

## Elucidating the phylogeny and taxonomic position of the genus *Spiniferomonas* Takahashi (Chrysophyceae)

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**Abstract:** *Spiniferomonas* represents a strictly single-celled plastid-bearing genus of silica-scaled chrysophytes, with a well-characterised morphology and ultrastructure, as well as a pretty known ecology based on scale records. However, the taxonomic status of this genus remains ambiguous due to the absence of relevant sequence data. *Spiniferomonas* has historically been classified in the Synurales, Paraphysomonadales, or “Chrysophaerellaceae” based upon interpretations of the silica scale morphology. In this study, we have aimed to genetically characterize a newly established *Spiniferomonas trioralis* culture as well as culture of unicellular *Chrysophaerella coronacircumspina* to elucidate the taxonomy of *Spiniferomonas* and to resolve the relationship between the two genera. Using SSU rDNA and rbcL concatenated sequences, we show that *Spiniferomonas* and *Chrysophaerella* are sister taxa in a well-supported monophyletic lineage of silica-scaled chrysophytes within the order Chromulinales.

**Key words:** Chromulinales, Chrysophyceae, *Chrysophaerella coronacircumspina*, silica-scaled chrysophytes, *Spiniferomonas trioralis*, rbcL, SSU rDNA, taxonomy

## INTRODUCTION

Chrysophytes (Chrysophyceae, Stramenopiles) represent a morphologically diverse but monophyletic group of mainly heterokont flagellates (ANDERSEN 2004; YANG et al. 2012). They are important primary producers or consumers of bacteria-sized microorganisms commonly observed in freshwater lentic ecosystems (FINLAY & ESTEBAN 1998; WOLFE & SIVER 2013; KRISTIANSEN & ŠKALOUD 2017). Chrysophytes excel in cell shape variability. This is mostly due to the naked character of many of their species lacking a cell wall or any other solid cell coverings. Even species living in loricas may produce both flagellated as well as amoeboid stages (STARMACH 1985). It is therefore not surprising that there are many enigmatic chrysophytes (KRISTIANSEN 1988; KRISTIANSEN et al. 2001) with little known ecology and no molecular data. However, some of these naked genera were recently re-examined using molecular genetics techniques, including heterotrophic *Spumella* Cienkowski (FINDENIG et al. 2010; GROSSMANN et al. 2016), and plastid-bearing *Ochromonas* Vysotskii (ANDERSEN et al. 2017), *Kre-*

*mastochryopsis* Bourrelly (REMIAS et al. 2020), and *Uroglena* Ehrenberg encompassing a genus *Eusphaerella* Skuja (PUSZTAI & ŠKALOUD 2019, 2021).

The only solid structure present in the life cycle of all chrysophytes, is the stomatocyst (SANDGREN 1981, 1983), although the cysts of a number of taxa are still unknown. Some chrysophytes additionally possess surface solid structures, such as scales, spines, and bristles. These organisms, known as silica-scaled chrysophytes, form at least three independent lineages based on molecular genetic data. The genera *Neotessella* Jo, Kim, Shin, Škaloud et Siver (ŠKALOUD et al. 2013b; Jo et al. 2016), *Mallomonas* Perty (Jo et al. 2011; SIVER et al. 2015; ČERTNEROVÁ et al. 2019), and *Synura* Ehrenberg (ŠKALOUD et al. 2020; JADRNÁ et al. 2021) belong to the order Synurales. Genera *Paraphysomonas* De Saedeleer and *Lepidochromonas* Kristiansen (SCOBLE & CAVALLIER-SMITH 2014; KAPUSTIN & GUIRY 2019) belong to the Paraphysomonadales. Finally, *Chrysophaerella* Lauterborn (ŠKALOUD et al. 2013b; ŠKALOUDOVÁ & ŠKALOUD 2013) is a member of the order Chromulinales.

The strictly single-celled plastid-bearing genus

*Spiniferomonas* (TAKAHASHI 1973; NICHOLLS 1985) has not yet been molecularly characterized and its phylogenetic position is therefore unknown. Its scales and bristles resemble those of *Chrysosphaerella*, which encompass both multi-celled as well as single-celled species, although the latter are very rare. NICHOLLS (1984) distinguished *Spiniferomonas* and *Chrysosphaerella* by the inability of *Spiniferomonas* to form colonies typical for *Chrysosphaerella* species. Conversely, KRISTIANSEN & TONG (1989) transferred some single-celled species from *Spiniferomonas* to *Chrysosphaerella* on the basis of similar ultrastructure of silica scales. To resolve this long-standing taxonomic bias, we established and genetically characterized the cultures of single-celled *Spiniferomonas* and *Chrysosphaerella* species: *S. trioralis* Takahashi and *C. coronacircumspina* Wujek et Kristiansen. By resolving the phylogenetic position of these two taxa, we were able to elucidate the taxonomy of the genus *Spiniferomonas*.

