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# The genus *Jaagichlorella* Reisigl (Trebouxiophyceae, Chlorophyta) and its close relatives: an evolutionary puzzle

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## Abstract

The genus *Chlorella* (in its traditional sense) is polyphyletic and belongs to at least twelve independent lineages of the Trebouxiophyceae and Chlorophyceae. Most of the aquatic species belong to the *Chlorella* and *Parachlorella* clades (within the so-called *Chlorella*-lineage of the Trebouxiophyceae), or to the genera *Scenedesmus* and *Mychonastes* (within the DO-group of the Chlorophyceae) according to phylogenetic analyses of the SSU and ITS rDNA sequences. In contrast to the aquatic species, the terrestrial strains investigated so far form a monophyletic lineage (*Watanabea*-clade) within the *Trebouxia*-lineage of the Trebouxiophyceae. Several genera with *Chlorella*-like morphology (*Chloroidium*, *Heterochlorella*, *Watanabea*, *Kalinella*, *Viridiella* and others) belong to the *Watanabea* clade. We studied 22 strains isolated from soil, bark, and artificial hard substrates, which have been traditionally identified as *Chlorella luteoviridis* or as unidentified *Chlorella*. To clarify the taxonomical status and intrageneric diversity of this group, we used an integrated approach (molecular phylogeny of SSU and ITS rDNA sequences, secondary structures, DNA barcoding, and morphology) including the ecological distribution. All investigated strains showed a low phenotypic plasticity, but a high genetic diversity, which could be only resolved in complex phylogenetic analyses based on the secondary structures of the investigated genes. Considering these results, we reestablished the genus *Jaagichlorella* for *Heterochlorella* and *Heveochlorella*, and proposed new combinations (*J. luteoviridis*, *J. hainangensis*, *J. roystonensis*, and *J. sphaerica*) as well as the new species, *J. africana*.

**Keywords:** *Chlorella, Heterochlorella, Heveochlorella, Jaagichlorella*, molecular phylogeny, integrative approach, systematics, terrestrial algae, hard substrate, biofilms

## Introduction

DNA sequences have given new insights into the evolution of microalgae and the results of phylogenetic analyses have lead to taxonomic changes within the systematics of these organisms. This is especially true for microalgae that lack traditional morphological characters, such as the unicellular coccoid green algae belonging to the genus *Chlorella* Beij., which have been revised and into twelve independent lineages of the Chlorophyceae and Trebouxiophyceae (Huss *et al.* 1999; Krienitz *et al.* 2004; Darienko *et al.* 2010, 2016; Pröschold *et al.* 2011; Krienitz *et al.* 2015).

*Chlorella*-like algae are very common in all types of habitats including extreme environments such as desert soil crusts, acidic soils, or artificial substrates on buildings (e.g. Albertano *et al.* 1991; Darienko & Hoffmann 2003; Büdel *et al.* 2009; Hallmann *et al.* 2016). As shown in previous studies, those algae from freshwater habitats mostly belong to different genera of the *Chlorella* lineage (Krienitz *et al.* 2012), whereas all terrestrial species (lithophilic, aerophilic and soil including lichen symbionts) previously assigned as *Chlorella* represent several genera, which were not closely related to *Chlorella vulgaris* Beij., the type species of the genus (Friedl 1997; Huss *et al.* 1999; Krienitz *et al.* 2004; Darienko *et al.* 2010; Pröschold *et al.* 2011; Krienitz & Bock 2012).

In studies of algal biodiversity on hard substrates using traditional cultural techniques and modern phylogenetic approaches, we discovered that one of the most common green coccoid alga was similar in morphology and phylogeny to *Chlorella luteoviridis*. This species was originally described by Chodat (in Conrad and Kufferath 1912) and emended including the type figures by himself (Chodat 1913) and Kufferath (1913). The authentic strain (= 'type culture') of

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*Chlorella luteoviridis* (SAG 211-2a) was found in a small pond in a forest in Belgium. Phylogenetic analyses of the SSU rDNA sequences have shown that these algae belonged to the *Watanabea*-clade (Trebouxiophyceae), which is sister to *Chloroidium* Nadson (Darienko *et al.* 2010, 2018). Therefore, Neustupa *et al.* (2009) transferred the strain SAG 211-2a to their new genus *Heterochlorella* Neustupa, Némcová, Eliás & Škaloud (*H. luteoviridis* (Chodat) Neustupa, Némcová, Eliás & Škaloud). However, the taxonomic status of algae related to *H. luteoviridis* remained unclear, because of high genetic variations among these strains shown in previous studies (Krienitz *et al.* 2004; Neustupa *et al.* 2009; Darienko *et al.* 2010). Morphologically similar species to *Heterochlorella luteoviridis* were also described from terrestrial habitats. Reisigl (1964) found in soil collected from the rhizosphere of *Kobresia myosuroides* (Vill.) Fiori (= *Elyna myosuroides* (Vill.) Fritsch), a species which he described as *Jaagichlorella geometrica* Reisigl. This species differed by smaller cell sizes than *Heterochlorella luteoviridis*. Tschermak-Woess (1988) described a photobiont of the lichen *Pseudocyphellaria carpoloma* (Delise) Vainio, which she named *Chlorella sphaerica* Tschermak-Woess. This species was independently deposited into two culture collections (SAG and UTEX). However, Darienko & Pröschold (2018) demonstrated that both depositions were not identical. Whereas the strain SAG 11.88 does not contain *Chlorella sphaerica* and was described as *Diplosphaera epiphytica* by Darienko & Pröschold (2018), the phylogenetic position of UTEX 2485 (authentic strain of *Chlorella sphaerica*) remained unresolved. The UTEX strain was morphologically similar to *Jaagichlorella geometrica* as demonstrated by Tschermak-Woess (1988).

Zhang *et al.* (2008) isolated a strain with similar cell size to *Jaagichlorella* from the bark of the rubber tree (*Hevea brasiliensis* Müll. Arg.) and described it as *Heveochlorella hainangensis* Zhang, Huss, Sun, Chang & Pang. Ma *et al.* (2013) described a second species of *Heveochlorella*, *H. roystonensis*, that was isolated from the bark of the royal palm (*Roystonea regia* (Kunth) O.F. Cook). A similar species isolated from the bark (*Gigantochloa* sp.) was established by Neustupa *et al.* (2009): *Kalinella bambusiana* Neustupa, Némcová, Eliás & Škaloud.

Despite the cell size, all these species are morphologically similar to *H. luteoviridis*, but show many molecular variations in their SSU rDNA sequences (Huss *et al.* 1999; Zhang *et al.* 2008; Darienko *et al.* 2010; Ma *et al.* 2013). In addition, some studies show differences among several strains in physiological growth conditions such as color of colonies on agar media and growth on different media containing glucose and other carbohydrates. This resulted in separation into new species or varieties: *Chlorella mutabilis* Shihira & Krauss, *C. nocturna* Shihira & Krauss (Shihira & Krauss 1965), *C. luteoviridis* var. *aureoviridis* Meyer (1932, 1933), *C. luteoviridis* var. *lutescens* (Chodat 1913). All these taxa are available in public culture collections. Some of these strains originated by Beijerinck (1904), who described *Chlorella variegata* Beij. based on variable color of colonies grown on agar. The strains in public culture collections (CCAP, SAG, and UTEX) designated as *C. variegata* were mixed up and later been transferred by Fott & Nováková (1969) either to *C. protothecoides* Krüger (= *Auxenochlorella protothecoides* (Krüger) Kalina & Puncocharova) or to *C. luteoviridis* (Darienko & Pröschold 2015b). The species name *Chlorella variegata* was proposed as synonym of *C. protothecoides* by Fott & Nováková (1969).

The aim of this study is to clarify the taxonomical status and intrageneric diversity of this group using an integrative approach. We studied 22 strains called "*Chlorella*" *luteoviridis* from public culture collections, nine of them were probably isolated from freshwater bodies of uncertain origin and five from biofilms on various hard substrates (see Table 1). We also propose the transfer of *Heterochlorella luteoviridis* and the species of *Heveochlorella* and *Chlorella sphaerica* to *Jaagichlorella*.

## Material and Methods

### Strains, cultivation and genetic information

In this study, the SSU and ITS rDNA of 22 ellipsoidal *Chlorella*-like strains were sequenced to establish a new phylogeny. The strains were obtained either from international algal collections, or new strains were isolated from green biofilms on artificial hard substrates. The information on their origins are summarized in Table 1. All new strains were deposited at the Sammlung von Algenkulturen at the University of Göttingen (SAG, Göttingen, Germany). They were grown on modified Bold Basal medium (3N-BBM+V; medium 26a in Schlösser 1997) on agar at 20°C under a 12:12h light-dark regime (light intensity: 50 $\mu E/m^2s$).

Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). The SSU and ITS rDNA were amplified using the Taq PCR Mastermix Kit (Qiagen GmbH, Hilden, Germany) with the primers EAF3 and ITS055R (Marin *et al.* 2003). The sequences of all strains were aligned according to their secondary structures of SSU and ITS rDNA (folding protocol described in detail in Darienko *et al.* 2016) and included into two data sets:

**Table 1**. Strains used in this study

| Strain | Origin | Species | Accession | Intron position | ITS-1 haplotype | ITS-2 haplotype | ITS-2 Barcode |
|---|---|---|---|---|---|---|---|
| SAG 211-2a | Belgium, pool in forest of Oisquercq near Bruxelles | *Jaagichlorella luteoviridis* | MH780927 | 516 | JLUT1 | JLUT | BC-1 |
| SAG 211-2b | Netherlands, Baarn, freshwater | *Jaagichlorella luteoviridis* | MH780928 | 516 | JLUT1 | JLUT | BC-1 |
| SAG 211-5a | unknown | *Jaagichlorella luteoviridis* | MH780929 | 516 | JLUT2 | JLUT | BC-1 |
| SAG 211-5b | unknown | *Jaagichlorella luteoviridis* | MH780930 | 516 | JLUT2 | JLUT | BC-1 |
| SAG 211-3 = CCAP 211/3 | freshwater | *Jaagichlorella luteoviridis* | MH780931, MH780933 | 516 | JLUT2 | JLUT | BC-1 |
| SAG 211-4 = CCAP 211/4 | Belgium, freshwater, Botanic Garden Bruxelles | *Jaagichlorella luteoviridis* | MH780932, MH780934 | 516 | JLUT2 | JLUT | BC-1 |
| CAUP H1963 | Netherlands, Delft | *Jaagichlorella luteoviridis* | MH780937 | 516 | JLUT2 | JLUT | BC-1 |
| CCAP 211/10A | freshwater | *Jaagichlorella luteoviridis* | MH780935 | 516 | JLUT2 | JLUT | BC-1 |
| CCAP 211/10E | Netherlands, Delft | *Jaagichlorella luteoviridis* | MH780936 | 516 | JLUT2 | JLUT | BC-1 |
| SAG 2213 | Namibia, surface of concrete wall | *Jaagichlorella africana* | MH780938 | - | JAFR | JAFR | BC-2 |
| SAG 2214 | Namibia, surface of concrete wall | *Jaagichlorella africana* | MH780939 | - | JAFR | JAFR | BC-2 |
| SAG 2133 | Germany, epilithic on roof tile | *Jaagichlorella roystonensis* | MH780940 | - | JROY1 | JROY1 | BC-3a |
| SAG 2196 | Germany, surface of rocks and buildings | *Jaagichlorella roystonensis* | MH780941 | - | JROY1 | JROY1 | BC-3a |
| SAG 2198 | Japan, Sena, epilithic on tombstone | *Jaagichlorella roystonensis* | MH780942 | 1046 | JROY2 | JROY2 | BC-3a |
| ITBB A3-8 | China, Hainan Province, epiphytic on the palm tree | *Jaagichlorella roystonensis* | JN003601, JX290371 | 516, 943 | JROY3 | JROY3 | BC-3b |
| SAG 2360 | China, Hainan Province, near Haikou, epiphytic on the rubber tree | *Jaagichlorella hainangensis* | MH780943 | 156, 943 | JHAI | JHAI | BC-4 |
| UTEX 2485 | New Zealand, Waweira Scenic Reserve, photobiont of *Pseudocyphellaria carpoloma* | *Jaagichlorella sphaerica* | MH780945 | 516 | JSPH | JSPH | BC-5 |
| SAG 2549 | Austria, Vienna, epilithic on stonewall | *Jaagichlorella geometrica* | MH780944 | 1046? | JGEO | JGEO | BC-6 |
| CAUP H7901 = SAG 2320 | Singapore, park, epiphytic on *Gigantochloa* sp. | *Kalinella bambusicola* | MH780946 | 516, 943 | KBAM | KBAM | BC-7 |
| CAUP H7902 | Slovenia, near Ankaran, epiphytic on bark of *Laurus nobilis* | *Kalinella apyrenoidosa* | MH780947 | - | KAPY1 | KAPY1 | BC-8 |
| SAG 2203 | Japan, epiphytic on bark of tree | *Kalinella apyrenoidosa* | MH780948 | - | KAPY2 | KAPY2 | BC-8 |

