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Evidence for the Correlation between rDNA Variation and Satellite Heteromorphy in Somatic Chromosome Complements of *Lobelia fulgens* 'Queen Victoria' (Subfamily Lobelioïdeae, Family Campanulaceae)

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齊藤由紀子[1, 2]・國府方吾郎[1, 2]・Michael Möller[3]：*Lobelia fulgens* 'Queen Victoria' における染色体上 rDNA 部位と付随体の異形性

The genus *Lobelia* (Campanulaceae) comprises of more than 360 species, primary distributes from tropical to temperate regions (Mabberley 1997). *Lobelia fulgens* Humpl. & Bonpl. ex Willd. is part of the cardinal-flower *L. cardinalis*-complex of sect. *Lobelia* subsect. *Lobelia* (Thompson and Lammers 1997). The taxonomy of this complex is complicated. Two species are recognized in cultivation which are roughly equivalent to two subspecies of McVaugh (1943) and Bowden (1982); hardier broad-leaved plants are known as *L. cardinalis* L., frost-tender narrow-leaved plants are called *L. splendens* Willd. or sometimes *L. fulgens*. Thompson and Lammers (1997) regarded *L. fulgens* as an independent species. It is one of the most popular ornamental species and several open pollinated cultivar selections exist showing dark bronze leaves (*e.g.*, 'Illumination', 'Queen Victoria').

The chromosome number has been established to ca. 22% of *Lobelia* species (197 reports; Lammers 1993). A cladistic analysis of chloroplast restriction sites indicated three broad cytoevolutionary groups: 1) woody diploids with x = 21 in Chile and diploids with x = 14 in Africa, Asia and Hawaii; 2) herbaceous diploids with several series of dysploid chromosome numbers mainly in Africa and Australia; and 3) widespread and herbaceous taxa based on a derived n = 7 (with frequent polyploids, Stace and James 1996), in which *L. fulgens* belongs.

Despite the numerous cytological investigations were carried out on the genus, a detailed karyotype study has been performed only on eight Brazilian *Lobelia* species of subgenus *Tupa* (Ruas *et al.* 2001). All species analyzed showed the chromosome number of 2n = 28, suggesting the tetraploid origin of the species and the basic number of x = 7. Their karyotype commonly consists of mainly metacentric and submetacentric chromosomes.

The aim of the present study is to investigate the chromosome number of four individuals of *L. fulgens* 'Queen Victoria' by a standard aceto-orcein squash method and by the fluorescent *in situ* hybridization (FISH) using ribosomal DNA (rDNA) probes to investigate the distribution and condensation pattern of rDNA sites on somatic chromosomes.

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Materials and Methods

Aceto-orcein squash method

Four individuals of *Lobelia fulgens* were cultivated in the living research collection of the Royal Botanic Garden Edinburgh (Table 1). Root tips were pretreated in 2 mM 8-hydroxyquinoline at 20°C for 4 h, and fixed in acetic ethanol (1 : 3) at 4°C for at least 2 h. They were macerated in 45% acetic acid at 60°C for 10 sec, and stained in 2% aceto-orcein at 20°C for 2 h, then squashed under a cover slip. This aceto-orcein squash method resulted in well-spread chromosomes that were suitable for the present study.

PCR amplification and DNA labelling

Total DNA isolated from *L. fulgens* by the DNeasy Plant Mini Kit (QIAGEN Cat. No. 69104) was used as a template for polymerase chain reaction (PCR) FISH probes. The PCR amplification of a part of the 45S rDNA array was performed using primer NS1 (5'-GTA GTC ATA TGC TTG TCT-3') and NS4 (5'-CTT CCG TCA ATT CCT TTA AG-3') designed by White *et al.* (1990). These were respectively located at the 5'end of 18S (NS1) and 2/3 downstream in 18S (NS4), resulting in an 18S rDNA fragment of approximately 1150 bp. The PCR profile for 30 cycles of DNA amplification was 1 min at 94°C, 1 min at 56°C and 1 min at 72°C. The amplified DNA fragments were labelled with digoxigenin- (DIG) dUTP by the Nick Translation Kit following the manufacturer's protocol (Roche, Cat. No. 976776). The labelled probe was dissolved with 50% formamide and 10% dextran sulfate (w/v) in 2 × SSC, and adjusted to a final DNA-concentration of 5 μg/ml. This hybridization mixture was denatured at 75°C for 10 min before being immediately chilled in ice-cold water for 10 min.