## MATERIALS AND METHODS

*S. trioralis* and *C. coronacircumspina* were collected with a plankton net with 20 µm mesh during our massive sampling campaign mainly focused on *Synura*, *Mallomonas*, and *Uroglena*-like taxa. More than 1,000 localities were investigated, mainly in Europe. We have found and sampled several single-celled *Spiniferomonas*-like taxa, but just two long-term cultures were successfully established. Both cultures originated from the mesotrophic forest ponds near the city of Liberec, Czech Republic. *S. trioralis* was sampled and isolated in autumn (13 Nov 2020) from the upper pond of the Kunratické rybníky pond system (50°44'59.41" N, 15°6'4.85" E) with pH 7.2 and conductivity 364 µS.cm<sup>-1</sup> at the time of sampling. *C. coronacircumspina* was sampled and isolated in spring (16 Apr 2020) from the lower pond of the Pivovarské rybníky pond system (50°45'14.37" N, 15°5'27.98" E), with pH 6.0 and conductivity 397 µS.cm<sup>-1</sup> at the time of sampling. Standard measurements of abiotic factors at the sampling site were carried out using a combined pH/conductometer (WTW 340i; WTW GmbH, Weilheim, Germany). Single-cell isolation and culture technique were carried out as described in PUSZTAI et al. (2016), except for the use of TES-buffered WC liquid medium (pH ~7.5; ANDERSEN 2005). For both cultures, the identity of the alga was determined by TEM (transmission electron microscopy) examination of the scales and spines. A drop from the algal culture was placed on a Formvar-coated copper grid and dried. Grids were rinsed with five drops of distilled water and dried again. Finally, the grids were examined with a JEOL 1011 TEM equipped with a Veleta CCD camera with acquisition software (Olympus Soft Imaging Solution GmbH, Münster, Germany).

DNA isolation was carried out as described in ŠKALOUDOVÁ & ŠKALOUD (2013). Nuclear SSU rDNA and chloroplast *rbcL* loci were amplified by PCR, providing a sufficient genus-level taxonomic resolution within the Chrysophyceae (ANDERSEN et al. 2017; KRISTIANSEN & ŠKALOUD 2017). The amplification of SSU rDNA was performed as described by ŠKALOUD et al. (2013), using the primers 18SF and 18SR (KATANA et al. 2001). The amplification of the *rbcL* gene

was performed according to PUSZTAI et al. (2019), using the primers *Chryso\_rbcL\_F4* and *Chryso\_rbcL\_R7*. The PCR products were purified and sequenced at MacroGen Inc. in Amsterdam, the Netherlands.