(i) 29 SSU rDNA sequences (1787 bp) of representatives of all members of the *Watanabea*-clade *sensu* Darienko *et al.* (2010) and (ii) a concatenated data set containing the 23 SSU and ITS rDNA sequences (2655 bp) of the investigated strains (identical SSU rDNA sequences among the investigated strains were only represented by one in Fig.1). The GenBank accession numbers of the new sequences as well as the group I intron positions, if present, are given in Figure 1 and Table 1, respectively. For the phylogenetic analyses presented in Figures 1 and 2, the data sets with unambiguously aligned base positions were used (introns if present were excluded from the data sets). To test which evolutionary model fits best for both data sets, we calculated the log-likelihood values of 56 models using Modeltest 3.7 (Posada, 2008) and the best models according to the Akaike criterion by Modeltest were chosen for the analyses. The settings of the best models are given in the figure legends. The following methods were used for the phylogenetic analyses: distance, maximum parsimony, maximum likelihood, and Bayesian inference. Programs used included PAUP version 4.0b10 (Swofford, 2002), RAxML version 7.0.3 (Stamatakis, 2006), MrBayes version 3.2.3 (Ronquist *et al.*, 2012), and PHASE package 2.0 (Jow *et al.*, 2002, Higgs *et al.*, 2003, Hudelot *et al.*, 2003, Gibson *et al.*, 2005, Telford *et al.*, 2005).

To test alternative tree topologies, the best tree (maximum likelihood tree using TIM+I+G model; see details in the legend presented in Fig. 1) was manipulated with the program TreeView version 1.6.6 (Page, 1996). These user-defined trees including the best tree were loaded into PAUP to calculate the likelihood scores for these trees. The probabilities of these trees were calculated using the approximately unbiased (AU), different bootstrap (non-scaled - NP; directly from the replicates - BP) and Bayesian (PP) tests (all with 10000 replicates) with the program CONSEL version 0.20 (Shimodaira & Hasegawa, 2001). All p-values below 0.05 mean that the user-defined trees are significantly worse compared to the best tree and therefore rejected.

**ITS-2 secondary structures, ITS-2/CBC approach and CBC analysis**

The secondary structures of ITS-2 sequences were folded using the computer programs Mfold (Zuker, 2003), and CONTRAfold (Do *et al.*, 2006) using the three constraints as described in Darienko *et al.* (2016). The secondary structure models of ITS-2 derived from these folding results were then used for species delimitation. For the ITS-2/CBC approach, the conserved region of ITS-2 was extracted following the procedure that was introduced for *Coccomyxa* by Darienko *et al.* (2015a): it includes (1) 15 base pairs of the 5.8S/LSU stem, (2) five base pairs of Helix I, (3) eleven base pairs of Helix II including the pyrimidine-pyrimidine mismatch, and (4) all base pairs of Helix III excluding the site loops, if present. The resulting data set was then manually aligned according to the secondary structures of these conserved regions (see Figs S1). These alignments have been translated into base pair alignment by using a number code for each base pair (**1** = A-U; **2** = U-A; **3** = G-C; **4** = C-G; **5** = G•U; **6** = U•G; **7** = pyrimidine-pyrimidine mismatch; **8** = deletion/insertion or single bases). The number-coded alignment was used for phylogenetic analysis calculated with PAUP (neighbor-joining method). The barcodes for each species were compared to detect for compensatory base changes (CBCs), hemi-CBCs (HCBCs), insertions/deletions, and single or unpaired bases.

In addition, the secondary structures of ITS-1 and ITS-2 were analyzed with the program CBCAnalyzer (Wolf *et al.* 2005) to determine CBCs and HCBCs among the variable regions.

**Identification and morphology**

The species were identified using the monographs of Fott & Nováková (1969), Shihira & Krauss (1965), Andreyeva (1975), Komárek & Fott (1983), and Ettl & Gärtner (2014). In addition, the authentic strains were compared to the original descriptions (Conrad & Kufferath 1912; Chodat 1913; Kufferath 1913; Meyer 1932, 1933; Reisigl 1964; Zhang *et al.* 2008; Neustupa *et al.* 2009, 2013; Ma *et al.* 2013). For the light microscopical investigations, the Olympus BX60 and Polyvar (Fa. Reichert & Jung; Vienna, Austria) microscopes (equipped with Nomarski DIC optics) were used. The microphotographs presented in Figures 3–6 were taken with a Prog Res C14 plus camera using the Prog Res Capture Pro imaging system (version 2.9.0.1), both from Jenoptik, Jena, Germany) and IM50 camera (Leica Microsystems, Heerbrugg, Switzerland) using the Cell^D image (Soft Imaging System, Münster, Germany) imaging system, respectively.

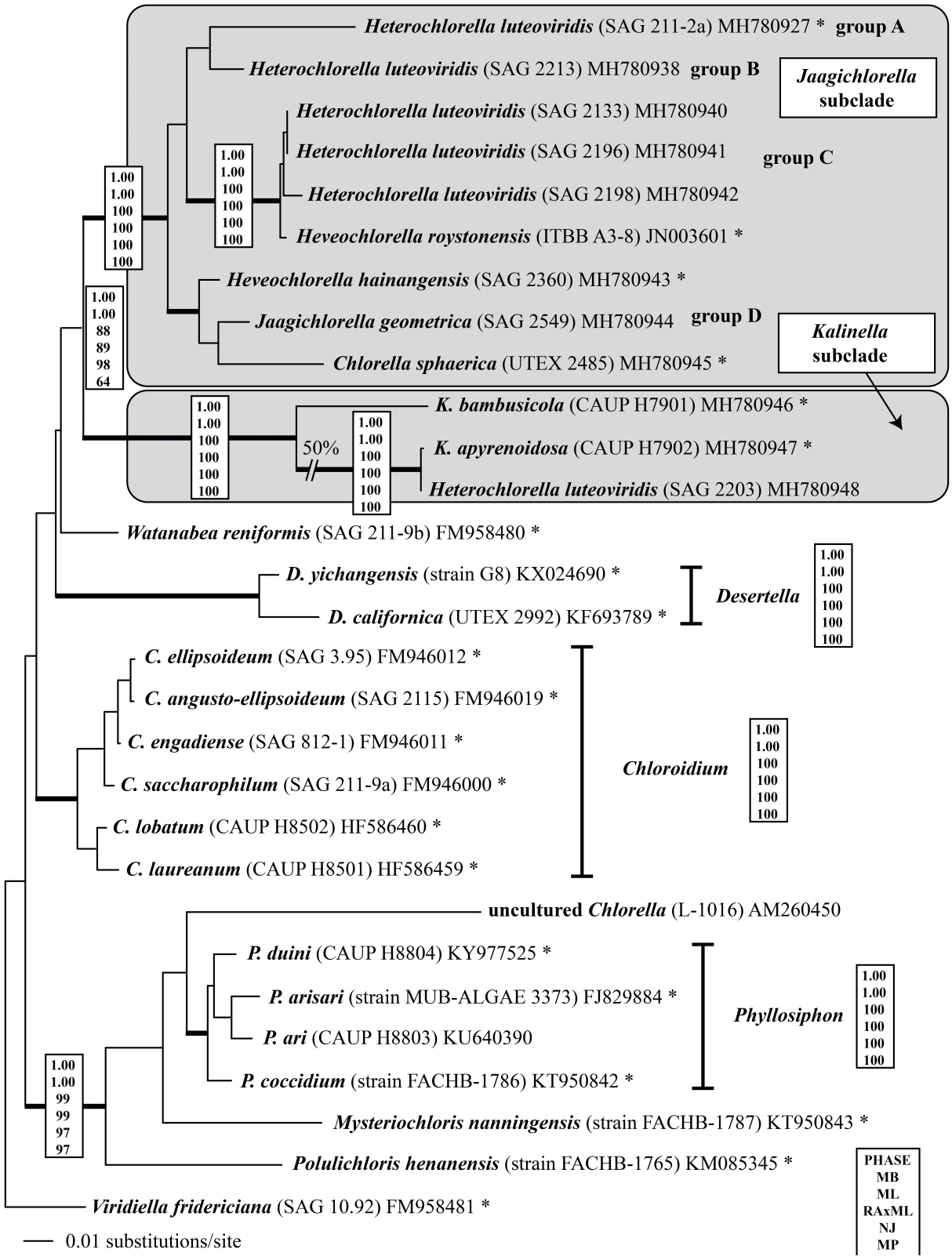


**FIGURE 1.** Molecular phylogeny of representatives belonging to the *Watanabea* clade based on SSU rDNA sequence comparisons. The phylogenetic tree shown was inferred using the maximum likelihood method based on the data set (29 taxa: 1787 aligned positions for SSU) using PAUP 4.0b10. For the analyses the best model was calculated by Modeltest 3.7. The setting of the best model was given as follows: TIM+I+G (base frequencies: A 0.2413, C 0.2367, G 0.2935, T 0.2285; rate matrix A-C 1.0000, A-G 2.1970, A-U 1.2024, C-G 1.2024, C-U 5.128, G-U 1.0000) with the proportion of invariable sites (I = 0.4935) and gamma shape parameter (G = 0.6539); The branches in bold are highly supported in all analyses (Bayesian values > 0.95 calculated with PHASE and MrBayes; bootstrap values > 90% calculated with PAUP using maximum likelihood, neighbor-joining, maximum parsimony and RAxML using maximum likelihood). The authentic strains of species are marked with an asterisk.

