (PCoA3) principal components accounted for 98.36, 0.96, and 0.64% of the total variance. The bi-plot, created on the basis of the first two PCoAs (99.32% of the total variability), supported the clustering pattern of the UPGMA dendrogram, which also divided the studied populations into two main clusters: Groups I and II (Figure 3). The PCoA provided a better graphical illustration and a clear separation of the *L. radiata* populations.

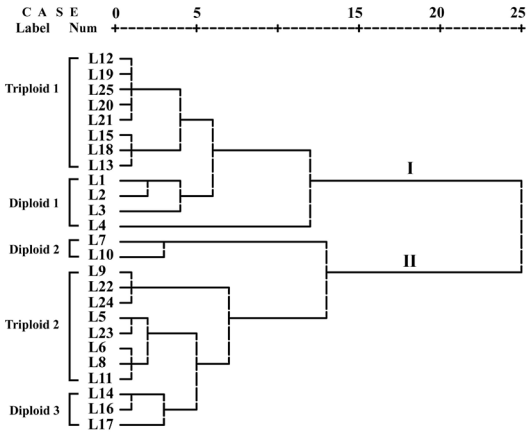

Figure 2. UPGMA cluster analysis of 25 *Lycoris radiata* populations.

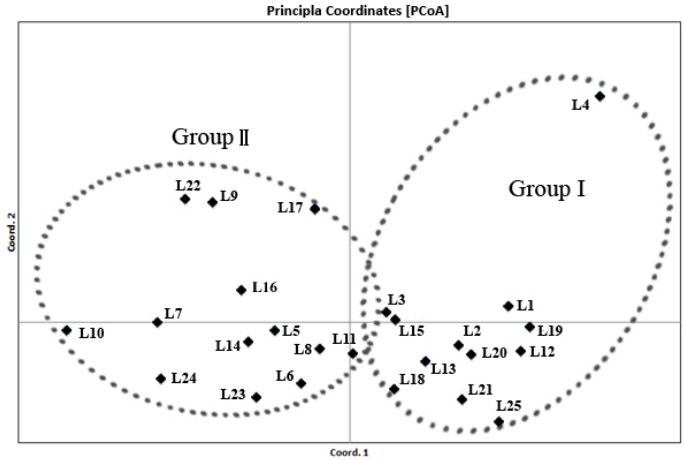

Figure 3. Principal coordinates analysis of 25 *Lycoris radiata* populations.

DISCUSSION

Several cytological studies conducted on *L. radiata*, worldwide, have revealed that *L. radiata* is a complex species that can be diploid (2n = 2x = 22) or triploid (2n = 3x = 33; Takemura, 1962; Chen and Li, 1985; Kurita, 1989; Sun et al., 1998). The basic chromosome number, x, of *L. radiata* is 11. This completely sterile species is very common and wide spread in China. However, according to our field observations, the fertile species is isolated, in the East China region. Zhou et al. (2007) reported a tetraploid cytotype of *L. radiata* for the first time; they observed that in these strains, the tepals did not recurve and the margins did not undulate, characteristics very distinct from those of the diploids and triploids. However, the discovery of the new tetraploid cytotype raised some questions. In our extensive field investigations, we did not find the species with the tetraploid cytotype, probably because of the small range of distribution of the tetraploids.

The usual karyotype of *L. radiata* consists only of rod-shaped chromosomes with subterminal and terminal constriction (with only one arm). However, several studies have shown some abnormal karyotypes of *L. radiata*, such as 2n = 33 = 1m + 31t + 1B, 2n = 32 = 1m + 31t (Bose, 1963; Kurita, 1988); 2n = 22 = 4st + 18t (Chen and Li, 1985; Sun et al.,1998); 2n = 23 = 6st + 14t + 2T + 1B, 2n = 22 = 1m + 12st + 8t + 1B (Shao et al., 1994); 2n = 24 = 6m + 8sm + 6st + 4t (Qin et al., 2004); 2n = 21 = 1m + 6st + 4t + 9T + 1B, 2n = 21 = 1M + 10st + 9T + 1B (Zhou et al., 2004); 2n = 21 = 1m + 20st, 2n = 25 = 1m + 20st + 2t + 2T (Zhou et al., 2007). In addition, Qin et al. (2004) observed long oval chromosomes. The karyotypes observed in our studies were different from those reported previously. The present data document the most extensive karyotypes known to date for *L. radiata*, with 11 karyotypes (Table 2) and different cytotypes observed in the same population. On the basis of previously reported results and those present in this study, it can, therefore, be concluded that the chromosome numbers and karyotypes of *L. radiata* vary in different populations.