FISH using rDNA probes

Fixed root tips were digested in a mixture of 2% cellulase "Onozuka" RS (Yakult) and 1% pectolyase "Y-23" (Seisin) dissolved in distilled water (w/v ; pH 4.5) at 36°C for 20 min, and then washed in distilled water for 10 min. A single digested root tip was placed on a glass slide, the meristematic apex dissected and squashed in 45% acetic acid under a glass cover slip. The cover slip was removed from the slide by the dry-ice method. The air-dried root meristem preparation was treated with 0.1% RNase (w/v) in 2 × SSC at 37°C for 1 h. After washing in 2 × SSC at room temperature for 10 min, it was submerged in 4% paraformaldehyde (w/v) in phosphate-buffered saline (PBS) at room temperature for 5 min, and then rinsed in distilled water at room temperature for 10 min. The preparation was then

Table. 1. Appearance and frequencies of satellite rDNA sites in four individuals of *Lobelia fulgens*

Acc. no.*	2n	No. examined**		Appearance of satellite			
		roots	complements	1 satellite		2 satellites	
				No.	%	No.	%
20000008A	14	8 out of 9	13	13	100.0	0	0.0
20000008A	14	1 out of 9	3	0	0.0	3	100.0
20000008A combined	14	9	16	13	81.2	3	18.8
20000008B	14	4	10	0	0.0	10	100.0
20000008C	14	2	22	0	0.0	22	100.0
20000008D	14	3	24	0	0.0	24	100.0

*Accession number of the Royal Botanic Garden Edinburgh.
**Number of roots and chromosome complements investigated in the present study.

dehydrated through an ethanol series (75%, 80% and 100% ethanol at room temperature for 3 min each), and dried at 36°C for 30 min. The preparation was covered with 15 µl of the hybridization mixture, and mounted with a silicone-coated cover slip before being sealed with rubber solution. The preparation was then denatured at 80°C for 10 min and placed overnight in a humid chamber at 37°C for DNA hybridization to occur. Following the hybridization, the slide was rinsed in 4 × SSC at 40°C for 10 min twice. The hybridization signals on the chromosomes were detected with 20 µg/ml Anti-digoxigenin-fluorescein, Fab-fragmentavidin (Roche, 157013) in 1% bovine serum albumin dissolved in 4 × SSC at 37°C for 1 h. The slide was rinsed in 4 × SSC at room temperature for 10 min twice in a dark box, and received 100 µl of an antifade solution with 1 µg/ml propidium iodide (PI) for counter-staining at 4°C for 30 min before being mounted with a coverslip. The hybridization signals fluoresced yellow while the non-hybridized region fluoresced red when visualized using a double band excitation filter (Zeiss, filter set No. 23).

Description of chromosomes

Chromosomes at mitotic metaphase were classified by arm ratio (R = long arm length/short arm length ratio) following Levan *et al.* (1964). Median- (R = 1.0 to 1.7), submedian- (R = 1.8 to 3.0), subterminal- (R = 3.1 to 7.0) and terminal- (R = 7.1 to ∞) centromeric position were designated and symbolized as "**m**", "**sm**", "**st**" and "**t**", respectively.

Results and Discussion

The four individuals of *Lobelia fulgens* 'Queen Victoria' investigated (RBGE 20000008A - D)