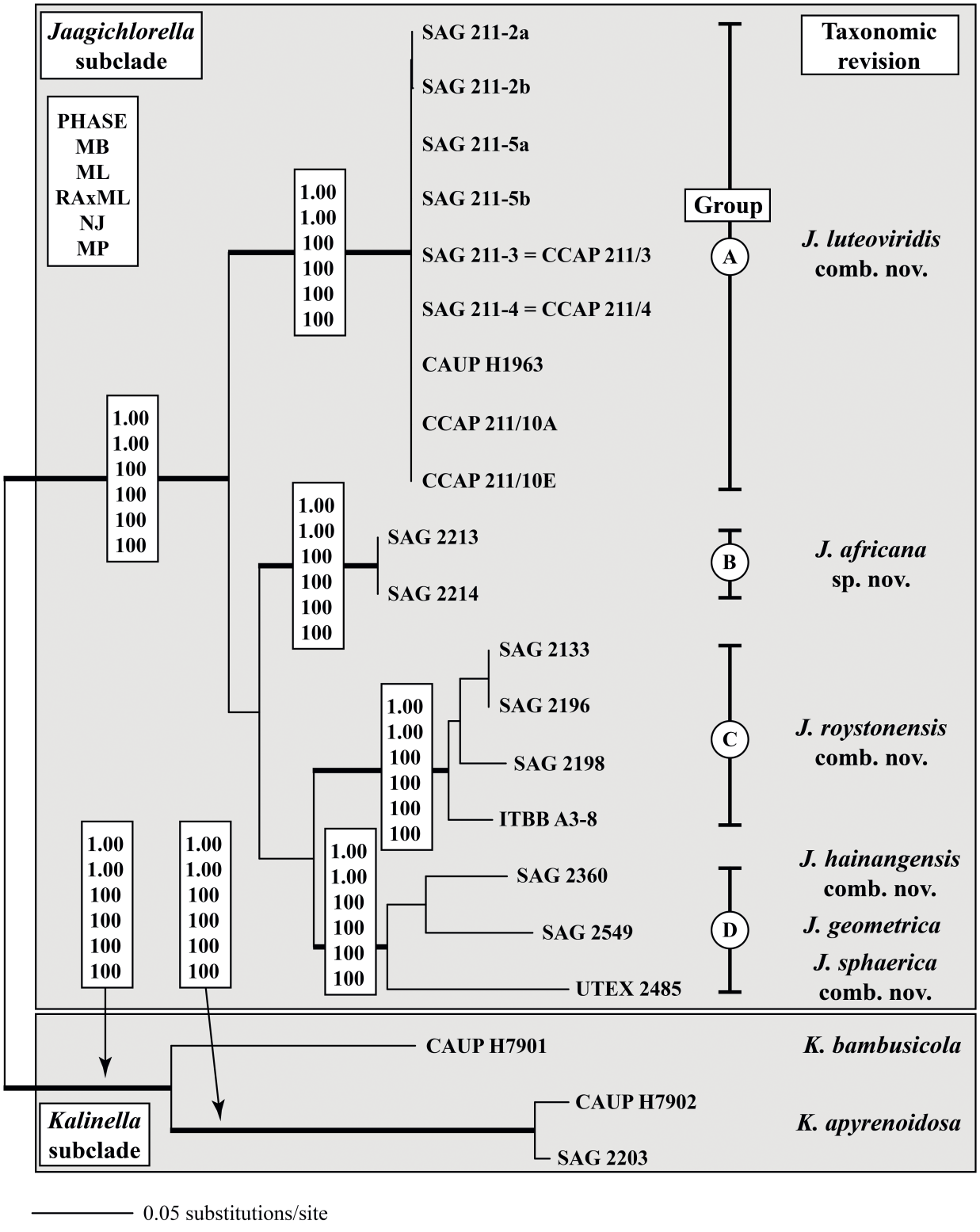


**FIGURE 2.** Molecular phylogeny of *Jaagichlorella* and *Kalinella* based on SSU and ITS rDNA sequence comparisons. The phylogenetic tree shown was inferred using the maximum likelihood method based on the data set (2655 aligned positions of 23 taxa) using PAUP 4.0b10. For the analyses the best model was calculated by Modeltest 3.7. The setting of the best model was given as follows: GTR+I+G (base frequencies: A 0.2324, C 0.2449, G 0.2794, T 0.2433; rate matrix A-C 1.5591, A-G 2.0795, A-U 1.4741, C-G 0.5739, C-U 4.8359, G-U 1.0000) with the proportion of invariable sites (I = 0.3875) and gamma shape parameter (G = 0.4780). The branches in bold are highly supported in all analyses (Bayesian values 1.00 calculated with PHASE and MrBayes; bootstrap values 100% calculated with PAUP using maximum likelihood, neighbor-joining, maximum parsimony and RAxML using maximum likelihood).

## Results

### Molecular phylogeny and ITS-2 DNA Barcoding of *Jaagichlorella* and its relatives

The phylogenetic analyses of SSU rDNA sequences (data not shown here; see Darienko *et al.* 2010) have revealed that all investigated strains of this study form a monophyletic lineage called *Watanabea*-clade within the Trebouxiophyceae. Within this clade, ten independent lineages could be revealed based solely on the SSU rDNA sequences. All strains with '*C. luteoviridis*' morphology (spherical cell shape, band-like chloroplast with pyrenoid, unequal sized autospores; Fig. 1) formed two separated lineages (*Jaagichlorella* and *Kalinella* subclades). The authentic strains of *Heterochlorella luteoviridis*, '*Chlorella*' *sphaerica*, *Heveochlorella hainangensis* and *H. roystonensis* belong to the *Jaagichlorella* subclade. Both species of *Heveochlorella* did not form a monophyletic lineage within this clade. Along with these strains, new isolates (SAG 2549, SAG 2213, SAG 2133, SAG 2196, and SAG 2198) also belong to the *Jaagichlorella* subclade. Only the strain SAG 2203 together with the strains CAUP H7901 and CAUP H7902 represent the *Kalinella* subclade. Both subclades were highly supported in all bootstrap and Bayesian analyses (marked in boxes in Fig. 1) and represent separated genera (see below).

To get a better resolution among these subclades, a concatenated dataset of SSU and ITS rDNA sequences was analyzed using the phylogenetic methods described in Material & Methods (Fig. 2). The phylogenetic analyses revealed a subdivision into four groups (A-D) among the *Jaagichlorella* subclade, which were highly supported in all bootstrap and Bayesian analyses. The group A contained eleven strains including the authentic strains of *Heterochlorella luteoviridis, Chlorella aureoviridis* and *C. luteoviridis* var. *lutescens, C. mutabilis* and *C. nocturna*. Surprisingly these strains were almost identical in their SSU and ITS rDNA sequences. Only variations in the intron regions could be detected. The two isolates from Namibia (SAG 2213 and SAG 2214), originally also identified as *Heterochlorella luteoviridis*, formed the group B. The group C included the authentic strain of *Heveochlorella roystonensis*, as well as three strains originally designated as *Heterochlorella luteoviridis*. Interestingly, the other authentic *Heveochlorella* strain (SAG 2360 *H. hainangensis*) formed together with the authentic strain of *Chlorella sphaerica* and a strain (SAG 2549) identified as *Jaagichlorella geometrica* the group D. The phylogenetic position of strain SAG 2203 among *Kalinella* presented in Fig.1 was confirmed in the analyses using the concatenated data set of SSU and ITS.

All these results raised the question about the generic concept of these strains. The type species of the genera *Heterochlorella*, *Heveochlorella* and *Jaagichlorella* belong to the groups A or D. The second species of *Heveochlorella* is member of group C and a new group B has been discovered. To test the robustness of this grouping, we created alternative user-defined topologies of the tree presented in Fig. 1, calculated the log-likelihood values using the best model found with ModelTest, and compared those with different tests such as the approximately unbiased test (AU) implemented in CONSEL (Table 2). The tests clearly revealed that all user-defined trees were significantly worse ($p < 0.05$) than the best tree shown in Fig. 1. For example, both species of *Heveochlorella* clearly belonged to two different groups (C and D). The monophyly of *Heveochlorella* (user-defined trees 9 and 10) was rejected by the AU tests. The collapse of the common branches for both subclades (trees 1 and 2) as well as the formation of each group to separate genera (trees 2-8) were also significantly rejected. Considering these results, the type species of the three genera *Jaagichlorella*, *Heterochlorella*, *Heveochlorella* together with other new isolates clearly belong to one genus, *Jaagichlorella* (proposed below in the taxonomic consequences). The close relationship of the *Kalinella* subclade to *Jaagichlorella* was confirmed in all analyses (Figs 1–2, Table 2). *Kalinella* was the sister group to *Jaagichlorella*, and remained therefore as second genus because of the long branch in our analyses shown in Fig. 1.

As demonstrated in Figs 1 and 2 as well as in Table 2, the two subclades *Jaagichlorella* (with their four groups A-D) and *Kalinella* represent genera. To decide how many species among both genera can be distinguished, we analyzed the ITS-2 secondary structures of all strains using the ITS-2/CBC approach established by Darienko *et al.* (2015a). The secondary structures of ITS-2 for each haplotype are presented in the supplemental Figures S1. Eleven ITS-2 haplotypes among the 23 investigated strains were found and the distance phylogeny (using the neighbor-joining method) of these haplotypes were calculated (see Table 1 and Fig. 3). The ITS-2/CBC approach of the conserved region revealed eight species (with nine barcodes: BC-1 to BC-8; Fig. 3) based on the CBC/HCBC pattern, which were described in detail below. The six species of *Jaagichlorella* and the two of *Kalinella* differed in their conserved region of ITS-2 by 16 CBCs/15HCBCs and 11 CBCs/5HCBCs, respectively. Additional CBCs and HCBCs could be detected in the variable region of ITS-2 and the whole ITS-1 (Table 3). Summarizing, the SSU and ITS rDNA sequences of all species compared to each other showed a high genetic variability, but almost no changes in the paired regions among the multiple strains of *Jaagichlorella luteoviridis* could be discovered.

