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Kalinella bambusicola gen. et sp. nov. (Trebouxiophyceae, Chlorophyta), a novel coccoid *Chlorella*-like subaerial alga from Southeast Asia

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SUMMARY

The traditional green algal genus *Chlorella*, which comprised coccoid algae surrounded by a smooth cell wall and reproducing solely by autosporulation, has proved to be polyphyletic and extremely diverse in phylogenetic terms. We studied a new subaerial *Chlorella*-like strain CAUP H7901 and morphological, ultrastructural, and molecular phylogenetic investigations indicated that it represents a new lineage of the trebouxiophycean *Watanabea* clade, dissimilar from other members of this group. The alga has globular coccoid cells with a single parietal pyrenoid-bearing chloroplast. The pyrenoid is transected by multiple radial thylakoid bands. The alga reproduces exclusively by means of asexual autospores of unequal size. In 18S rDNA sequence phylogenies, it was nested within the *Watanabea* clade close to lineages containing *Chlorella saccharophila*, *Chlorella luteoviridis*, *Heveochlorella hainangensis*, and two uncharacterized strains, but alternative positions within the *Watanabea* clade could not be rejected by an approximately unbiased (AU) test. Here we describe this organism as a new genus and species *Kalinella bambusicola* gen. et sp. nov. Furthermore, we describe *Heterochlorella* gen. nov. to accommodate a species previously referred to as *Chlorella luteoviridis*.

Key words: green algae, *Heterochlorella*, *Kalinella*, phylogeny, taxonomy, trebouxiophyceae.

INTRODUCTION

Coccoid green algae with globular or elliptical vegetative cells reproducing exclusively by asexual autospores and surrounded by a smooth cell wall were traditionally classified in the genus *Chlorella* Beijerinck (Fott & Nováková 1969; Ettl & Gärtner 1995). More than 100 species of the genus were described using traditional morphological methods (Komárek & Fott 1983; Punčochářová 1994). However, scarcity of morphological characters and small dimensions of vegetative cells hampered suitable identification and discrimination of individual taxa (Ettl & Gärtner 1995). Heterogeneity of the genus *Chlorella* was suggested by ultrastructural (Kalina & Punčochářová 1987; Ikeda & Takeda 1995; Němcová & Kalina 2000; Yamamoto *et al*. 2005) and biochemical studies (Kessler & Soeder 1962; Kessler 1992; Takeda 1993). However, the real phylogenetic diversity of *Chlorella*-like algae has been revealed primarily by molecular methods (Friedl 1995; Huss *et al*. 1999; Krienitz *et al*. 2003, 2004; Yamamoto *et al*. 2003; Karsten *et al*. 2005; Aslam *et al*. 2007). Nowadays, *Chlorella*-like morphologies are known to have evolved several times in the Chlorophyceae and the Trebouxiophyceae (Krienitz *et al*. 2003, 2004). *Chlorella vulgaris* Beijerinck, the type species of the genus represented by its authentic strain SAG 211-11b, is a member of a trebouxiophycean lineage containing taxa that occur mostly in freshwater habitats. Consequently, the generic name *Chlorella* has to be reserved for taxa belonging to this group (Krienitz *et al*. 2004). As a result of polyphyly of the traditionally defined genus *Chlorella*, several independent *Chlorella*-like lineages were recently described as independent trebouxiophycean genera (Krienitz *et al*. 2004; Aslam *et al*. 2007; Zhang *et al*. 2008).

Apart from freshwater or marine ecosystems, *Chlorella*-like algae have frequently been found in different terrestrial habitats (Ettl & Gärtner 1995). A number of terrestrial *Chlorella*-like strains were found to belong to an independent trebouxiophycean lineage containing morphotypes corresponding to *C. saccharophila* (Krüger) Migula, *C. angusto-ellipsoidea* Hanagata et Chihara and *C. trebouxioides* Punčochářová (Krienitz *et al*. 2004; Aslam *et al*. 2007). Taxonomic description of this group as a new genus was suggested, though not formally published, by Kalina (1996) and by Friedl *et al*. (2007). However, phylogenetic and taxonomic revision of this species complex will be published soon

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(T. Friedl, pers. comm., 2007). Recent molecular phylogenetic studies (e.g. Huss *et al*. 2002; Krienitz *et al*. 2004; Zhang *et al*. 2008) illustrated that *Chlorella luteoviridis* Chodat, a species occurring mostly in terrestrial habitats, forms a separate trebouxiophycean lineage, probably sister to the *C. saccharophila* clade. Krienitz *et al*. (2004) referred to the *C. luteoviridis* lineage as '*Heterochlorella*' in their phylogenetic tree. However, this generic name was not formally established and taxonomically described. Other trebouxiophycean algae that are related to these lineages are coccoid terrestrial species *Watanabea reniformis* Hanagata *et al., Dictyochloropsis reticulata* Tschermak-Woess (Huss *et al*., 1999; Aslam *et al*. 2007), and *Viridiella fridericiana* Albertano *et al*. that inhabits extremely acidic environments (Huss *et al*. 2002). All of these species form a lineage that was informally called the *Watanabea* clade, which was defined as one of the trebouxiophycean lineages containing mostly terrestrial taxa (Karsten *et al*. 2005).

In the present study, we investigated the *Chlorella*-like strain CAUP H7901 isolated originally from a bamboo stem in a park in Singapore. We characterize the morphology, ultrastructure and phylogenetic position of this alga and describe it as a new trebouxiophycean genus and species. In addition, to resolve the untenable position of '*C.*' *luteoviridis* in the *bona fide* genus *Chlorella*, we propose here to validate the generic name *Heterochlorella* and the combination *Heterochlorella luteoviridis*.