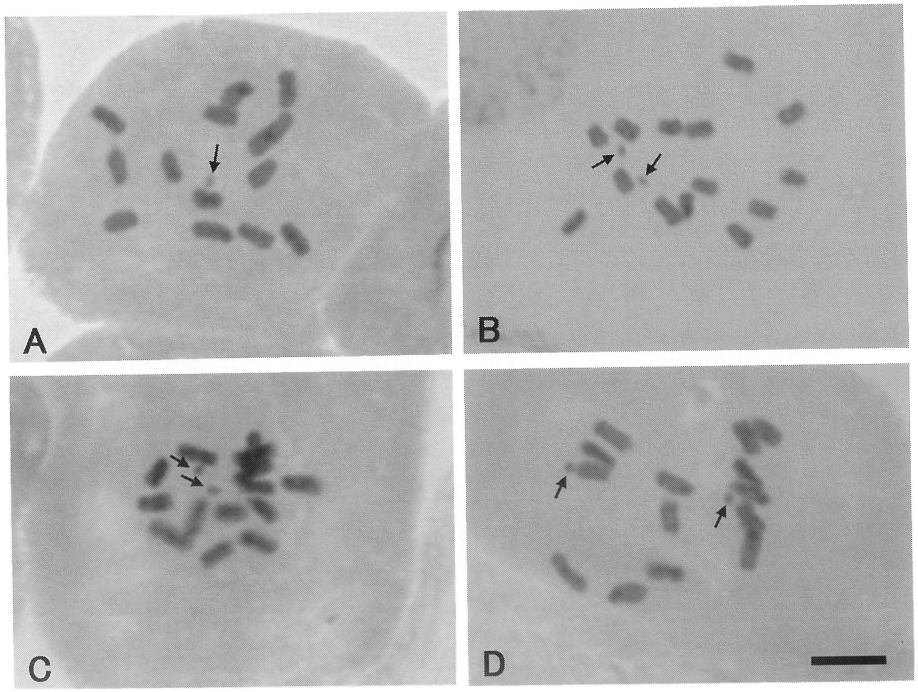

Fig. 1. Aceto-orcein stained chromosomes at mitotic metaphase in four individuals of *Lobelia fulgens* 'Queen Victoria' investigated. **A**. 20000008A. **B**. 20000008B. **C**. 20000008C. **D**. 20000008D. Arrows show the satellites. Bar = 10 µm.

commonly showed the chromosome number of 2n = 14 (Fig. 1). This is a first count for this species. In sect. *Lobelia* subsect. *Lobelia* including *L. fulgens*, this is the predominant diploid number. Out of 22 species investigated so far 18 share this diploid number (Lammers 1993), including *L. cardinalis* which closely related to *L. fulgens* (Thompson and Lammers 1997). The basic chromosome number of x = 7 found in *L. fulgens* is highly conserved in section *Lobelia* species (Knox and Kowal 1993, Lammers 1993, Stace and James 1996).

Ideograms of the four individuals, 20000008A, B, C and D, based on orcein-stained chromosomes are shown in Fig. 2. The fourteen chromosomes of the four individuals are arranged according to length, from the longest to shortest. Because of intrinsic variation in measurements, homologous pairs of chromosomes do not necessarily pair up. The lengths of the chromosomes ranged from 6.1 to 2.9 μm. The four individuals commonly showed the karyotype consisting of 4 **sm** and 12 **m** chromosomes. The longest pair of chromosomes was submetacentric. The second pair of **sm** chromosomes were found in a middle position (from the 4th to 10th). The arm ratios of the two **sm** chromosomes were very close to 1.7, the highest extreme of the range of **m** chromosome defined by Levan *et al.* (1964).

Three individuals of 20000008B, C and D had a satellite on two out of 12 **m** chromosomes (Fig. 2B - D), while that of 20000008A had a single satellite on one out of 12 metacentric chromosomes in a complement (Fig. 2A). The satellite-chromosomes (sat-chromosomes) of the four individuals were