Mookerjea (1955) observed that the karyotypes of *L. radiata* were very variable, and also consisted of satellite chromosomes; because the author could not explain the great variability in the karyotypes of *L. radiata*, the opinions expressed could not gain sufficient attention at that time. Based on the results from our studies on the 25 *L. radiata* populations, we concur with the observations of Mookerjea (1955) that the karyotypes in *L. radiata* show great variability among different populations and even within the same population. In addition, we observed the satellite chromosomes in the L1 population, confirming the results of Mookerjea (1955).

Polyploidy, defined as the possession of at least three complete set of chromosomes, is a widespread phenomenon in the flowering plants. It occurs in 30-35% of the angiosperms, according to Stebbins (1971), whereas Coghlan et al. (2005) estimated that 50-70% of all the species could be polyploid. Polyploidy has significant effects on the biochemistry, ecophysiology, and morphology of plants (Balao et al., 2009). Of the 25 populations surveyed, 15 were triploids. Clearly, polyploidy is prevalent in *L. radiata*. However, in the present study, except for one diploid population containing some triploid plants, the remaining populations had a single cytotype. This indicates an effect of minority cytotype exclusion and/or a widespread lack of gene-flow between the ploidy levels in nature. Balao et al. (2009) used sample chromosome counts and flow cytometry to determine the overall genome size and the ploidy levels in 244 individuals belonging to 25 populations of *Dianthus broteri*. Extensive variation in chromosomes numbers (four levels of ploidy) was detected with each population reported to have a single ploidy level. Hardy et al. (2001) and Husband and Sabara (2004) also reported that *Centaurea jacea* and *Chamerion angustifolium* plants, which possessed different ploidy levels, were effectively isolated, reproductively. Levin (1975) reported

that cytological races within a polyploid species could be spatially/ecologically segregated, which was explained on the basis of minority cytotype exclusion or the varying ecological tolerances (Mandáková and Münzbergová, 2006).

The possible origin of the triploid strains of *L. radiata* remains debatable and has attracted the attention of many researchers. Allozyme analysis by Chung (1999) in eight Korean populations of the sterile triploid revealed that all the 24 allozyme loci surveyed were monomorphic in all the populations. He suggested that only a few bulbs of *L. radiata* were introduced from China, and secondarily, from Japan and Korea, and they were naturalized in the South Korean peninsula via a strong vegetative reproduction by the rapid formation of new bulbs. These results suggested that there were few genetic variations among the triploid strains in Japan and Korea and that they might have evolved from a common ancestor introduced from China. In addition, Hayashi et al. (2005) also reported that an unidentified triploid could be the mother clone of all the triploid strains in Japan. Inter-simple sequence repeat markers analysis by Lv (2006) conducted in a few species of *Lycoris* indicated that no special bands were amplified in the different triploid populations. They suggested that the triploid *Lycoris* species was highly identical, genetically. Our results demonstrate that the triploid strains, which were distinctly divided into two parts (Triploid 1 and Triploid 2; Figure 2), indicate that the strains of *L. radiata* showed a genetic differentiation and may not be a highly identical species, genetically. The two groups of triploid strains might have originated from different diploid strains of *L. radiata*. Whether the triploids were derived from different diploids or not, is a question that needs further investigation using allozyme, molecular, and *in situ* hybridization methods.

Conflicts of interest

The authors declare no conflict of interest.

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