MATERIALS AND METHODS

In the present study, we investigated two algal strains:

1 The strain CAUP H7901, isolated in 2007 from a sample of algal growth on *Gigantochloa* sp. in a park in Singapore;
2 The strain SAG 211-2a, the authentic strain of *Chlorella luteoviridis*, isolated originally from a pool in a forest of Oisquercq near Bruxelles, Belgium.

The strains were cultivated on agar-solidified Bold's Basal Medium (BBM) culture medium (Bischoff & Bold 1963). They were grown under a 12:12 h LD (light : dark) regime at 20°C with irradiance of 40 μmol m^{-2} s^{-1}. Living cells were examined by an Olympus BX 51 light microscope using differential and phase contrasts and the microphotographs were taken using Olympus Z5060 digital microphotography equipment. Material for transmission electron microscopy (TEM) observations was prepared from a mixed sample from cultures of different age (2 days, 1 week, 2 months). This method was chosen in order to detect changes in the amount and deposition of starch in the chloroplast (Neustupa *et al*. 2007). The samples were fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetroxide in 0.05 M phosphate buffer and 1% uranyl acetate in methanol. After dehydration in ethanol, the strains were embedded in a Spurr's resin (Spurr 1969) via isobutanol. Ultrathin sections, cut with a diamond knife on a Reichert–Jung Ultracut, were post-stained with lead citrate, and examined with the TEM JEOL 1011.

Detailed chloroplast morphology was investigated using a Leica TCS SP2 laser scanning confocal microscope equipped with an Ar-Kr laser at the 488 nm excitation line and an acousto optical beam splitter (AOBS) filter-free system collecting emitted light between 498 and 700 nm. The Leica 63×/1.4 N.A. oil immersion and 63×/1.2 water immersion objectives fitted on the Leica DM IRE2 inverted microscope were used. Optical sections of chloroplasts were captured and used for 3D reconstruction of their morphology. Chlorophyll autofluorescence was exploited to visualize the chloroplast structure. Final processing and 3-D reconstructions of confocal images were done in the Leica Confocal Software, version 2.61 (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) and the Image J 1.34p program (Abramoff *et al*. 2004).

For isolation of genomic DNA, cells were scraped from an agar plate with a clean spatula, transferred to an eppendorf tube, resuspended in distilled water and harvested by centrifugation. Total DNA was extracted using the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany). The 18S rRNA gene was amplified by polymerase chain reaction (PCR) in two overlapping parts. The first was obtained using the universal forward (F) primer according to Katana *et al*. (2001) and the green algal-specific reverse primer vivi1650R (T. Friedl, pers. comm., 2007). The second part was amplified using a D72F forward primer (5′-GACGCAATCACCGAGCCTT-3′, designed according to a sequence of the F-vivi1650R product) and the universal reverse primer ITS4 (White *et al*. 1990). PCR products were resolved by electrophoresis on vertical agarose gels. Bands of expected size were dissected and the DNA was purified using the QIAquick Gel Extraction Kit (Qiagen, Düsseldorf, Germany). The purified F-vivi1650R fragment was sequenced from both ends using sequencing primers as in Katana *et al*. (2001), whereas the D72F-ITS4 fragment was sequenced only at its 5′ end by the D72F primer to obtain the sequence of the 3′ end of the 18S rRNA gene. Sequencing reads were assembled with the CAP3 assembler server (http://pbil.univ-lyon1.fr/cap3.php) and manually edited by visual inspection of sequencing chromatograms. The newly obtained sequence, excluding the region representing the F primer, was deposited at GenBank with the accession number EU346910.

Basic Local Alignment Search Tool (BLAST) searches and a preliminary maximum likelihood (ML) analysis indicated that the 18S rDNA sequence of the