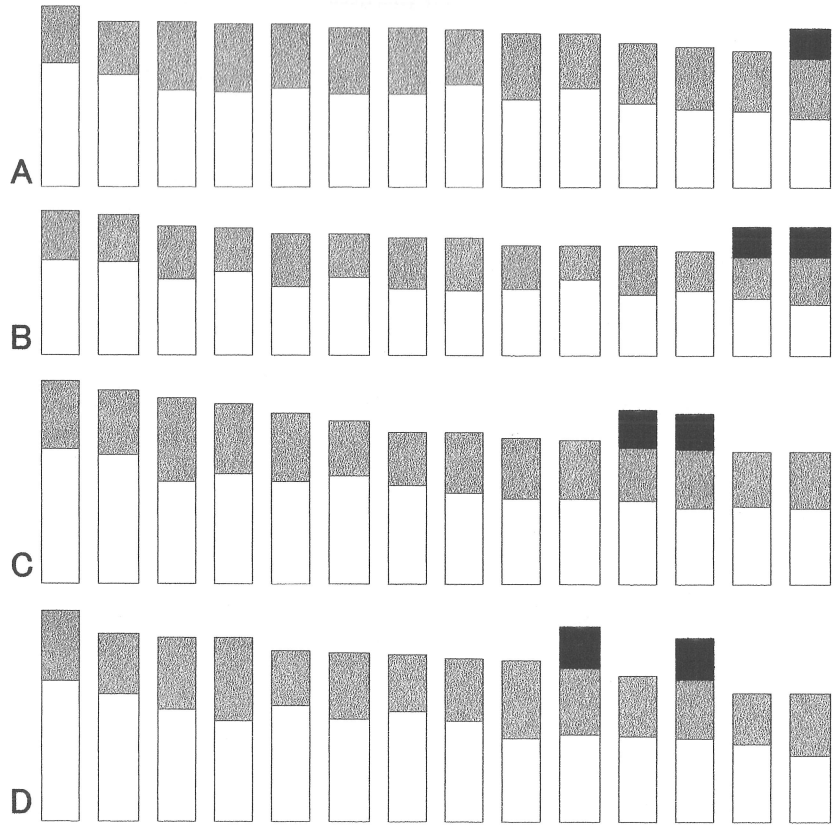

Fig. 2. Ideograms at mitotic metaphase in four individuals of *Lobelia fulgens* 'Queen Victoria' investigated. **A.** 20000008A. **B.** 20000008B. **C.** 20000008C. **D.** 20000008D. Open areas show the long arm, grayish areas show the short arm and solid areas show the satellite.

among the smallest and fell in position 11 to 14 (Fig. 2). Cytotaxonomically there were no statistical significant differences among the four individuals investigated, except for the sat-chromosomes.

FISH analyses revealed a single rDNA site per genome residing on the terminal of the two homologous **m** chromosomes in all chromosome complements observed for all four individuals investigated. The extent of rDNA sites in sat-chromosomes included not only the satellite itself, but the satellite stalk (Figs. 3C and 4B) as well as a small portion of the short arm of the **sm** chromosome (Figs. 3E and 4D).

Besides the variation in number of satellites, all homomorphic complements of all the individuals showed a statistically significant difference between the length of the secondary constriction in a pair of sat-chromosomes ($p<0.05$; Student's *t*-test). In three individuals, 20000008B, C and D, all 56 cells in nine roots investigated showed the two satellites on a **m** chromosome (frequency 100%; Figs. 3C - E and 4B - D). However, only a single satellite rDNA site was found on a **m** chromosome of 20000008A. The second rDNA site was present as a small signal at the terminal of the short arm of a **m** chromosome only; *i.e.*, the complement showed heteromorphy in rDNA satellites (Figs. 3A and 4A). This phenomenon was not, however, fully consistent: in eight root tip preparations observed, all 18 complements observed exhibited this heteromorphy. Three complements from the ninths root tip of 20000008A showed no heteromorphism, and a satellite was observed on both **m** chromosomes (frequency 16.7%; Fig. 3B). This was interpreted by the presence of a chimeric protomeristem, resulting in roots with and without cells having homomorphic pairs of satellite chromosomes in 20000008A. Further detailed investigations are necessary to verify this hypothesis.

The heteromorphism of the satellite morphology in 20000008A may be due to several factors, related to functional, or structural differences in the organization of the rDNA gene array:

1) *artifact of preparation*: one of the two satellites might easily break off from the short arm during squashing. However, this is unlikely as none of the complements of any other three individuals showed such weakness of the stalk. Furthermore, the 'broken-off' satellite was never found in the complements with a heteromorphic pair, neither in aceto-orcein or FISH preparations. In FISH, satellites should be visible even when folded beneath other chromosomes.

2) *Structural factors*: a) *loss of satellite*: one of the two satellites might have been lost during previous cell cycles except for a small fragment on the terminal of the short arm. This is a possible explanation if the individual was a chimera (see above). b) *different length of rDNA sites*: this is possible for the same reason given above, if the individual was a chimera. Variation in copy numbers of rDNA genes have been demonstrated in FISH, by employing different staining techniques ($AgNO_3$, CMA and FISH) (de Souza *et al.* 2004).