**Table 2**. Comparisons of the maximum likelihood tree in Figure 1 with user-defined trees by approximately unbiased tests using maximum likelihood method.

| Tree | -ln L | Diff -ln L | AU | NP | BP | PP |
|---|---|---|---|---|---|---|
| **1** | 9110.58125 | (best) | 0.999 | 0.998 | 0.998 | 1.000 |
| **2** | 10468.98668 | 1358.40543 | < 0.001* | < 0.001* | 0 * | 0 * |
| **3** | 9145.23515 | 34.65391 | < 0.001* | < 0.001* | < 0.001* | < 0.001* |
| **4** | 9152.36315 | 41.78191 | 0.003* | 0.001* | 0.001* | < 0.001* |
| **5** | 9161.94182 | 51.36057 | 0.001* | < 0.001* | 0 * | < 0.001* |
| **6** | 9146.10080 | 35.51955 | 0.001* | 0.001* | 0.001* | < 0.001* |
| **7** | 9145.23520 | 34.65395 | < 0.001* | < 0.001* | < 0.001* | < 0.001* |
| **8** | 9188.65517 | 78.07393 | < 0.001* | < 0.001* | 0 * | < 0.001* |
| **9** | 9274.71459 | 164.13335 | < 0.001* | < 0.001* | 0 * | < 0.001* |
| **10** | 9341.48164 | 230.90040 | < 0.001* | < 0.001* | 0 * | < 0.001* |

**User-defined trees (Figure 1)**
**Tree 1** = best tree (Fig. 1)
**Tree 2** = collapse the common branch of *Jaagichlorella*/*Kalinella* subclades
**Tree 3** = collapse the common branch of *Jaagichlorella* subclade
**Tree 4** = group A sister to the *Kalinella* subclade
**Tree 5** = group B sister to the *Kalinella* subclade
**Tree 6** = group C sister to the *Kalinella* subclade
**Tree 7** = group D sister to the *Kalinella* subclade
**Tree 8** = *J. geometrica* sister to the *Kalinella* subclade
**Tree 9** = monophyly of *Heveochlorella* - SAG 2360 sister to ITBB A3-8
**Tree 10** = monophyly of *Heveochlorella* - ITBB A3-8 sister to SAG 2360

***P < 0.05 significantly rejected**

**Table 3.** Compensatory base changes (CBCs) and Hemi-CBCs among the ITS-1 and ITS-2 rDNA sequences of the *Jaagichlorella* and *Kalinella* strains. The bottom left corner shows the CBCs (HCBCs) of ITS-1, the upper right those of ITS-2. The names in the column indicate the haplotypes of ITS-1, those in the row the haplotypes of ITS-2. The asterisk marked that *J. luteoviridis* has only one haplotype in ITS-2.

| CBC (HCBC) | ITS-2<br>ITS-1 | JLUT* | JLUT* | JAFR | JROY1 | JROY2 | JROY3 | JHAI | JGEO | JSPH | KBAM | KAPY1 | KAPY2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *J. luteoviridis* | JLUT1 | | 0 (0) | 9 (4) | 7 (3) | 9 (5) | 6 (2) | 6 (3) | 11 (2) | 7 (3) | 9 (5) | 11 (7) | 11 (7) |
| | JLUT2 | 0 (0) | | 9 (4) | 7 (3) | 9 (5) | 6 (2) | 6 (3) | 11 (2) | 7 (3) | 9 (5) | 11 (7) | 11 (7) |
| *J. africana* | JAFR | 8 (6) | 8 (6) | | 11 (1) | 9 (3) | 9 (3) | 6 (1) | 9 (1) | 8 (1) | 7 (3) | 9 (5) | 9 (5) |
| *J. roystonensis* | JROY1 | 9 (5) | 9 (5) | 7 (4) | | 0 (1) | 1 (2) | 5 (1) | 8 (1) | 5 (2) | 6 (3) | 10 (6) | 10 (6) |
| | JROY2 | 6 (5) | 6 (5) | 6 (4) | 3 (2) | | 1 (4) | 6 (1) | 7 (2) | 7 (2) | 9 (3) | 11 (7) | 11 (7) |
| | JROY3 | 5 (7) | 5 (7) | 5 (6) | 1 (3) | 1 (3) | | 5 (1) | 7 (1) | 5 (2) | 7 (3) | 9 (6) | 9 (6) |
| *J. hainangensis* | JHAI | 6 (7) | 6 (7) | 6 (5) | 10 (5) | 9 (4) | 9 (6) | | 3 (2) | 3 (3) | 6 (2) | 11 (5) | 11 (5) |
| *J. geometrica* | JGEO | 5 (5) | 5 (5) | 5 (3) | 7 (7) | 7 (6) | 8 (6) | 1 (4) | | 5 (3) | 7 (3) | 11 (6) | 11 (6) |
| *J. sphaerica* | JSPH | 6 (5) | 6 (5) | 4 (3) | 8 (4) | 6 (3) | 7 (3) | 3 (4) | 2 (4) | | 7 (3) | 13 (3) | 13 (3) |
| *K. bambusicola* | KBAM | 3 (1) | 3 (1) | 2 (2) | 1 (1) | 1 (1) | 0 (2) | 4 (1) | 1 (0) | 1 (1) | | 7 (4) | 7 (4) |
| *K. apyrenoidosa* | KAPY1 | 3 (3) | 3 (3) | 2 (1) | 3 (0) | 1 (0) | 0 (0) | 0 (0) | 0 (0) | 2 (0) | 3 (0) | | 0 (0) |
| | KAPY2 | 6 (7) | 6 (7) | 5 (3) | 6 (1) | 4 (1) | 3 (1) | 3 (0) | 3 (1) | 5 (2) | 3 (2) | 0 (0) | |

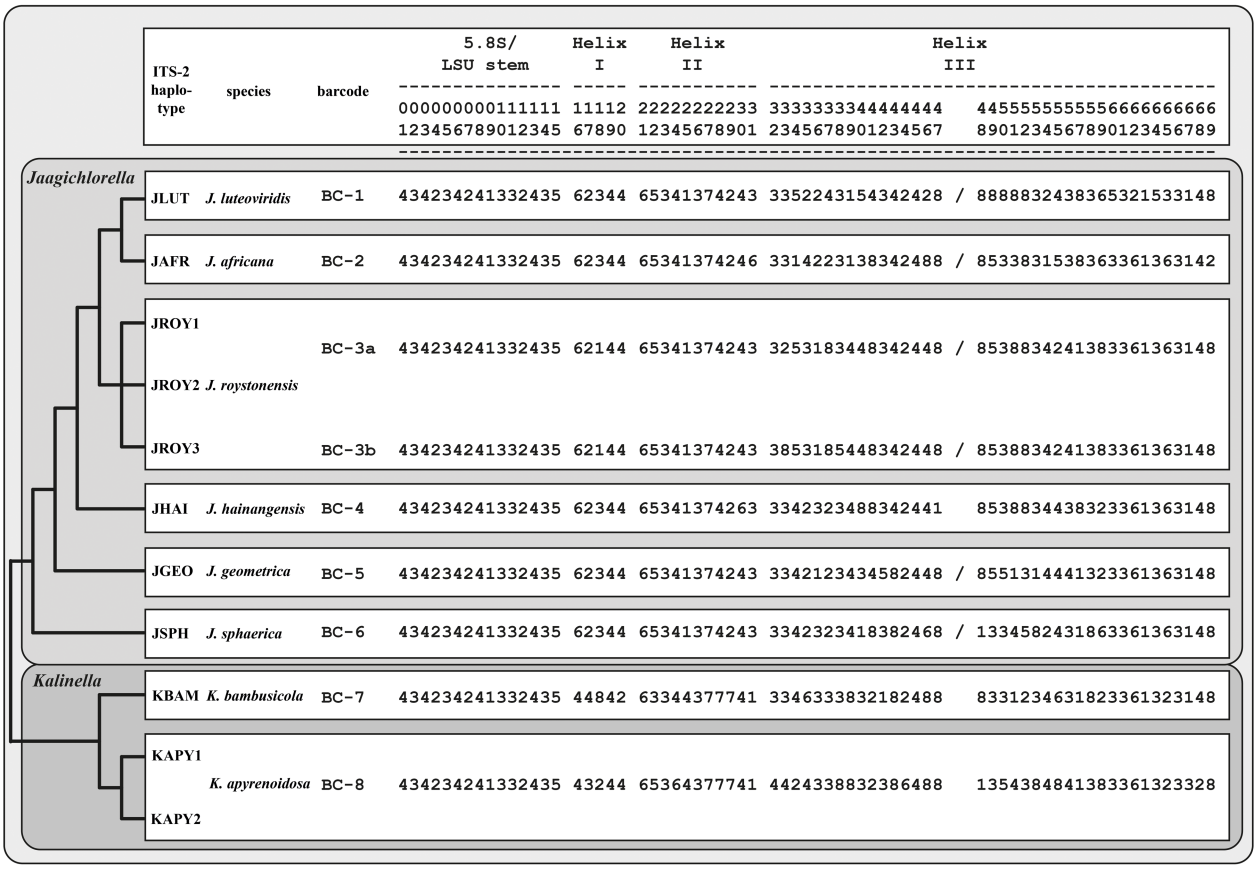


**FIGURE 3**. Comparison of the conserved region of ITS-2 among the species of *Jaagichlorella* and *Kalinella*. Extraction of this region and translation into a number code for its usage as barcode. The '/' indicated the position of the site loop in Helix III, which we excluded from the barcode. Number code for each base pair: 1 = A-U; 2 = U-A; 3 = G-C; 4 = C-G; 5 = G•U; 6 = U•G; 7 = pyrimidine-pyrimidine mismatch; 8 = deletion, single or unpaired bases. The phylogenetic tree shown was calculated using the neighbor-joining method based on the data set (69 number-coded positions of 11 haplotypes) using PAUP 4.0b10.

**Phenotypic plasticity**

In contrast to the high genetic variability, all investigated strains showed only little variation in their morphology. All strains had mostly spherical or slightly ellipsoidal or irregular cell shape with cup-shaped chloroplast, often removed in the bottom part from cell wall and then became band-shaped (according to Fott & Nováková 1969) or disc-shaped (according to Shihira & Krauss 1965). The cell sizes varied among the investigated strains by around 3 µm in young cells, and between 8.2–9.1 µm by mature vegetative cells. All investigated strains of both genera (*Jaagichlorella* and *Kalinella*) were characterized by production of unequal size autospores, even or odd in numbers in the autosporangia. Using the identification keys (Fott & Nováková, 1969), the strains of groups A, B, and C fitted in morphology with the description of *Chlorella luteoviridis*. Only two strains of group D (SAG 2549 and SAG 2360) were smaller in size and therefore identified as *Jaagichlorella geometrica* and *Heveochlorella hainangenis* by comparison with the original descriptions of Reisigl (1964) and Zhang *et al.* (2008), respectively. The strain of *Chlorella sphaerica* (UTEX 2485) fitted with the original diagnosis provided by Tschermak-Woess (1988), which was confirmed by Darienko & Pröschold (2018).

Comparing the morphology of the strains belonging to groups A-D (*Jaagichlorella*) in detail, the following variations were discovered:

**Group A (Fig. 4A–I):** All strains showed a very thin, slightly yellowish chloroplast, which covered maximum a 1/3 of the cell. Chloroplast saucer-shaped, very often was removed in bottom part and becomes band-shaped, adjoined to the cell wall from one side; very often occupied middle position in cell. Pyrenoid(s) one or sometimes two (depending on the strain) were surrounded by several starch grains (4-5). Interestingly, one or several large vacuoles can be observed in cells of all ages (including autospores in sporangia), which sometime occupied almost 2/3 of the cell volume.