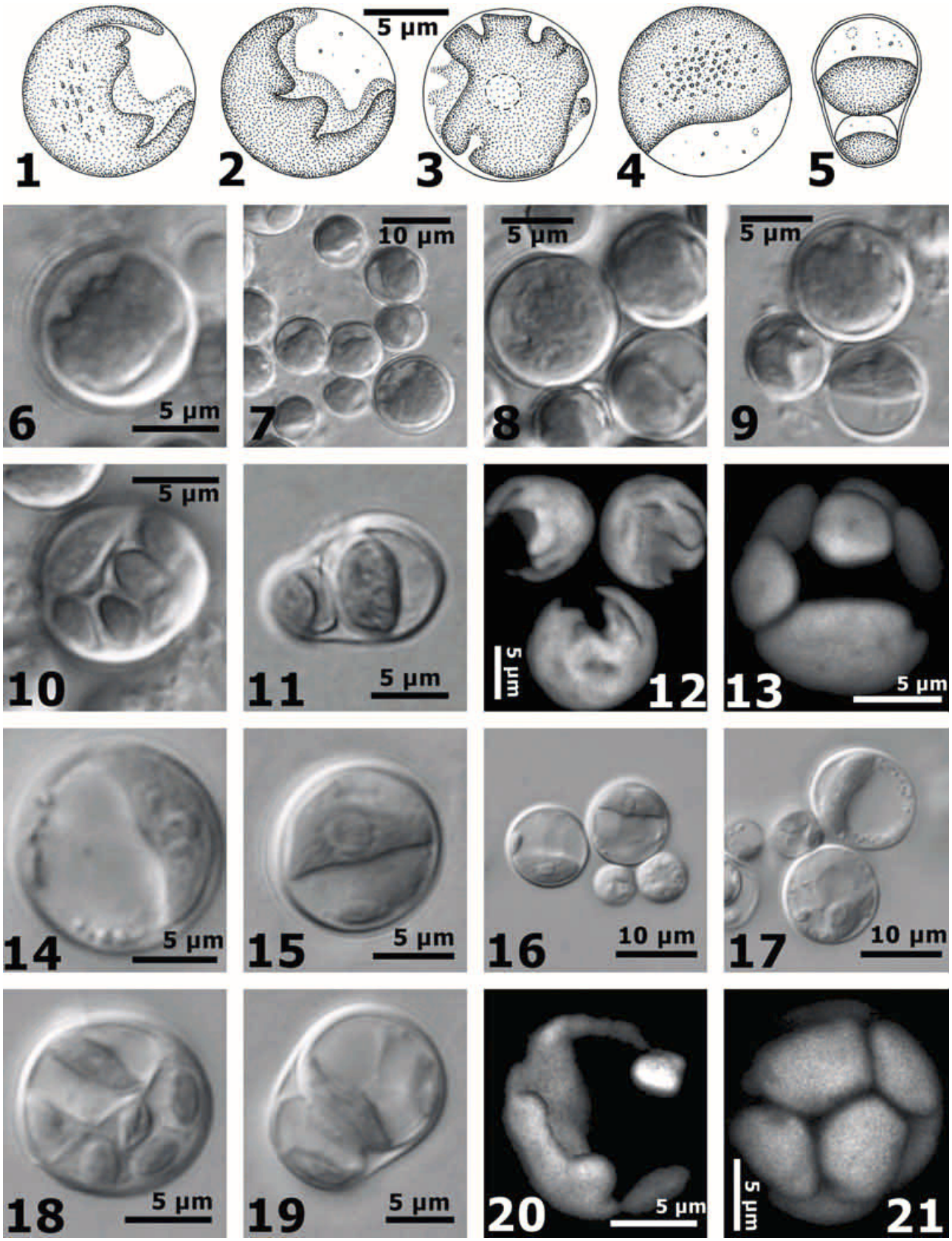
CAUP H7901 strain branches within the Trebouxiophyceae. The sequence was therefore added to an alignment of representative trebouxiophycean 18S rDNA sequences available from the DNA Data Bank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL) and GenBank databases (295 sequences at the time of the analysis) build using ClustalX (Thompson *et al*. 1997) and manual curation with GeneDoc (K.B. Nicholas and H.B. Nicholas, http://www.psc.edu\biomed\genedoc) guided by the secondary structure model for the *Chlamydomonas reinhardtii* 18S rRNA molecule available from the European Ribosomal RNA Database (http://www.psb.ugent.be/rRNA/secmodel/Crei_SSU.html; Wuyts *et al*. 2000). The ML analyses were carried out with RAxML 7.0.3 (Stamatakis 2006), using a generalized time reversible (GTR) model of nucleotide evolution with rate parameters estimated from the data. The Γ correction with four rate categories plus invariant positions and the alpha parameter estimated from the data were used to cope with a site-to-site rate variation. Three independent runs of the program were attempted and the result with the highest log likelihood was considered for further analyses. The ML bootstrap analysis was carried out on 100 replicates with RAxML using the same substitution model and the –f i (thorough bootstrap) option. For a distance bootstrap analysis (1000 replicates), bootstrap replicates of the data were created with SEQBOOT and distance matrices were computed using DNADIST in the PHYLIP 3.62 software package (J. Felsenstein, University of Washington) with F84 model of nucleotide evolution, transition/transversion ratio 2.0, one rate category and no Γ correction. Replicate trees were then reconstructed using BioNJ (Gascuel 1997) and a majority-rule consensus tree was obtained using CONSENSE from the PHYLIP package. The Bayesian analysis was carried out using MrBayes 3.1 (Huelsenbeck & Ronquist 2001). Two parallel MCMC runs were carried out for 1 million generations each with one cold and three heated chains using the GTR+Γ+I evolutionary model (with parameters estimated from the data). Trees were sampled every 100 generations. After visual inspection of log-likelihood values of sampled trees, the initial 2501 trees of each run were discarded as a very conservative 'burn-in' and posterior probabilities of tree bipartitions were calculated on the basis of the consensus of the remaining 15 000 (2×7500) trees. ML trees with alternative tree topologies specified by a predefined constraint (–g option) were inferred with RAxML using the procedure as for the unconstrained ML tree. Per-site log likelihoods for the alternative trees were computed with RAxML (–f g option) and fed into CONSEL (Shimodaira & Hasegawa 2001) to calculate *P*-values of the approximately unbiased (AU) and weighted Shimodaira-Hasegawa (wSH) tests (Shimodaira & Hasegawa 1999; Shimodaira 2002).

RESULTS

The vegetative cells of the CAUP H7901 strain are regularly globular (3.8–) 4.6–10.5 (–12.0) μm in diameter (Figs 1–9). They have a single compact, cup-shaped chloroplast with slightly waved margins (Figs 2,3,6,12,13) and a single nucleus. The pyrenoid is barely visible via light microscope, but it is well developed in most cells and surrounded by a starch envelope composed of numerous starch grains (Figs 22,25). Multiple radial bands composed of two or four thylakoids enter the pyrenoid matrix (Fig. 24). In the longitudinal cross-section they mostly appear as parallel bands (Fig. 25). The cell wall is smooth and thin, without any mucilage. It is composed of two layers with slightly different electron density. Old vegetative cells are regularly filled up with globular vacuoles. Reproduction takes place entirely by means of two to eight globular autospores (Figs 5,10,11,23). In most cases, there is a single large autospore and one, three or seven smaller autospores produced within a single sporangium (Figs 10,11).