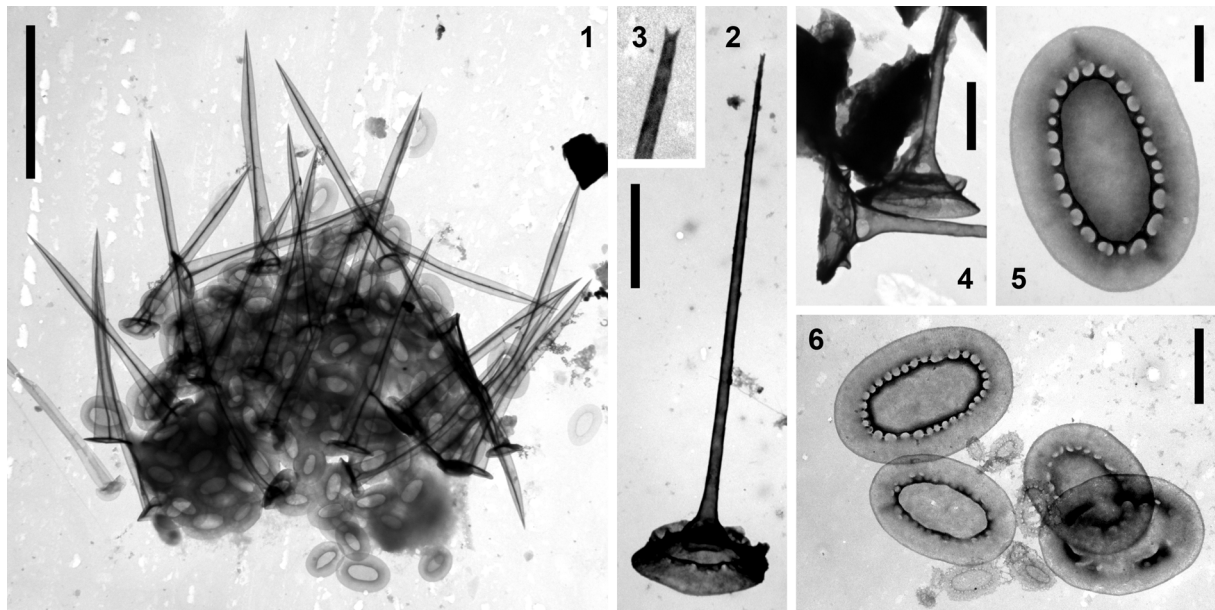
A concatenated SSU rDNA and *rbcL* alignment was produced, processed and analysed as described in PUSZTAI et al. (2019). The newly determined sequences were aligned to other sequences of Chrysophyceae from the GenBank database. The sequences were selected according to ANDERSEN et al. (2017) and KRISTIANSEN & ŠKALOUD (2017) to encompass all chrysophycean lineages. This selection was extended by all available sequences of *Chrysosphaerella* using BLAST (ALTSCHUL et al., 1990) and by newly acquired sequences of rare but unmistakable chrysophyte taxa *Dermatochrysis reticulata* (Meyer) Entwisle et Andersen isolated in summer (15 Jun 2017) from the Big pond, Newfoundland (46°59'1.97" N, 53°31'45.59" W). For each of the alignment partitions, the most appropriate substitution model was estimated using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.4 (DARRIBA et al. 2012). This procedure selected the following models: (1) GTR + I + G for SSU rDNA; (2) GTR + G for the first codon position of the *rbcL* gene; (3) TVM + I + G for the second codon position of the *rbcL* gene; and (4) GTR + G for the third codon position of the *rbcL* gene. The phylogenetic tree was inferred by Bayesian inference (BI) as described in PUSZTAI et al. (2019). Bootstrap analyses were performed as described in PUSZTAI et al. (2016). The GenBank accession numbers of all strains used in this study are provided in Supplementary Table S1.

## RESULTS

We successfully established long-term cultures of *Spiniferomonas trioralis* and *Chrysosphaerella coronacircumspina* (Figs 1–6). *Spiniferomonas trioralis* scales were typical, consisting of oval plate-like scales with a simple concentric ridge, and its spines had a disc-shaped base with a shaft tapering to a pointed apex (Fig. 1). *C. coronacircumspina* scale-case consisted of typical scales and spines (Figs 2–6) but possessed significantly higher variability (Fig. 6) than was observed in *Spiniferomonas*. Scales were oval, plate-like, with a characteristically decorated concentric ridge (Figs 5, 6). Spines were tubular in cross section, with a funnel-shaped double-disc base with a hole and a shaft tapering to a bifurcated apex (Figs 2–4).

A two-gene phylogenetic analysis based on nuclear SSU rDNA and plastid *rbcL* molecular loci clearly demonstrated *S. trioralis* and *C. coronacircumspina* represent closely related members of the order Chromulinales (Fig. 7). *S. trioralis* was inferred in sister position to all, colonial as well as single-celled, *Chrysosphaerella* species.

*Chrysosphaerella coronacircumspina* is the first single-celled species of the genus to be genetically characterized, and it is sister to all previously sequenced colonial species, including *C. brevispina* Korshikov, *C. longispina* Lauterborn, and *C. rotundata* Škaloudová et Škaloud. Accordingly, *Spiniferomonas* and *Chrysosphaerella* represent sister genera forming a monophyletic,



Figs 1–6. Scale–case ultrastructure (TEM) of *Spiniferomonas trioralis* and *Chrysosphaerella coronacircumspina*: (1) *S. trioralis* whole scale case consisting of characteristic scales and spines; scales oval, plate with a simple concentric ridge; spines triangular in cross section, with a simple disc–shaped base and a shaft tapering to a pointed apex; (2) *C. coronacircumspina* tubular spine in cross section, with a funnel–shaped double–disc base and a shaft tapering to a bifurcated apex; (3) *C. coronacircumspina* bifurcated apex of spine in detail; (4) *C. coronacircumspina* spines with well visible funnel–shaped double–disc base with a hole; (5) *C. coronacircumspina* oval, plate scale with a characteristically decorated concentric ridge; (6) *C. coronacircumspina* scales of different size and silicification. Scale bars represent 0.5  $\mu\text{m}$  (5), 1  $\mu\text{m}$  (6), 2  $\mu\text{m}$  (4), 3  $\mu\text{m}$  (2), and 5  $\mu\text{m}$  (1).

well–supported lineage.