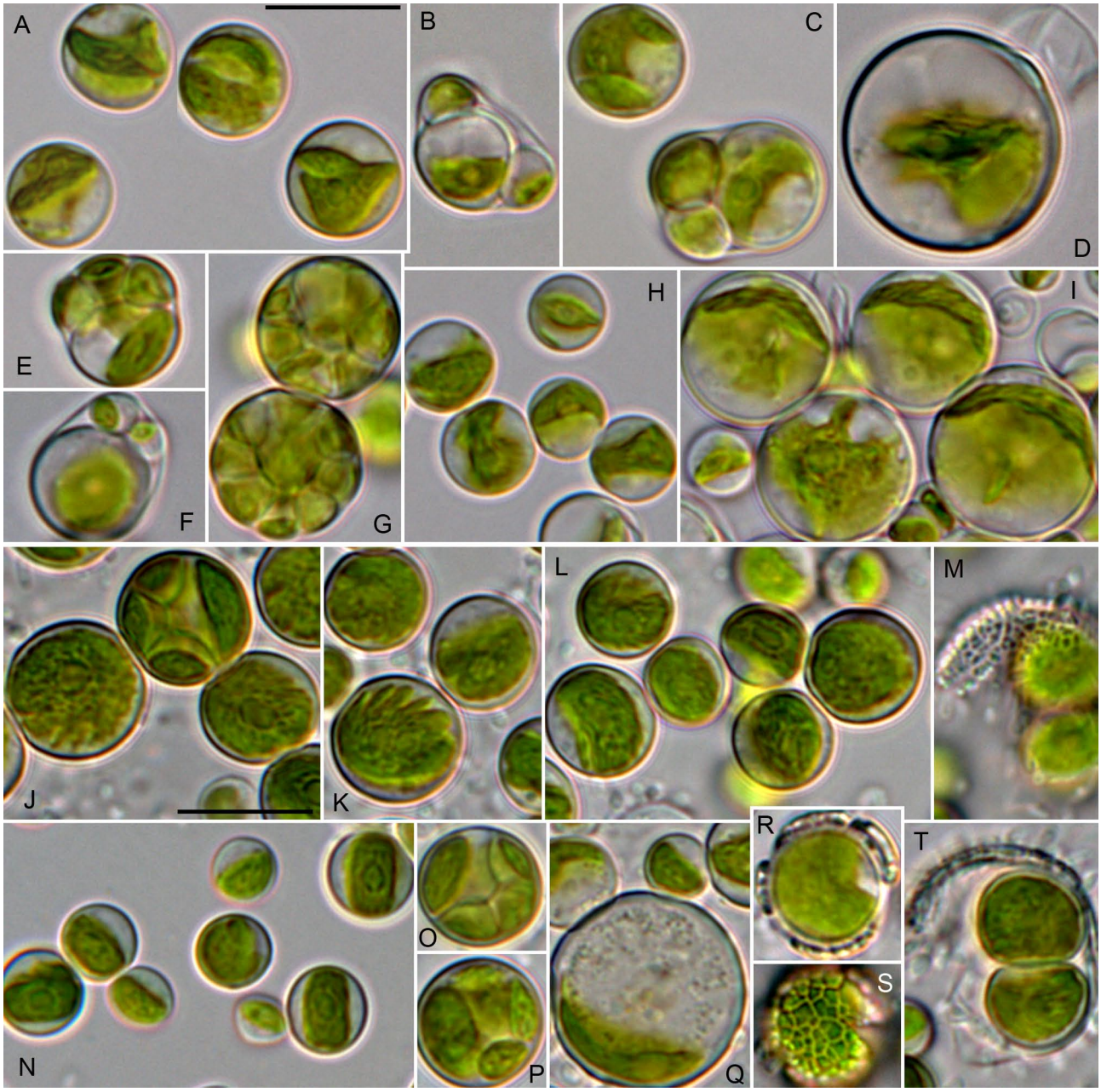


**FIGURE 4**. Morphology and phenotypic plasticity of *Jaagichlorella luteoviridis* (SAG 211-2a; **A.–I.**) and *J. africana* (SAG 2213; **H.–T.**); scale bar = 10 µm.

**Group B (Fig. 4J–T):** The cells of two strains were spherical in shape containing a massive cup-shaped dark-green chloroplast, which covered 2/3 of the cell. On the edge of the chloroplast small incisions on the margin are visible, which is only slightly removed from the cell wall. The single pyrenoid is surrounded by many starch grains. The cell wall is relative thick with an outer thin black-layer, sometimes outside partially loosened. Numerous, small vacuoles and inclusions are present. No big vacuoles were observed. They differed from strains of groups A and C by a large chloroplast.

**Group C (Fig. 5A–G'):** The strains of this group are also characterized by spherical or slightly ellipsoidal cell shape with a saucer- to cup-shaped chloroplast containing a pyrenoid surrounded by several starch grains. The chloroplast is sometimes slightly removed from the cell wall, which never occupies the middle position of the cells, and is thicker covering a larger cell volume (in contrast to the strains of group A). Vacuoles were much smaller and occupied much less cell volume. The cell wall was thicker, and showed more contrast, sometimes with one-side thickening.

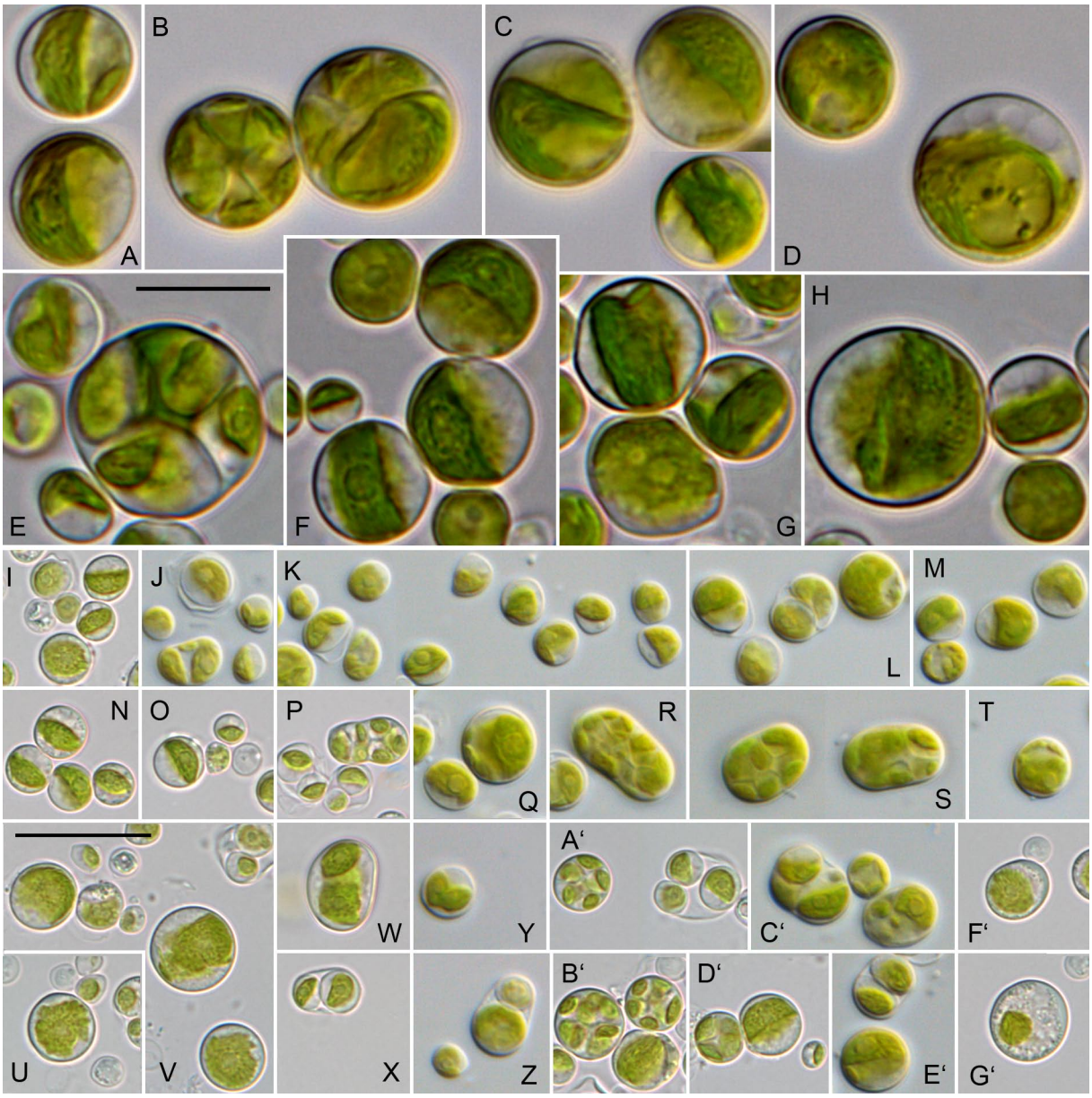


**FIGURE 5**. Morphology and phenotypic plasticity of *Jaagichlorella roystonensis* var. *epilithica* (SAG 2133; **A.–H.**) and *J. roystonensis* var. *handai* (SAG 2198; **I.–G‘.**); scale bar = 10 µm.

**Group D (Fig. 6A–J‘, 7A–J):** Morphologically, the three strains SAG 2549, SAG 2360, and UTEX 2485 were almost identical with those of group C and differed only in their smaller cell sizes. Comparing the morphology of SAG 2549, SAG 2360, and UTEX 2485 with the original description of *Jaagichlorella geometrica* (Reisigl 1964), *Heveochlorella hainangensis* (Zhang *et al.* 2008), and *Chlorella sphaerica* (Tschermak-Woess 1988) showed no differences.

The strains belonging to the *Kalinella* subclade were slightly different in morphology from each other. The authentic strain of *Kalinella bambusicola* (CAUP H7901 = SAG 2320) is similar in morphology to the strains of groups A-C (*luteoviridis* morphology). However, the authentic strains of both *Kalinella* species, *K. bambusicola* (CAUP H7901) and *K. apyrenoidosa* (CAUP H7902) were almost identical in morphology with those described in their original diagnoses of Neustupa *et al.* (2009) and (2013), respectively. The strain SAG 2203 is slightly different in morphology (Fig. 7) compared to *K. apyrenoidosa.* The mature vegetative cells were smaller (6.4–7.3 µm) in size, and have a relative thick cell wall, often with an apical small thickening. The chloroplast covered most of the cell, and is mantle-shaped with slightly wavy margin. The chloroplast is usually not removed from the cell wall. The light zone in the chloroplast (pyrenoid?) is small indistinct, without good visible starch grains. The strain CAUP H7902 is morphologically almost identical with SAG 2203.