3) *Functional variation*: a) *hybrid origin*: the individual of 20000008A might be an undetected hybrid between parents with differently sized rDNA sites. However, there is no morphological evidence pointing to a hybrid origin; all four individuals have identical vegetative and generative gross characteristics. Furthermore 20000008A is fully fertile. b) *differential amphiplasty*: one of two nucleolar organizer regions (NOR) sites might be more condensed than the other, which might be linked to differential transcription of rDNA genes (*i.e.*, the selective inactivation of rDNA sites). Differential chromosome condensation patterns were firstly observed in hybrids. It was termed 'amphiplasty' when all chromosomes are affected, and 'differential amphiplasty' when only particular chromosomes are affected (Viegas *et al.* 2002). Navashin (1934) first observed differential amphiplasty in a certain *Crepis* species where one parental set of rDNA clusters were affected. The genetic mechanisms underlying this 'nucleolar dominance' are still unclear, but are possibly linked to length variation of control regions in the intergeneric

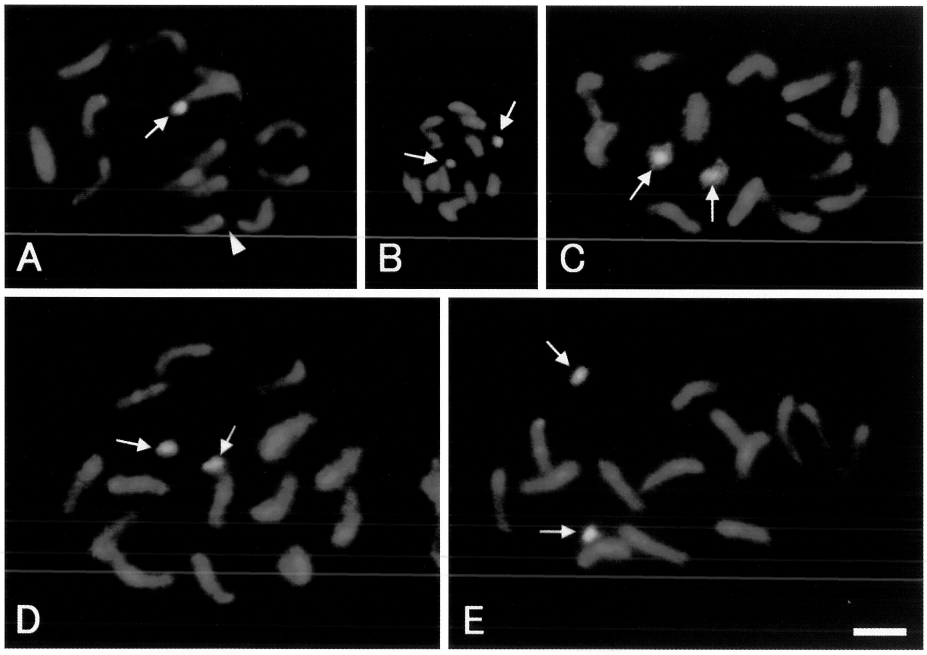

Fig. 3. FISH detected rDNA sites in chromosomes at mitotic prometaphase in four individuals of *Lobelia fulgens* 'Queen Victoria'. **A**. 20000008A with a heteromorphic pair of sat-chromosomes. **B**. 20000008A with a homomorphic pair of sat-chromosomes. **C**. 20000008B. **D**. 20000008C. **E**. 20000008D, all with homomorphic sat-chromosomes. Arrows show rDNA sites on satellite. Arrowheads show rDNA site fragments. Bar = 10 μm.