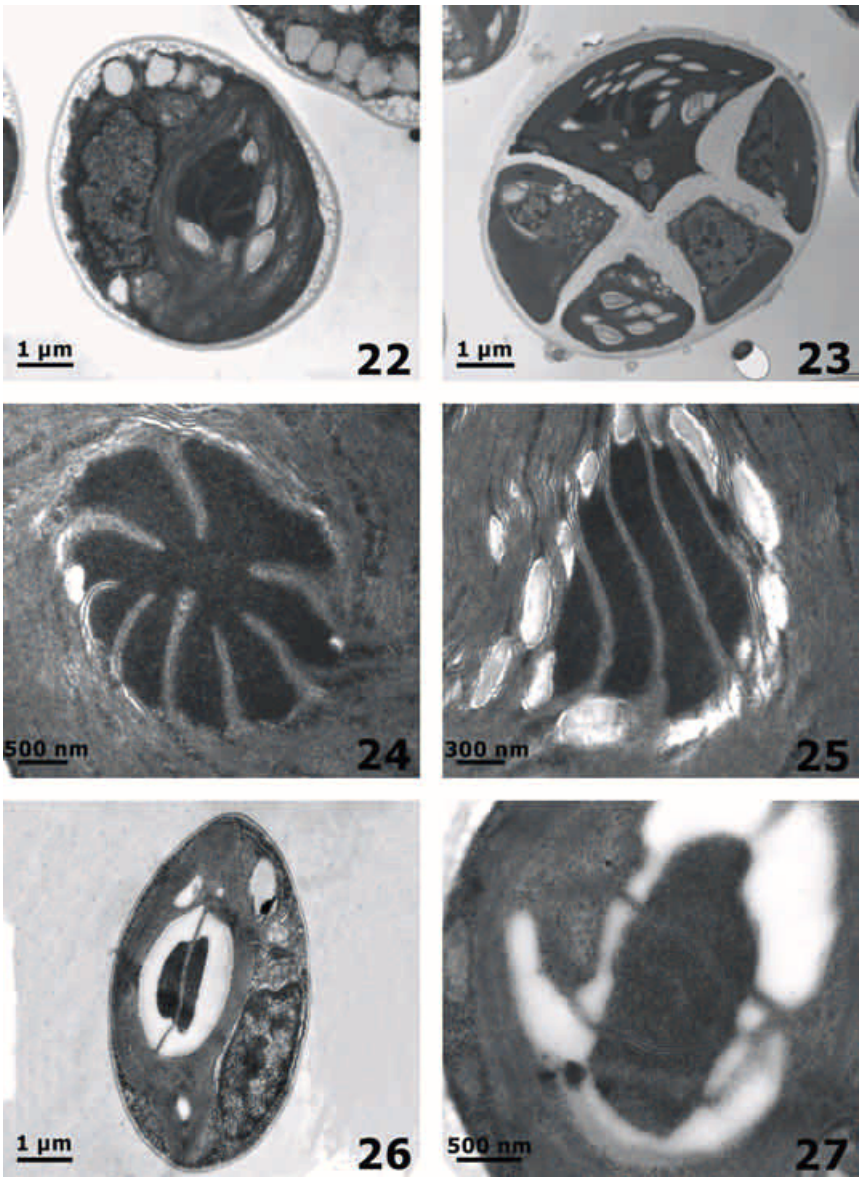
The morphological characters of vegetative cells of the '*C*'. *luteoviridis* SAG 211-2a strain (Figs 14–21) correspond to previous observations (Fott & Nováková 1969; Ettl & Gärtner 1995). Characteristically, chloroplasts in cells often are loosened from the cell wall in their parietal part (Figs 15–17). A single band of thylakoids composed of two or four thylakoids transect the pyrenoid matrix (Figs 26,27).

In order to establish the phylogenetic position of the CAUP H7901 strain, we determined a partial sequence of the 18S rRNA gene. The sequenced part of the gene (2234 bp) comprises 1773 bp of the 18S rDNA sequence itself and 461 bp of a putative group I intron S516 (following the nomenclature proposed by Johansen & Haugen 2001). According to BLAST searches, the best hits represented sequences attributed to the class Trebouxiophyceae, but the level of sequence similarity reached only up to 90% of identical positions. An ML analysis with a selection of representatives of major green algal lineages revealed that the CAUP H7901 strain is undoubtedly nested within this class (data not shown). We therefore conducted a phylogenetic analysis focusing on the position of the new sequence within the Trebouxiophyceae (Fig. 28). The resulting tree has a topology generally congruent with previously published analyses of similar datasets (e.g. Krienitz *et al*. 2003; Henley *et al*. 2004; Fawley *et al*. 2005; Karsten *et al*. 2005; Aslam *et al*. 2007). The CAUP H7901 strain is found to branch in a moderately-to-strongly supported cluster (79%/96%/ ML/NJ BS/0.98 BPP) together with two strongly supported lineages, both also representing *Chlorella*-like organisms. One of them, hereafter referred to as the *Heveochlorella*/*Heterochlorella* lineage, comprises the



Figs 1–21. Morphology of *Kalinella bambusicola* (strain CAUP H7901) and *Heterochlorella luteoviridis* (strain SAG 211-2a). 1–4. Vegetative cells of *K. bambusicola*. 5. The autosporangium of *K. bambusicola* containing two unequal autospores. 6–9. Vegetative cells of *K. bambusicola*. 10,11. Autosporangia of *K. bambusicola* illustrating unequal size of the daughter cells. 12. The 3-D confocal reconstruction of the chloroplast in vegetative cells of *K. bambusicola*. 13. The 3-D confocal reconstruction of the chloroplasts in an autosporangium of *K. bambusicola*. 14–17. Vegetative cells of *H. luteoviridis*. 18,19. Autosporangia of *H. luteoviridis* illustrating unequal size of the daughter cells. 20. The 3-D confocal reconstruction of the chloroplast in the vegetative cell of *H. luteoviridis*. 21. The 3-D confocal reconstruction of the chloroplasts in the cell of *H. luteoviridis* shortly before autosporogenesis.