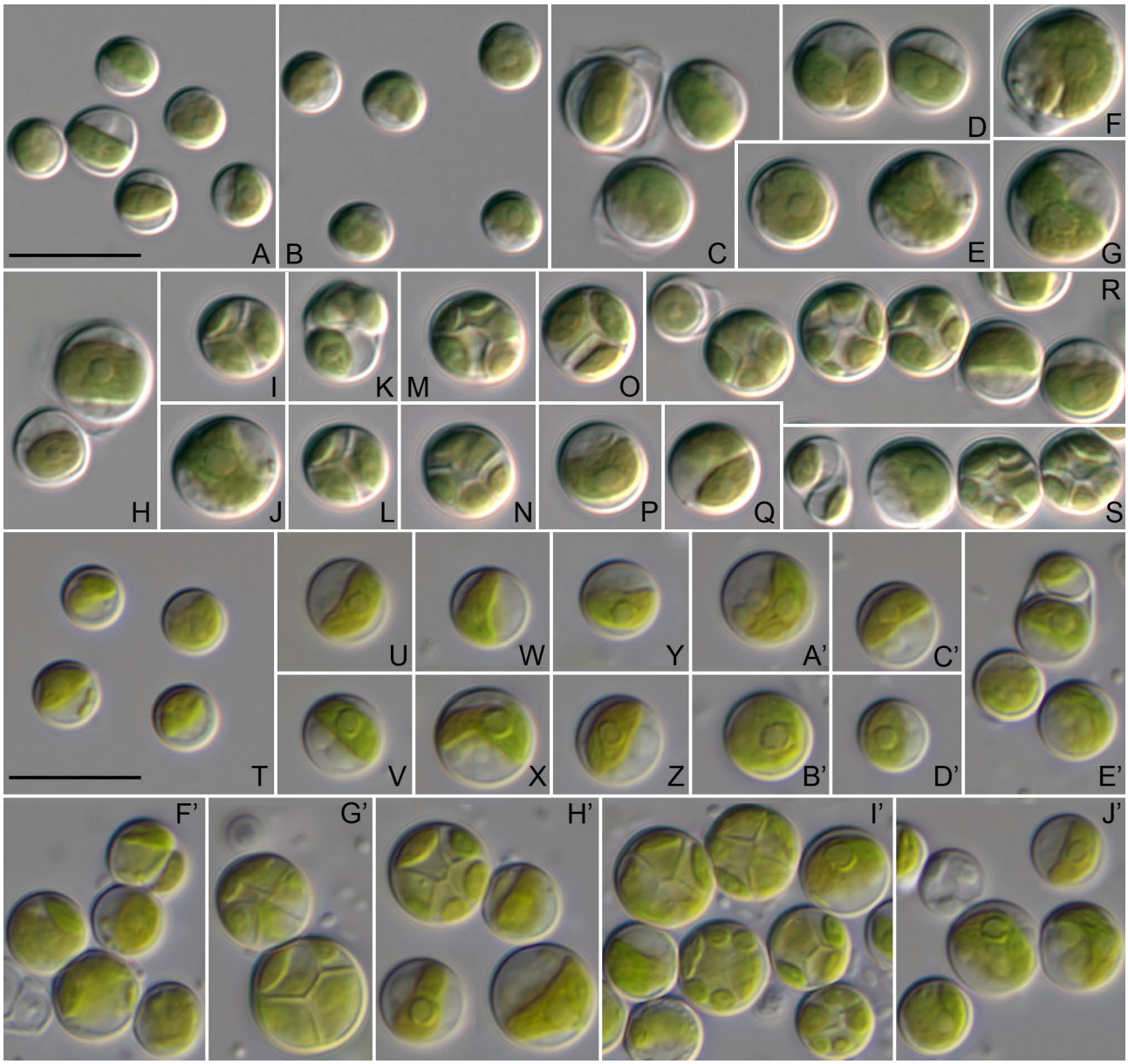


**FIGURE 6**. Morphology and phenotypic plasticity of *Jaagichlorella hainangensis* (SAG 2360; **A.–S.**) and *J. geometrica* (SAG 2549; **T.–J'.**); scale bar = 10 µm.

## Discussion

### *Jaagichlorella*, *Heterochlorella*, *Heveochlorella* and *Kalinella*: an evolutionary puzzle

As shown in the figures and tables, all investigated strains showed a *Chlorella luteoviridis* morphology. These algae have little phenotypic plasticity, but were characterized by very high genetic variability reflected in the high evolutionary rates among the members of the *Watanabea* clade (Trebouxiophyceae). As a result of phylogenetic analyses, taxa with similar morphology were described as new genera and species among this clade: *Kalinella* with its two species, *K. bambusicola* and *K. apyrenoidosa* (Neustupa *et al.* 2009, 2013), and *Heveochlorella*, with *H. hainangensis* and *H. roystonensis* (Zhang *et al.* 2008, Ma *et al.* 2013). In addition, the new generic name *Heterochlorella* was proposed for *Chlorella luteoviridis* (Neustupa *et al.* 2009). The phylogenetic analyses of the new sequences presented in this study shined a new light on the generic concept of closely related taxa. The two species of *Heveochlorella* belonged to two different groups (C and D of the *Jaagichlorella* subclade; Fig. 1). Both groups are closely related to the groups A (*Heterochlorella*) and B (new lineage, described below as *Jaagichlorella africana*). The group D contains beside *Heveochlorella hainangensis, Chlorella sphaerica* and a strain, which could be clearly identified as *Jaagichlorella*

*geometrica*. As a consequence of our findings, which are highly supported in all bootstrap and Bayesian analyses and the probability tests of user-defined trees, the three genera *Jaagichlorella*, *Heterochlorella* and *Heveochlorella* need to be revised (see proposal below). As shown in Figs 1–2, the strains belonging to the *Jaagichlorella* subclade represent six species of one genus. According to the International Code for Nomenclature (ICN), the oldest generic name has priority, in our case *Jaagichlorella*, which was described by Reisigl (1964). Both other genera are therefore later synonyms (proposals see below). The only alternative scenario, the recognition of each group (A-D) as separated genera, was rejected by approximately unbiased tests presented in Table 2. The other argument against the establishment of new generic names for these groups is that the genetic biodiversity among the *Watanabea* clade has not fully been discovered. For example, Sanders *et al.* (2016) showed that different lichen species contained new undescribed taxa of *Heveochlorella*, which also belong to the *Jaagichlorella* subclade and therefore to the genus *Jaagichlorella*. Unfortunately, only partial SSU rDNA sequences without any documented morphology are available, but the combination with our data in this study showed that new lineages can still be discovered (Fig. 8). We cannot decipher if these new lineages represent new species because no ITS rDNA sequences of these specimens are available in GenBank.

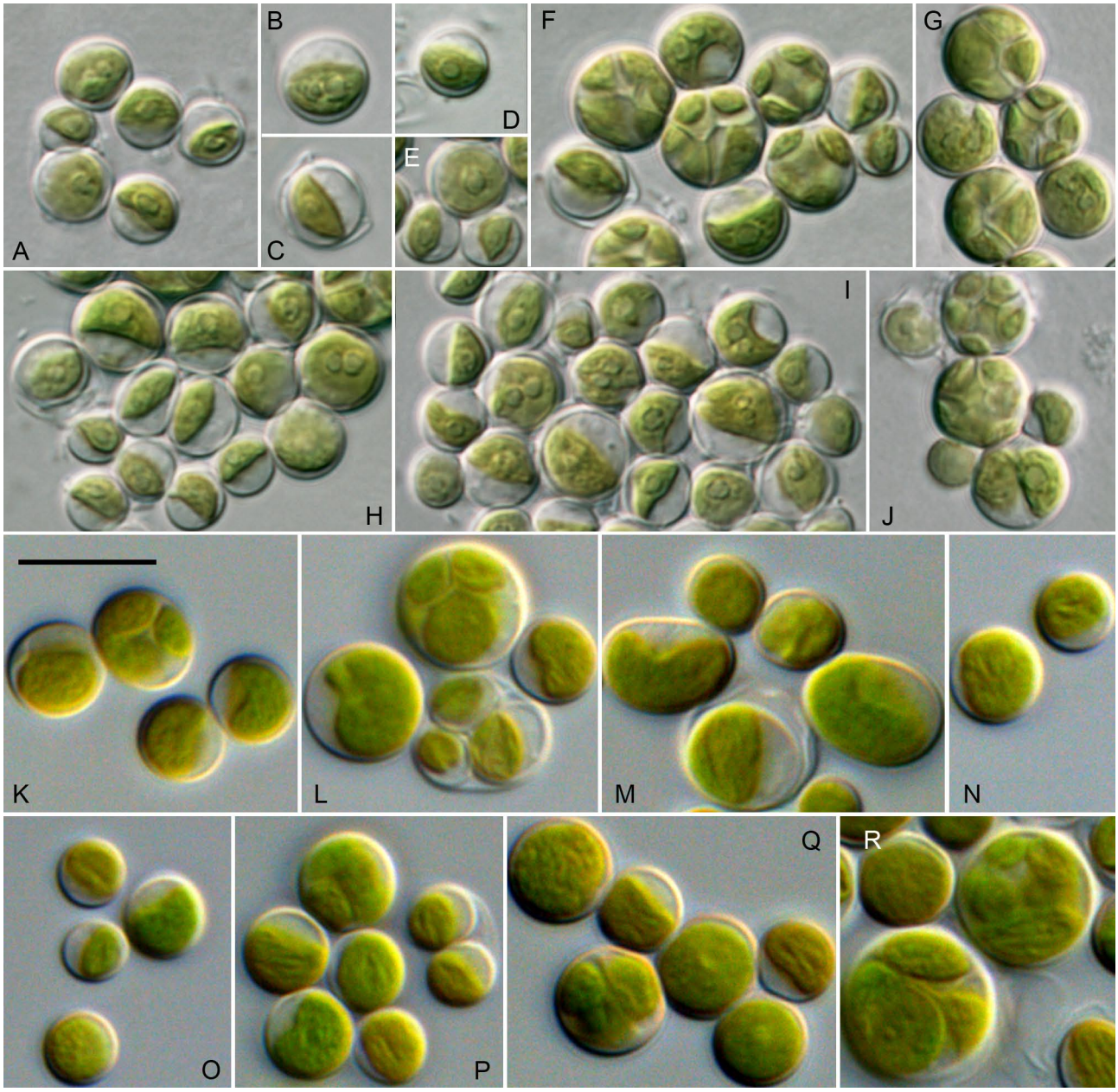


**FIGURE 7**. Morphology and phenotypic plasticity of *Jaagichlorella sphaerica* (UTEX 2485; **A.–J.**) and *Kalinella apyrenoidosa* var. *japonica* (SAG 2203; **K.–R.**); scale bar = 10 µm.