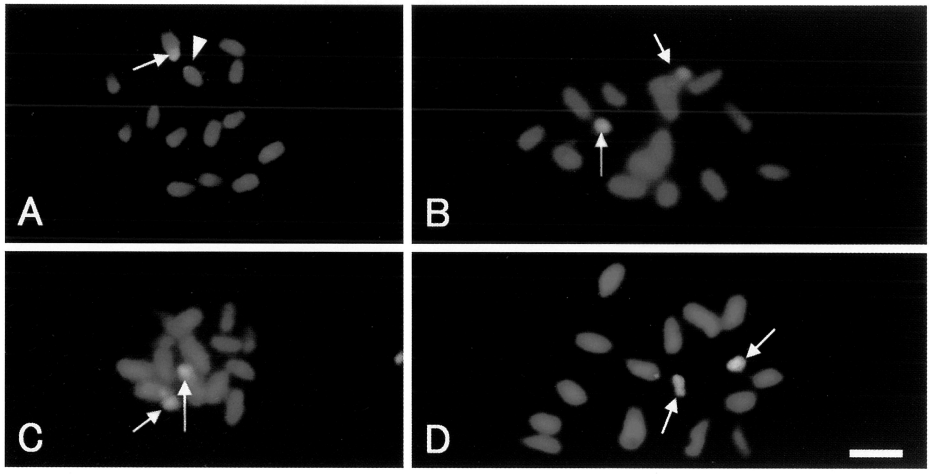

Fig. 4. FISH detected rDNA sites in chromosome at mitotic metaphase in four individuals of *Lobelia fulugens* 'Queen Victoria'. **A**. 20000008A with a heteromorphic pair of sat-chromosomes. **B**. 20000008B. **C**. 20000008C. **D**. 20000008D. Arrows show rDNA sites on satellites. Arrowhead shows rDNA site fragments. Bar = 10 μm.

spacers of rDNA genes (Viegas *et al.* 2002). Although the phenomenon has been mainly observed and studied in hybrids, rDNA heteromorphy has also been reported in species of diverse genera, such as *Galtonia* (Liliaceae; Forrest and Jong 2004) and *Glycine* (Leguminosae; Singh *et al.* 2001). Caperta *et*

al. (2002) found heteromorphic length in pairs of NORs in rye (= *Secale cereale* L.), and linked their heteromorphism strongly to the transcription of rDNA genes. The difference of lengths of the satellite stalks in homomorphic sat-chromosomes may be an indication of the presence of differential amphiplasty in the genus *Lobelia*.

At the present stage, it is difficult to ascertain which mechanism is responsible for the phenomenon reported here for the individual of 20000008A. If the individual was a chimera, with root tip cells having one and two satellites, a variation in copy number of rDNA gene arrays at the terminal NOR site would result from heteromorphic satellites. This structural variation would segregate in a Mendelian fashion in selfing offspring of this plant. The involvement of a mechanism similar to differential amphiplasty can be tested by crossings between heteromorphic and homomorphic individuals (with two satellites) or species. If the latter is homozygous dominant the offspring should all be heteromorphic with one satellite.

Whether differential amphiplasty or variation in rDNA gene arrays is responsible for the heteromorphy of rDNA satellites in the individual of 20000008A of *L. fulgens* 'Queen Victoria' requires further investigations including detailed cytological observations on progenies from crosses between homomorphic and heteromorphic individuals and hybridization between the species to elucidate possible genetic control mechanisms, and silver staining of chromosome preparations to distinguish active and inactive NORs.

Summary

Somatic chromosomes of four individuals of *Lobelia fulgens* cultivar 'Queen Victoria' were observed by the aceto-orcein staining method and the fluorescent *in situ* hybridization (FISH) method using 18S ribosomal DNA (rDNA) probes. The four individuals all showed the somatic chromosome number of 2n = 14 and the karyotype consisted of six **m**, six **sm** and two **st** chromosomes. Three individuals were homomorphic for their satellite morphology, but one showed the distinct heteromorphism in satellite appearance. The underlying genetic mechanisms require further detailed investigations.

摘　要

Lobelia fulgens の園芸品種 'Queen Victoria' 4個体の体細胞分裂中期染色体をアセトオルセイン染色法と 18S ribosomal DNA (rDNA) をプローブとした蛍光 *in situ* hybridization 法を用いて観察した。4個体は全て2n = 14で6個の中部動原体形染色体，6個の次中部動原体型染色体，2個の付随体をもつ中部動原体型染色体から構成されていた。1対の中部動原体型染色体においてrDNAとして検出される付随体の出現について，4個体のうち3個体では同形であったが，残る1個体（20000008A）では9本中8本の根端分裂組織で異形であり，残る1本で同形であった。このことより，20000008A個体内において，付随体の出現に関する異形と同形がキメラとして存在することが示唆された。この付随体の異形がみられる要因を調べるためには銀染色法などを含めた更なる研究が必要である。

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