Figs 22–27. Ultrastructure of *Kalinella bambusicola* (strain CAUP H7901) and *Heterochlorella luteoviridis* (strain SAG 211-2a). 22. The vegetative cell of *K. bambusicola* with a parietal chloroplast. 23. The autosporangium of *K. bambusicola*. 24. The cross-section of a pyrenoid of *K. bambusicola*; thylakoids centripetally penetrate the pyrenoid matrix. 25. The longitudinal section of a pyrenoid of *K. bambusicola* with several parallel thylakoids that bisect the pyrenoid matrix; the pyrenoid is surrounded by separate starch grains. 26. The vegetative cell of *H. luteoviridis* with the pyrenoid bisected by a single thylakoid band and a starch envelope composed of two plates surrounding the pyrenoid. 27. The pyrenoid of *H. luteoviridis* bisected by a band of four thylakoids and surrounded by a less compact starch envelope.



recently described *Heveochlorella hainangensis* (Zhang *et al*. 2008), the authentic strain SAG 211-2a of *Chlorella luteoviridis* (i.e. *Heterochlorella luteoviridis*, see below), a strain (MES A5-4) also referred to as *C. luteoviridis* in the database entry of the corresponding sequence, and one undescribed MBIC10057 '*Chlorella*' sp. strain. The second lineage that seems to be closely related to the investigated CAUP H7901 strain comprises three '*Chlorella*' species ('*C*'. *saccharophila* SAG 211-9a, '*C.*' *angustoellipsoidea* MES A7-4, and '*C.*' *trebouxioides* MES-A1-2) and is hereafter designated as '*C.*' *saccharophila* lineage. The mutual relationship among CAUP H7901, the *Heveochlorella*/*Heterochlorella* lineage and the '*C.*' *saccharophila* lineage has received some statistical support from the ML method only (78%). *Watanabea reniformis* SAG

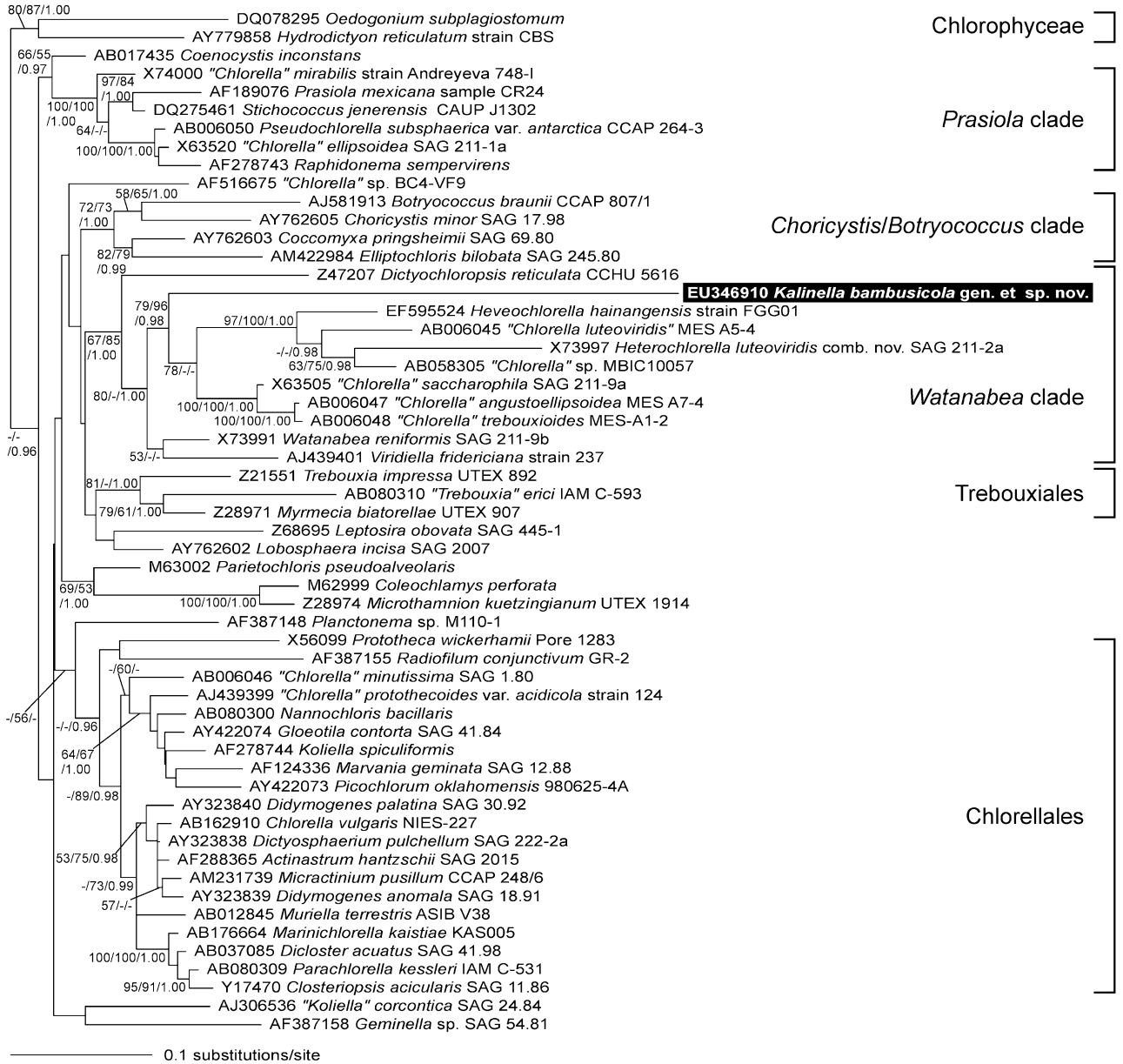


Fig. 28. Phylogenetic position of *Kalinella bambusicola* gen. et sp. nov. based on 18S rRNA gene sequence data (56 taxa, 1691 nucleotide positions). The maximum likelihood (ML) tree shown (GTR+Γ+I, loglk = –12 599.225776, $\alpha = 0.514663$, *invar* = 0.498446) was constructed with RAxML (see *Materials and Methods*). ML/neighbor joining (NJ) bootstrap values/Bayesian posterior probabilities are indicated for nodes where higher than 50%/0.95. The numbers preceding taxon names correspond to DNA Data Bank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL) and GenBank accession numbers of the respective sequences.