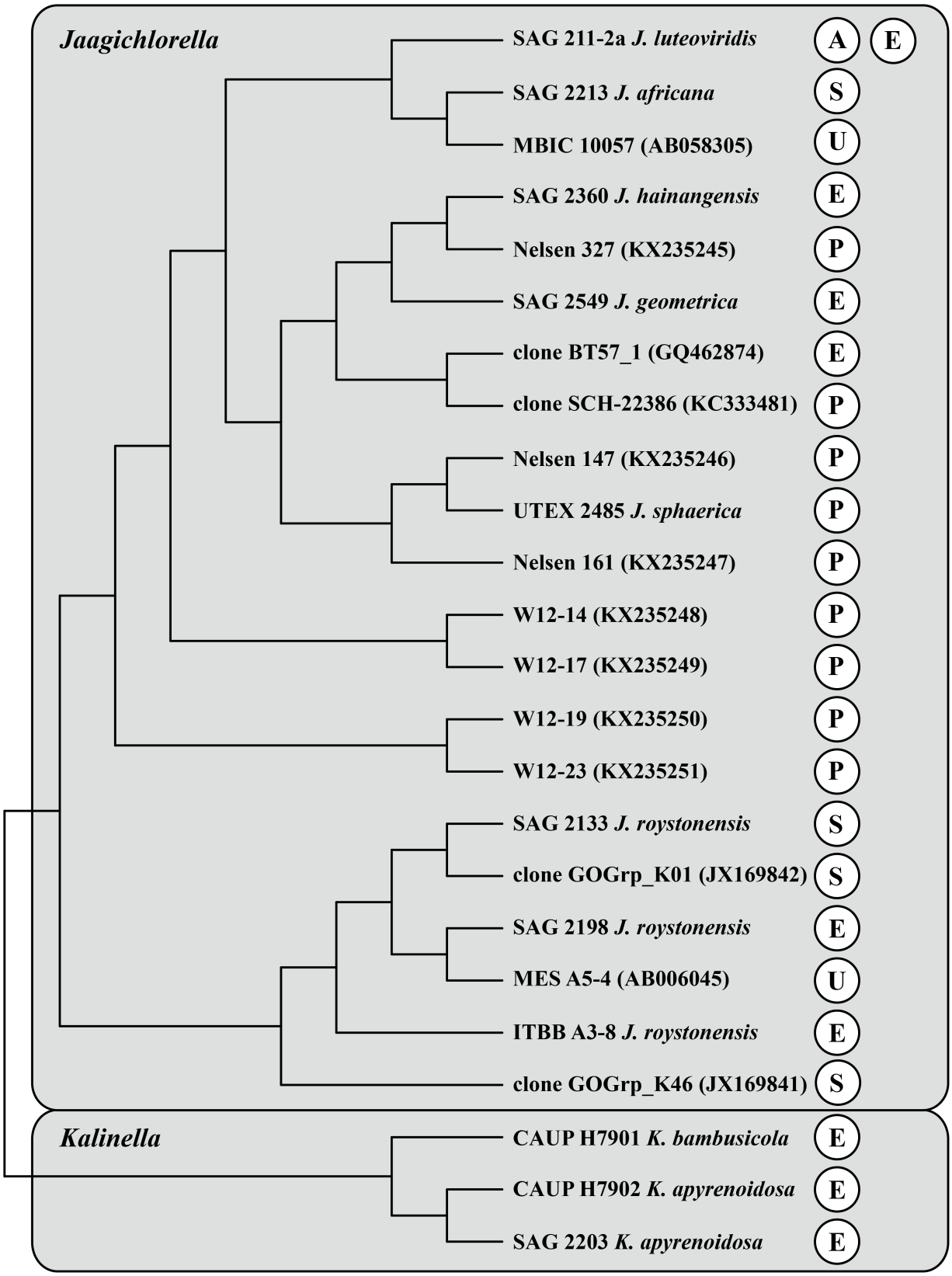


**FIGURE 8.** Molecular phylogeny of *Jaagichlorella* and *Kalinella* based on partial SSU rDNA sequence comparisons. The phylogenetic tree shown were inferred using the neighbor-joining method based on the data set (346 aligned positions of 24 taxa) using PAUP 4.0b10. The accession numbers of the partial sequences found in GenBank is given after the names of the isolates/clones. The letters after each sequence indicates their origin (A = aquatic; E = epiphytic; P = photobiont of lichen; S = epilithic on rocks or artificial hard substrates; U = unknown).

Using traditional identification keys such as Komárek & Fott (1983) or Ettl & Gärtner (2014), most of the investigated strains were identified as *Chlorella luteoviridis*. This included the strains, which were described as *Kalinella bambusicola* and *K. apyrenoidosa*. Our analyses revealed that both species are the sister group of *Jaagichlorella*. We cannot decide at this stage if the genus *Kalinella* should also be synonymized, this needs further investigations especially by including of more strains. Therefore, we kept *Kalinella* as a separate genus despite its close affiliation to *Jaagichlorella*. Summarizing, both genera were characterized by morphology (spherical cells, unequal size of autospores, and chloroplast saucer- or cup-shaped, presence of pyrenoid with/without starch layers) and molecular phylogeny (SSU and ITS rDNA sequences including their secondary structures). As demonstrated in this study, genera should be characterized using an integrative approach, recognizable by different features (morphology, reproduction, and molecular signatures), which includes the study of old literature to avoid double descriptions of species and genera as demonstrated here for *Heterochlorella* and *Heveochlorella*.

**Historical overview about the described species with *Chlorella luteoviridis* morphology**

*Chlorella luteoviridis* was described by Chodat (in Conrad & Kufferath, 1912; Chodat, 1913; Kufferath 1913) from Kufferath's materials, received from Belgium. The type description is valid according to the ICN; however, the morphology of this taxon was poorly described (spherical cells, distinct pyrenoid, unequal size of autospores) and not illustrated, which was later provided by Chodat (1913) and Kufferath (1913) based on the same cultured material. This material, the authentic strain SAG 211-2a, was investigated in this study. It belongs to group A (Fig. 2). As demonstrated above, this group also contains the authentic strains of *C. luteoviridis* var. *lutescens* (SAG 211-4 = CCAP 211/4; described by Chodat in Conrad & Kufferath, 1912), *C. aureoviridis* (SAG 211-3 = CCAP 211/3; Meyer, 1932), *C. mutabilis* (SAG 211-5a; Shihira & Krauss, 1965) and *C. nocturna* (SAG 211-5b; Shihira & Krauss, 1965). These species differed only by the color of colonies grown on organic agar from *C. luteoviridis* and showed no morphological differences cultivated under standard conditions described in Material & Methods. Fott & Nováková (1969) and Andreyeva (1975) listed these species as synonyms of *C. luteoviridis*, which is supported by our phylogenetic analyses. In addition, three strains (CCAP 211/10A, CCAP 211/10E, and CAUP H1963) originally assigned as *C. variegata* also belonged to group A. This species was described from the sap of an *Ulmus* tree (Beijerinck,1904). The taxonomy of this species is very confusing. Despite its valid description according to the ICN, there is only information about growth on different organic media, but no information on morphology, which could be used for identification. The figures provided by Beijerinck showed a mixture of two different algae, which could be identified as *C. protothecoides* Krüger and *Chl. luteoviridis*, and *Prototheca* Krüger, a colorless green algae, which was designated as fungus by Beijerinck (1904). Most of the cells presented in the figure by Beijerinck had a bi-lobate chloroplast without a pyrenoid and the autospores were equal in size. This morphology clearly differed from those of *C. luteoviridis* and showed similarities to *C. protothecoides*. Adding to the confusion, Beijerinck isolated several strains of *C. variegata* and sent them to different investigators (names are not listed). These strains were probably later deposited in different culture collections, which explained why these strains, all named *C. variegata*, represented two different species: *C. luteoviridis* (CCAP 211/10A, CCAP 211/10C = CAUP H1963, CCAP 211/10E) and *C. protothecoides* (SAG 211-10a, SAG 211-10b, CCAP 211/10B; see details in Darienko & Pröschold 2015b), which were reported by Fott & Nováková (1969) and Andreyeva (1975).

*Chlorella luteoviridis* was transferred to the new genus *Heterochlorella* based on phylogenetic analyses of SSU rDNA sequences by Neustupa *et al.* (2009). Unfortunately, the intended combination is incomplete because the citation in the basionym was incorrect and no lectotype for *Chlorella luteoviridis* has been designated.

The other strains assigned as *C. luteoviridis* belonged to groups B and C. As demonstrated in Fig. 4, the two strains SAG 2213 and SAG 2214 were very similar in morphology to *C. luteoviridis*. Only little differences could be discovered as described above. In contrast, both strains belonging to group B were phylogenetically different from strains of group A, which were confirmed by ITS-2 barcodes presented in Fig. 3 and several CBCs and HCBCs in their secondary structures of ITS-1 and ITS-2 (Table 3). The strains of group C differed only by smaller cell size from the strains of the other groups. To this group belonged the strains SAG 2133, SAG 2196, and SAG 2198 as well as the authentic strain of *Heveochlorella roystonensis* (ITBB A3-8). Little genetic variations and few CBCs/HCBCs could be discovered among these strains, but no CBC within the conserved region (ITS-2 barcode) could be observed.

The authentic strains of *Heveochlorella hainangensis* (SAG 2360) and *Chlorella sphaerica* (UTEX 2485) formed together with a strain (SAG 2549), which was clearly identified as *Jaagichlorella geometrica*, the group D. The genus *Jaagichlorella* was established by H. Reisigl (1964) and is characterized by spherical or slightly ellipsoidal cells with a plate-shaped chloroplast with pyrenoid. According to Reisigl, the only difference to *Chlorella* is the type of chloroplast: plate-shaped by *Jaagichlorella* and cup-shaped by *Chlorella*. No comparison with *Chlorella luteoviridis*

was done by Reisigl (1964). Fott & Nováková (1969) noted that *Jaagichlorella geometrica* is very similar to *Chlorella luteoviridis* and mentioned it as a special form of *C. luteoviridis* with small cell size. Andreyeva (1975) and Komárek & Fott (1983) followed this conclusion. Recent publications on *Heveochlorella*, *Heterochlorella* and *Kalinella* ignored the existence of *Jaagichlorella*.

## Nomenclatural and taxonomical conclusions

As demonstrated in this study, all strains belonged to four groups (A-D), which represent six species. They are members of one genus, *Jaagichlorella*. Our findings require several nomenclatural changes, which are proposed below:

***Jaagichlorella*** Reisigl, 1964, *Österr. Bot. Z.* **111**: 467.

**Synonym:** *Heveochlorella* J. Zhang, V.A.R. Huss, X. Sun, K. Chang, & D. Pang, 2008, *Eur. J. Phycol.* **43**: 186; *Heterochlorella* J. Neustupa, Y. Nemcová, M. Eliáš, & P. Škaloud, 2009, *Phycol. Res.* **57**: 167.

**Emended description:** Vegetative cells are solitary, spherical, sometimes slightly irregular in shape, chloroplast parietal, saucer-shaped in young cells, band-like shaped in mature cells, often removed from the cell wall, containing a single pyrenoid surrounded by starch grains. Reproduction by unequal sized autospores. One autospore often has the double size of the others.

**Type species: *Jaagichlorella geometrica*** Reisigl

***Jaagichlorella luteoviridis*** (Chodat) Darienko & Pröschold *comb. nov.* (Fig. 4A–I)

**Basionym**: *Chlorella luteoviridis* Chodat in Conrad & Kufferath, 1912, *Bull. Soc. Roy. Bot. Belg.* **49**: 322; Chodat, 1913, *Matériaux Flore cryptogam. Suisse* **4**: fig. 101 (lectotype, designated here).

**Synonyms**: *Chlorella aureoviridis* Meyer, 1932, *Beih. Bot. Centralbl.* **49**: 510–511, fig.5 (lectotype, designated here); *Chlorella luteoviridis* Chodat var. *lutescens* Chodat in Conrad & Kufferath, 1912, *Bull. Soc. Roy. Bot. Belg.* **49**: 322; Chodat 1913, *Matériaux Flore cryptogam. Suisse* **4**: fig. 108 (lectotype, designated here); *Chlorella mutabilis* Shihira & Krauss 1965, *Chlorella*: 18, fig. 7,8; *Chlorella nocturna* Shihira & Krauss 1965, *Chlorella*: 19, fig. 9,10; *Chlorella vulgaris* var. *luteoviridis* (Chodat) Shihira & Krauss 1965, *Chlorella*: 22, fig. 15,16.

**Emended description:** Young cells are spherical 4.6–6.4 µm or sometimes slightly ellipsoidal 5.5 x 4.6 µm or 6.4 x 5.5 µm with relative thick cell wall. Chloroplast of young cells is saucer-shaped to band-shaped, removed from cell wall in some places, very often pleated, with a single pyrenoid surrounded by many starch grains. Often in young cells many colorless vacuoles are present.

Size by mature vegetative cells depends on the strain, but in average was 7.3–9.1 µm. Old cells by some investigated strains were up to 20.5 µm in diameter, but on average 10.0–12.7 µm.