## DISCUSSION

Silica–scaled chrysophytes represent a group that has a reasonably good species concept based on the ultrastructure of their scales, spines, and bristles (KRISTIANSEN & PREISIG 2007). However, molecular data are still lacking for many of their species or even for some genera. For example, the rather rare genera *Chrysodidymus* (now treated as a member of *Synura*) or *Neotessella* were revisited by polyphasic approach encompassing molecular genetic techniques quite recently (ŠKALOUD et al. 2013b; PUSZTAI et al. 2016). For the genus *Spiniferomonas*, the combination of its relative infrequency together with small cell size and subsequent oversight in samples probably caused its unclear phylogenetic position and taxonomic ambiguity. Considering the general fragility and difficulty in culturing of many chrysophytes (PUSZTAI & ŠKALOUD 2019), it is not surprising that the genus *Spiniferomonas* and the single–celled *Chrysosphaerella* species lacked in culture collections worldwide. Therefore, the establishment of their long–term cultures unblocked the possibility of elucidating their taxonomy by a robust phylogenetic framework as a standard (BOENIGK et al. 2012).

*S. trioralis* is a cosmopolitan, widely distributed, and the most common species of the genus *Spiniferomonas* in Europe (STARMACH 1985; ŠKALOUD et al. 2013a). It has been described, together with six other species including the type species of the genus, *S. bourrellii* Takahashi, from Japan and South Africa

as the most widespread species within the newly erected genus (TAKAHASHI 1973). According to the work of PREISIG & HIBBERD (1982, 1983), there were uncertainties as to whether or not the type species of *S. bourrellii* has a plastid. This issue was resolved by NICHOLLS (1985), who clearly demonstrated the presence of a plastid in the cells of *S. bourrellii* and confirmed the monophyly of the genus *Spiniferomonas*. Conversely, *C. coronacircumspina* is less common than the colonial species *C. brevispina*, but is still probably the most common unicellular species within the genus *Chrysosphaerella* in Europe (STARMACH 1985; ŠKALOUD et al. 2013a).

Our results confirmed the separation of *Spiniferomonas* and *Chrysosphaerella* assumed by KRISTIANSEN & TONG (1989) based on the priority of silica scale–case ultrastructure rather than on unicellular or colonial habit. According to KRISTIANSEN & TONG (1989), species with complex spine bases of a double–disk or at least with a septum and a nearby hole should belong to *Chrysosphaerella*, regardless of whether they are colonial or solitary. Species of the genus *Spiniferomonas* never possess such structures. Moreover, we carefully checked the original descriptions and it seems that further characteristic, not mentioned by KRISTIANSEN & TONG 1989, could be used to discriminate between *Spiniferomonas* and *Chrysosphaerella*: species of the latter genus possess mostly tubular spines with gently bifurcated tips. This character is most prominently visible in the colonial species *C. brevispina*, *C. longispina*, and *C. rotundata*