211-9b and *Viridiella fridericiana* strain 237 are found with varying statistical support (80%/<50%/1.00) as the taxa most closely related to the assemblage of CAUP H7901 and the two other *Chlorella*-like lineages. Finally, *Dictyochloropsis reticulata* CCHU 5616 appears as the next most closely related taxon and the clade formed by all of these lineages corresponds to the *Watanabea* clade circumscribed by Karsten *et al.* (2005). The monophyly of the *Watanabea* clade is likely, though statistical support in our analysis varies with the method used (67%/85%/1.00). However, the position of the *Watanabea* clade within the class Trebouxiophyceae is unresolved, as was seen in previously published phylogenies.

The branch of the CAUP H7901 strain in the 18S rDNA tree is relatively long, indicating an increased rate of evolution of the 18S rRNA gene in this lineage as compared with most other trebouxiophyceans. Indeed, inspecting the alignment of trebouxiophycean 18S rDNA sequences revealed that CAUP H7901 has a

Table 1. Testing alternative tree topologies with the approximately unbiased (AU) and weighted Shimodaira-Hasegawa (wSH) tests

Topology	Topological constraint (taxa in brackets forced to form a monophyletic group)	AU *P*-value	wSH *P*-value
1	Unconstrained ML topology (= Fig. 28)	0.709	0.983
2	(*Kalinella* + "*Chlorella*" *saccharophila* lineage)	0.539	0.846
3	(*Kalinella* + *Heveochlorella*/*Heterochlorella* lineage)	0.410	0.714
4	(*Kalinella* + *Watanabea reniformis*)	0.457	0.823
5	(*Kalinella* + *Viridiella fridericiana*)	0.069	0.559
6	(*Kalinella* + *Watanabea reniformis* + *Viridiella fridericiana*)	0.417	0.796
7	(*Kalinella* + *Dictyochloropsis reticulata*)	0.162	0.919
8	("*C*". *saccharophila* lineage + *Heveochlorella*/*Heterochlorella* lineage + *Watanabea reniformis* + *Viridiella fridericiana*)	0.475	0.470
9	(*Watanabea* clade except *Kalinella*)	0.129	0.356
10	(*Kalinella* + "*Chlorella*" *ellipsoidea*/*Pseudochlorella*/*Raphidonema* lineage)	0.001	0.014
11	(*Kalinella* + "*Chlorella*" sp. BC4-VF9	0.001	0.012
12	(*Kalinella* + Chlorellales)	0.012	0.022

number of unusual substitutions in otherwise highly conserved positions. These substitutions appear to be spread along the whole length of the gene without any conspicuous accumulation to a particular region. We sequenced the gene from both strands and the sequencing chromatograms lack signs of technical problems, so the determined sequence (including the unusual substitutions) is very likely genuine. Furthermore, when we checked some of these substitutions mapping into double-helical regions of the predicted secondary structure of the 18S rRNA molecule, we found that they were correlated. For instance, the helix 30 of the predicted secondary structure contains an A-U pair of complementary nucleotides absolutely conserved in all trebouxiophyceans in our database except CAUP H7901, which has the corresponding positions (1522 and 1527) substituted by a compensatory G-C pair.

The divergent nature of the 18S rDNA sequence of CAUP H7901 poses a challenge to inferring its precise position within the Trebouxiophyceae. Values of statistical support for the position as inferred at Figure 28, that is, within the *Watanabea* clade close to the *Heveochlorella*/*Heterochlorella* and '*C.*' *saccharophila* lineages, are suggestive but not completely convincing. We therefore tested alternative phylogenetic positions of CAUP H7901 using the AU and wSH tests (Shimodaira & Hasegawa 1999; Shimodaira 2002). We inferred a series of alternative trees imposing various topological constraints and calculated the corresponding *P*-values (Table 1). Forcing CAUP H7901 to form a monophyletic group with either the *Heveochlorella*/*Heterochlorella* lineage, the '*C.*' *saccharophila* lineage, *W. reniformis*, *V. fridericiana*, both *W. reniformis* and *V. fridericiana*, and *D. reticulata* (topologies 2–7, respectively) did not lead to trees with likelihood values significantly worse than the unconstrained tree (i.e. that at Fig. 28). When we enforced monophyly of the *Watanabea* cluster except CAUP H7901 and *D. reticulata* or except CAUP H7901 only (topologies 8 and 9, respectively), the resulting trees were also not rejected. On the other hand, enforcing monophyly of CAUP H7901 with some other lineages containing *Chlorella*-like representatives, for example, the '*C.*' *ellipsoidea*/*Pseudochlorella*/*Raphidonema* lineage from the *Prasiola* clade, '*Chlorella*' sp. BC4-VF9, or the Chlorellales (topologies 10–12, respectively), led in both tests to trees with *P*-values significantly lower (<0.05) than the unconstrained tree.