Reproduction is by 2-4-8 unequal autospores, producing in even or odd quantity (3 autospores per sporangia were observed). Autospores have saucer-shaped or band-shaped chloroplasts and some large vacuoles. Liberation of autospores by rupture of the sporangial cell wall. Remains of cell wall are usually bag-shaped. Often one autospore remains in the sporangial cell wall and develops to a mature vegetative cell or to a new autosporangia. Autosporangia are spherical (8.2–9.1 µm) or mostly irregular, because of unequal size of autospores and also varied in size (between 16.4 x 19.1 µm), but were in average 10.0 x 10.9 µm to 10.0 x 12.7 µm. Large autospores are often 6.4 µm in diameter, but sometimes up to 10.0 µm; small autospores around 3.7–4.6 µm in size. SSU and ITS rDNA sequences (GenBank: MH780927) and ITS-2 Barcode BC-1 in Fig. 3.

**Epitype** (designated here to support the lectotype): The authentic strain (SAG 211-2a) is cryopreserved in a metabolic inactive state at the Culture Collection of Algae (SAG), University of Göttingen, Germany.

***Jaagichlorella africana*** Darienko & Pröschold *sp. nov.* (Fig. 4J–T)

**Description:** Young cells are spherical (5.5–6.4 µm in diameter) or slightly ovoid to irregular (6.4 x 5.5 µm in size). Cell wall by young cells is relative thick. Chloroplast cup-shaped to saucer-shaped, covers 2/3 of the cell with a single, distinct pyrenoid surrounded by several starch grains. Mature vegetative cells are mostly spherical, 7.3–9.1 µm, sometimes 11.8 µm in size. Cell wall is relative thick. Chloroplast deep cup-shaped, often with small incisions or wavy

margins, in the bottom part sometimes slightly removed from the cell wall. Chloroplast thick, with dark-green color, which covers 2/3 or more of the cell. The single pyrenoid is distinct surrounded by many starch grains. Numerous, small vacuoles and inclusions are present. Old cells often have a reduced chloroplast, which is only slightly removed from the cell wall.

Reproduction is by 2-4-8 unequal autospores. Autospores have a saucer-shaped or band-shaped chloroplast with a visible pyrenoid. Liberation of spores by rupture of the sporangial cell wall. Remains of the sporangial cell wall are usually bag-shaped. Often one autospore remains in the cell wall and develops to a mature cell or a new sporangium. Autosporangia are spherical or often irregular, 9.1–11.8 µm, sometimes up to 15.5 µm in size. Large autospores between 6.4–11.8 µm, small autospores 3.7–5.4 µm in size. SSU and ITS rDNA sequences (GenBank: MH780938) and ITS-2 Barcode BC-2 in Fig. 3.

**Type locality:** Lithophytic on the surface of a concrete wall, Namibia.

**Holotype** (designated here): The authentic strain SAG 2213 is cryopreserved in a metabolic inactive state at the Culture Collection of Algae (SAG), University of Göttingen, Germany.

**Iconotype** (designated here to support the holotype): Fig. 4J in this study.

**Etymology**: The species epithet indicates the origin of this species.

***Jaagichlorella roystonensis*** (S. Ma, V. Huss, X. Sun & J. Zhang) Darienko & Pröschold *comb. nov.*

**Basionym:** *Heveochlorella roystonensis* S. Ma, V. Huss, X. Sun & J. Zhang in Ma *et al.*, 2013, *Eur. J. Phycol.* **48**: 205–206.

**Emended description:** SSU and ITS rDNA sequences (GenBank: JN003601, JX290371) and ITS-2 Barcode BC-3b in Fig. 3.

***Jaagichlorella roystonensis* var. *epilithica*** Darienko & Pröschold var. *nov.* (Fig. 5A–H)

**Description:** Young cells are spherical or sometimes irregular with relative thick cell wall, 6.4–7.3 µm in size. Chloroplast by young cells saucer-shaped, covers a little bit more than a half of the cell; with a single pyrenoid surrounded by many starch grains.

Mature vegetative cells are spherical 9.1–10.0 µm, sometimes 11.8 µm in diameter, with cup-shaped chloroplast, sometimes slightly removed from the cell wall in the bottom part of the cell and occupied the middle position of the cell; chloroplast relatively thick, green or yellowish-green. Cells with numerous, colorless vacuoles. Old cells are spherical, 10.9–13.6 µm in size, sometimes up to 17,3 µm, with reduced small chloroplast, cells contain several vacuoles and fill around 1/3 of the cell.

Reproduction is by 2-4-8 unequal autospores. Autosporangia are spherical or very often irregular, (8.2)–9.1–10.0 µm, sometimes they can reach 16.4–20.9 µm. Large autospores 6.4–7.3 µm, (up to 10.0 µm), small autospores 3.7–5.4 µm in size. SSU and ITS rDNA sequences (GenBank: MH780940) and ITS-2 Barcode BC-3a in Fig. 3.

**Type locality:** Lithophytic on rocks in Germany.

**Holotype** (designated here): The authentic strain SAG 2133 is cryopreserved in a metabolic inactive state at the Culture Collection of Algae (SAG), University of Göttingen, Germany.

**Iconotype** (designated here to support the holotype): Fig. 5C in this study.

**Etymology**: The epithet of this variety indicates its epilithic life style.

**Comment:** Differs from the type variety by ecology and differences in the variable regions of SSU and ITS rDNA sequences.

***Jaagichlorella roystonensis* var. *handai*** Darienko & Pröschold var. *nov.* (Fig. 5I–G‘)

**Description:** Differs from the variety *epilithica* by slightly irregular cell shape and one-sided thickening of the cell wall. SSU and ITS rDNA sequences (GenBank: MH780942) and ITS-2 Barcode BC-3a in Fig. 3.

**Type locality:** Bark of tree, Japan.

**Holotype** (designated here): The authentic strain SAG 2198 is cryopreserved in a metabolic inactive state at the Culture Collection of Algae (SAG), University of Göttingen, Germany.

**Iconotype** (designated here to support the holotype): Fig. 5Q in this study.

**Etymology**: The name of this variety was given in honor of the isolator, Dr. S. Handa.

**Comment:** Differs from the type variety by ecology and differences in the variable regions of SSU and ITS rDNA sequences.

***Jaagichlorella hainangensis*** (J. Zhang, V.A.R. Huss, X. Sun, K. Chang, & D. Pang) Darienko & Pröschold *comb. nov.* (Fig. 6A–S)

**Basionym:** *Heveochlorella hainangensis* J. Zhang, V.A.R. Huss, X. Sun, K. Chang, & D. Pang, 2008, *Eur. J. Phycol.* **43**: 187.

**Emended description:** SSU and ITS rDNA sequences (GenBank: MH780943) and ITS-2 Barcode BC-4 in Fig. 3.

**Comment:** The morphological features of this species are identical to *Jaagichlorella geometrica* (see emended diagnosis below), but differs in ecology, origin and CBCs/HCBCs in the conserved region of the ITS-2 sequences.

***Jaagichlorella geometrica*** Reisigl (Fig. 6T–J‘)

**Emended description:** Cells are solitary, spherical or slightly irregular. Young cells 3.6–4.2 µm in diameter, mature cells 6.0–8.0 µm. Chloroplast in young cells cup-shaped, in mature cells band-shaped removed from the cell wall, with a single pyrenoid surrounded by several starch grains. Reproduction by unequal sized autospores. Autosporangia between 6.0–10.0 µm in diameter, usually contain 4 autospores. SSU and ITS rDNA sequences (GenBank: MH780944) and ITS-2 Barcode BC-6 in Fig. 3.

**Lectotype** (designated here): Fig. 25 in Reisigl (1964).

**Epitype** (designated here to support the lectotype): The strain SAG 2549 is cryopreserved in a metabolic inactive state at the Culture Collection of Algae (SAG), University of Göttingen, Germany.

***Jaagichlorella sphaerica*** (Tschermak-Woess) Darienko & Pröschold *comb. nov.* (Fig. 7A–J)

**Basionym:** *Chlorella sphaerica* Tschermak-Woess, 1988, *Plant Syst. Evol.* **159**: 136.

**Emended description:** SSU and ITS rDNA sequences (GenBank: MH780945) and ITS-2 Barcode BC-5 in Fig. 3.

**Comment:** The morphological features of the authentic strain have been described by Darienko & Pröschold (2018). This species is characterized by its ecology (photobiont of the lichen *Pseudocyphellaria carpoloma*), origin (New Zealand) and CBCs/HCBCs in the conserved region of the ITS-2 sequences.

***Kalinella apyrenoidosa* var. *japonica*** Darienko & Pröschold var. *nov.* (Fig. 7K–R)

**Description:** Young cells are spherical or sometimes slightly irregular, 4.6–5.5 µm in size, with relatively thick cell wall. Chloroplast of young cells deep cup-shaped, without incisions covering 2/3 of the cell. Chloroplasts contain a thylakoid-free zone, which could be interpreted as pyrenoid, located in the middle of chloroplast, small, indistinct, without visible starch grains.

Mature vegetative cells are mostly spherical, only sometimes ovoid or irregular, 6.4–7.3 µm in diameter, cell wall with age becomes thicker and can produce small drop-shaped, apical thickness. Chloroplast is thick, dark-green, covering most of the cell, sometimes removed in the bottom part of the cell wall. Old cells are spherical, 8.2-9.1 µm in diameter.

Reproduction by unequal sized autospores, in even and odd numbers - 2 (3) - 4 (8). Autosporangia mostly spherical, sometimes irregular, 8.2–10.0 µm in size. Liberation of autospores by rupture of the mother cell wall. Remains of sporangial cell wall are usually bag-shaped and stick on the cell wall of the daughter cells. SSU and ITS rDNA sequences (GenBank: MH780948) and ITS-2 Barcode BC-8 in Fig. 3.

**Type locality:** Epiphytic algal growth on bark tree, Japan.

**Holotype** (designated here)**:** The strain SAG 2203 is cryopreserved in a metabolic inactive state at the Culture Collection of Algae (SAG), University of Göttingen, Germany.

**Iconotype** (designated here to support the holotype): Fig. 7P in this study.

**Etymology:** The epithet of this variety indicates its origin (Japan).

## Distribution of *Jaagichlorella* species

The six species of *Jaagichlorella* known have different distribution patterns. The strains of *J. luteoviridis* were isolated from aquatic habitats or from sap of the bark of trees. Interestingly, these strains are in culture collections for a long time and no recent sequences of this species have been published. The other species of *Jaagichlorella* were collected from different terrestrial habitats, *J. africana* from a concrete wall, *J. roystonensis* from bark of trees and artificial hard substrates, *J. hainangensis* from bark of tree, *J. sphaerica* is a photobiont of a lichen, and *J. geometrica*

was isolated from the rhizosphere of a plant. In addition, Sanders *et al.* (2016) found *Jaagichlorella* (designated as *Heveochlorella*) as a photobiont in different lichens. Our BLAST N search of ITS-2 sequences (100% coverage, >97% identity) revealed almost no new entries in GenBank. Only three entries (FJ028714, FJ028717, and FJ028720) of *Jaagichlorella geometrica* could be found using this algorithm. These uncultured clones are from the town hall of San Sebastian (Spain). This indicates that *J. geometrica* is probably widely distributed. Summarizing, it seems that species of *Jaagichlorella* belong to rare taxa distributed in aquatic and mainly in terrestrial habitats with the exception of soil. They have a worldwide distribution pattern, but can be rarely found, which maybe explains that *Jaagichlorella* has not been recorded using NGS approaches.

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## Supplemental material

Figures S1. ITS-2 secondary structures of the *Jaagichlorella* and *Kalinella* strains investigated in this study. The structures of the ITS-2 have been drawn with VARNA (Darty *et al.* 2009).