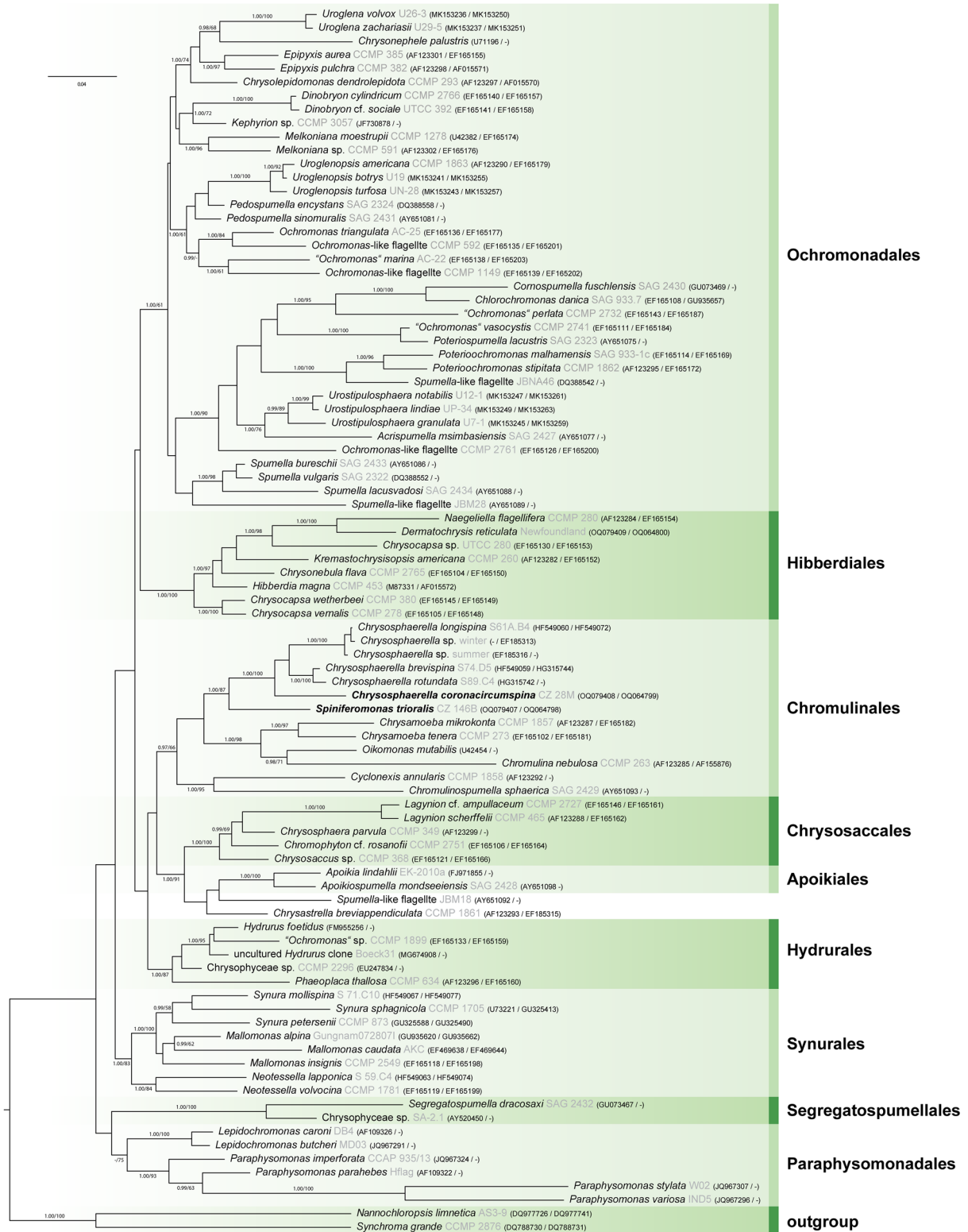


Fig. 7. Phylogeny of the Chrysophyceae obtained by Bayesian inference of the concatenated SSU rDNA and rbcL dataset. The analysis was performed under a partitioned model, using different substitution models for each partition. Values at the nodes indicate statistical support estimated by two methods: MrBayes posterior node probability (left) and maximum likelihood bootstrap (right). Newly obtained *Spiniferomonas trioralis* and *Chryso-sphaerella coronacircumspina* strains are marked in bold. Scale bar represents the expected number of substitutions per site.

(see ŠKALOUD et al. 2013a gallery for comparison). However, it is also always detectable in the unicellular species *C. septispina* (Nicholls) Kristiansen et Tong (NICHOLLS 1984), *C. annulata* Kristiansen et Tong (KRISTIANSEN & TONG 1989) and the newly sequenced *C. coronacircumspina* (Fig. 3).

According to the simplest tubular spine with only a septum and a nearby hole, the unicellular *C. septispina* and *C. annulata* are probably the basal species of the genus yet genetic data are missing to support this conclusion. On the other hand, there are several *Spiniferomonas* species possessing spines with a “transient morphology” similar to *Chryso-sphaerella*. *S. bourrellii* and *S. conica* Takahashi possess tubular spines with a funnel-shaped base, but with a single tip and no bifurcation. *S. silverensis* Nicholls have spines with a funnel-shaped base, not tubular but mostly triangular in cross-section, and this feature is typical of most *Spiniferomonas* species. Some of these evolutionary trends in changes in spine complexity and transitions between the two genera were originally suggested by NICHOLLS (1984), but without knowledge of their phylogeny. Such similar trends in scale ultrastructure and size changes are only partially known for *Mallomonas* and *Synura* silica-scaled taxa (ČERTNEROVÁ et al. 2019; JADRŇÁ et al. 2021; SIVER 2022). Therefore, molecular characterization of other *Spiniferomonas* and *Chryso-sphaerella* species is very important as it may provide a key to better understanding the evolution of silica-scaled chrysophytes and their armour in general.

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## Supplementary material

The following supplementary material is available for this article:

Table S1. Taxa selected according to ANDERSEN et al. (2017) and KRISTIANSEN & ŠKALOUD (2017) used in current Chrysophyceae phylogeny. Outgroup taxa selected according to YANG et al. (2012). Newly acquired strains are highlighted in bold.

This material is available as part of the online article (<http://fottea.czechphycology.cz/contents>)

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