DISCUSSION

As knowledge of the vast diversity of *Chlorella*-like green algae accumulated, their proper morphological discrimination has been considered increasingly difficult (Fott & Nováková 1969; Ettl & Gärtner 1995; Krienitz *et al*. 2004). We would certainly not be able to identify the phylogenetic and taxonomic affiliation of the CAUP H7901 strain solely on the basis of morphological and ultrastructural characters. However, several morphological characters led us to an assumption that this strain might be related to members of the *Watanabea* clade. The unequal size of autospores together with the parietal pyrenoid-bearing chloroplasts with the waved margins and the globular vegetative cells with the thin cell wall are features typical for most members of this group (even if it has also been ascertained in some other *Chlorella*-like algae such as *C. ellipsoidea* (Ettl & Gärtner 1995).

The phylogenetic analysis of 18S rDNA indeed revealed the position of CAUP H7901 within the *Watanabea* clade as a deep branch potentially closely related to the *Heveochlorella*/*Heterochlorella* and '*C.*' *saccharophila* lineages. However, the exact placement of CAUP H7901 within the *Watanabea* clade remains

uncertain because several alternative positions could not be rejected as statistically worse by the likelihood-based AU and wSH tests. We cannot even exclude the possibility that the CAUP H7901 lineage actually diverged prior to the remaining members of the *Watanabea* clade. However, it is unlikely that the CAUP H7901 strain is closely related to *Chlorella*-like organisms that belong to the *Prasiola* clade or the Chlorellales (or to the enigmatic undescribed '*Chlorella*' sp. BC4-VF9), because such relationships were rejected by both the AU and wSH tests. Given the present results, we consider the close relationship of CAUP H7901 with *Chlorella*-like members of the *Watanabea* clade as the best working hypothesis, which has to be tested further by analyzing more phylogenetically informative genes.

The intron S516 present in the 18S rRNA gene of CAUP H7901 is found also in some other representatives of the *Watanabea* clade, for example '*C.*' *luteoviridis* SAG 211-2a or '*C.*' *saccharophila* MBIC10092, but is absent from even closely related taxa (e.g. '*C. luteoviridis*' MES A5-4, *H. hainangensis* strain FGG01, '*C.*' *saccharophila* SAG 211-9a). The presence/absence of introns in the 18S rRNA gene is apparently of little taxonomic value, since they are gained and lost very frequently (Haugen *et al*. 2005).

The problems with placing the CAUP H7901 strain within the trebouxiophycean trees may in part be due to the apparently increased evolutionary rate of its 18S rRNA gene. Such a rate is not so unusual among trebouxiophyceans, as it is seen to a varying degree in some other taxa, most of which were excluded from the phylogenetic analysis presented at Figure 28 (e.g. species from the family Oocystaceae, and *Auxenochlorella protothecoides*). The potentially closely related *Heveochlorella*/*Heterochlorella* lineage, especially '*C.*' *luteoviridis* SAG 211-2a, also exhibits relatively divergent 18S rDNA sequences, suggesting that there might be some inherent propensity to speed up the evolution of the 18S rRNA gene in this line of descent.

The unsettled phylogenetic position of CAUP H7901 makes it necessary to discuss morphological and ultrastructural features of CAUP H7901 in the context of the whole *Watanabea* clade. The *Chlorella*-like members of the *Watanabea* clade differ in their pyrenoid structure. In the authentic strain SAG 211-2a of '*C.*' *luteoviridis* the pyrenoid is regularly transected by a single band of two or four thylakoids that divide the starch envelope into two halves (Němcová & Kalina 2000; Fig. 26), a pattern clearly different from the CAUP H7901 strain that has the multiple thylakoid bands (composed by two or four thylakoids) entering the pyrenoid in a radial fashion (Fig. 24). However, the pattern of thylakoids transecting the pyrenoid matrix is quite variable across other *Chlorella*-like members of the clade. There are multiple thylakoid bands entering the pyrenoids in other strains assigned to '*C.*' *luteoviridis*, for example, SAG 211-2b, MES A5-4 (Ikeda & Takeda 1995; Hanagata *et al*. 1996) and plural undulating single thylakoids transecting pyrenoids in members '*C.*' *saccharophila* clade (Ikeda & Takeda 1995; Hanagata *et al*. 1996). In *H. hainanensis*, the tube-like thylakoids traverse the pyrenoid matrix in multiple directions (Zhang *et al*. 2008). Thus, we believe that the pyrenoid ultrastructural features should be used for species delimitation rather than for characterization of higher taxa in these lineages.

The gross morphology of the CAUP H7901 strain differs from those of '*C.*' *luteoviridis* primarily by a compact chloroplast that is tightly nestled against the cell wall. On the other hand, in *C. luteoviridis* chloroplasts are rather flat and typically loosened from the cell wall in their parietal part (Fott & Nováková 1969; Ettl & Gärtner 1995). The vegetative cells of the SAG 211-2a strain of '*C.*' *luteoviridis* are spherical or slightly ellipsoidal (3–) 4.5–13 (–14) μm in diameter and therefore slightly larger than those of the CAUP H7901 strain. In addition, the pyrenoid of the SAG 211-2a strain is distinct, more or less spherical, covered with two or numerous starch grains (see, e.g. Figs 14–16). On the other hand, the pyrenoid of the CAUP H7901 strain is barely visible via light microscope as the starch grains surrounding the pyrenoid matrix are less developed. The *H. hainanensis*, strain FGG01, differs from the investigated CAUP H7901 strain primarily by having many small pyrenoids in chloroplasts and smaller cell dimensions (Zhang *et al*. 2008). The difference between CAUP H7901 and members of the '*C.*' *saccharophila* lineage is even more profound, with the former having regularly globular rather than ellipsoidal vegetative cells and autospores, and a distinctly parietal chloroplast that has been reported in a more or less central position in '*C.*' *trebouxioides* and '*C.*' *saccharophila* (Punčochářová 1994; Ettl & Gärtner 1995). *Watanabea reniformis* and *Viridiella fridericiana* differ from CAUP H7901 by lacking a pyrenoid and by a notable presence of elliptical and irregular autospores and young vegetative cells (Albertano *et al*. 1991; Hanagata *et al*. 1998). To the best of our knowledge, the suite of morphological characters of the CAUP H7901 strain is unlike any established morphospecies of coccoid green algae. In addition, the morphological and sequence data suggest a deep split of the CAUP H7901 strain from other members of the *Watanabea* clade. Thus, we decided to describe this strain as a new species, which deserves a separate generic status.

Furthermore, we suggest a taxonomic revision for '*C.*' *luteoviridis*, which cannot remain in the genus *Chlorella* that is typified by the distantly related *C. vulgaris*. According to the 18S rDNA phylogeny, a validly described species most closely related to '*C.*' *luteoviridis* is the recently reported *H. hainangensis* (Zhang *et al*. 2008). However, as already explained by Zhang

et al. (2008) *H. hainangensis* and '*C.*' *luteoviridis* should be most suitably viewed as representing distinct genera because of the level of divergence in the 18S rRNA gene and of the morphological traits differentiating these two taxa (the number and size of pyrenoids, the number and orientation of pyrenoid-transecting thylakoids). We subscribe to this view and validate here the generic name *Heterochlorella* that was for '*C.*' *luteoviridis* already informally used by Krienitz *et al*. (2004). Two strains, MBIC 10057 and MES A5-4, seem to be more closely related to the type species of *Heterochlorella* (*H. luteoviridis* SAG 211-2a) than to *H. hainangensis* based on their 18S rDNA sequence (Fig. 28). Unfortunately, the MBIC 10057 strain has been lost (Dr N. Kurano, Marine Biotechnology Institute, Japan, pers. comm., 2007) and the MES A5-4 culture is also not available from any of the public algal culture collections. Thus, we were not able to investigate these strains to assess their taxonomic status, but the degree of the 18S rRNA sequence divergence from the SAG 211-2a strain suggests that they may represent species of the genus *Heterochlorella* distinct from SAG 211-2a or even separate genera of the *Heterochlorella*/*Heveochlorella* lineage.

Phylogenetic differentiation of *Chlorella*-like coccoid green algae considerably exceeds their morphological diversity. Especially in subaerial conditions, where the drought stress drives selection towards the globular or globular-like (e.g. ellipsoidal) forms with low surface-to-volume ratio, gross morphology alone has little power in identification of individual species or lineages. However, using a combination of phylogenetic, ultrastructural and morphological markers, the diversity of these interesting organisms can be revealed and formally described. Physiological data (e.g. growth on mannitol that may possibly be limited to algae belonging to the *Watanabea* clade – see Huss *et al*. (1999); or Zhang *et al*. (2008) may also be useful for phenotypic assessment of individual lineages. The mannitol-growth data would be desirable for new members of the *Watanabea* clade to assess their applicability for its phenotypic and taxonomic characterization.

Kalinella gen. nov

Diagnosis latina
Cellulae vegetativae solitariae sphericae, nucleus unus, paries cellularis firma tenuis, non gelatinosa. Chloroplastus unicus, crasse poculiformis aut parietalis, pyrenoide unica praeditus. Propagatio asexualis per sphaeroidae autosporas, saepe in eadem cellula in aequalibus multiplicate. Reproductio per zoospores et reproductio sexualis non occurrunt.

Type species (designated here)
Kalinella bambusicola.

Etymology
'Kalina', in honor of Tomáš Kalina, our teacher and a prominent Czech scientist in the field of taxonomy and ultrastructure of *Chlorella*-like green algae; plus *ella*, small or diminutive.

Kalinella bambusicola sp. nov.

Diagnosis latina
Cellulae vegetativae sphericae (3.8–) 4.6–10.5 (–12.0) μm magnae. Nucleus unus, paries cellularis firma tenuis, non gelatinosa. Chloroplastus unicus, cellulas juvenes parietalis, cellulas maturas crasse poculiformis, interdum parietalis, cum margo undulatus. Pyrenoide unica, non perspicua, granis amylaceis dispersis circumcinctae. Propagatio asexualis per 2–8 sphaeroidae autosporas, saepe in eadem cellula in aequalibus multiplicate.

Holotype
The lyofilized material deposited at the CAUP (Culture Collection of algae of the Charles University in Prague, Department of Botany, Benátská 2, 12801, Praha 2, Czech Republic) under designation LYO-H 7901 (*hic designatus*). The living cultures (ex-holotypes) have been deposited as CAUP H 7901 ibidem.

Type locality
The epihytic algal growth on *Gigantochloa* sp. in Fort Canning Park, Singapore.

Etymology
The specific epithet 'bambusicola' refers to the occurrence of this aero-terrestrial species epiphytically on a bamboo stalk.

Heterochlorella gen. nov.

Diagnosis latina
Cellulae vegetativae solitariae sphericae, nucleus unus, paries cellularis firma tenuis, non gelatinosa. Chloroplastus unicus, parietalis aut cingularis, parietum cellulae partim tantum tegente, pyrenoide unica praeditus. Propagatio asexualis per sphaeroidae autosporas, saepe in eadem cellula in aequalibus multiplicate. Reproductio per zoospores et reproductio sexualis non occurrunt.

Type species (designated here)
Heterochlorella luteoviridis.

Etymology
In reference to the morphological similarity with the genus *Chlorella*. For the first time used informally by Dr

Lothar Krienitz and his co-workers (Krienitz *et al.* 2004).

Heterochlorella luteoviridis comb. nov.

Basionym
Chlorella luteoviridis Chodat 1912, Bull. de la Soc. royale de Botanique de Belgique, p. 322.

Authentic strain
SAG 211-2a.

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