



## Phylogeny and morphology of genus *Nephrocytium* (Sphaeropleales, Chlorophyceae, Chlorophyta) from China

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### Abstract

The genus *Nephrocytium* Nägeli is a common member of phytoplankton communities that has a distinctive morphology. Its taxonomic position is traditionally considered to be within the family Oocystaceae (Trebouxiophyceae). However, research on its ultrastructure is rare, and the phylogenetic position has not yet been determined. In this study, two strains of *Nephrocytium*, *N. agardhianum* Nägeli and *N. limneticum* (G.M.Smith) G.M.Smith, were identified and successfully cultured in the laboratory. Morphological inspection by light and electron microscopy and molecular phylogenetic analyses were performed to explore the taxonomic position. Ultrastructure implied a likely irregular network of dense and fine ribs on the surface of the daughter cell wall that resembled that of the genus *Chromochloris* Kol & Chodat (Chromochloridaceae). Phylogenetic analyses revealed that *Nephrocytium* formed an independent lineage in the order Sphaeropleales (Chlorophyceae) with high support values and a close phylogenetic relationship with *Chromochloris*. Based on combined morphological, ultrastructural and phylogenetic data, we propose a re-classification of *Nephrocytium* into Sphaeropleales, sharing a close relationship with *Chromochloris*.

**Keywords:** *Chromochloris*, *Nephrocytium agardhianum*, *Nephrocytium limneticum*, Oocystaceae, Sphaeropleales, ultrastructure

### Introduction

The genus *Nephrocytium* is commonly documented in phytoplankton assemblages around the world (Wang & Feng 1999; Cocquyt & Vyverman 2005; Shams *et al.* 2012). It was originally described as a distinct genus in Palmellaceae by Nägeli in 1849, based on *Nephrocytium agardhianum* as the type species (Nägeli 1849). The relationship between genus *Oocystis* A. Braun and *Nephrocytium* was always considered to be close, and they were included in the subfamily Nephrocytieae in De Toni 1888 (Stoyneva *et al.* 2007). Later, *Oocystis* became the type genus of Oocystaceae, representing a distinct family in most monographs (Stoyneva *et al.* 2007), and *Nephrocytium* was considered the only genus in the subfamily Nephrocytieae within Oocystaceae (Brunnthaler 1913). Komárek & Fott (1983) also classified *Nephrocytium* into Oocystaceae in their monograph of coccoid green algae and suggested that examination of the ultrastructure was necessary to confirm the taxonomic status. According to AlgaeBase, *Nephrocytium* is a cosmopolitan genus comprising 31 species registered in Oocystaceae. However, only 8 of the 31 species are taxonomically accepted (Guiry & Guiry 2016).

The genus *Nephrocytium* is easily recognized by its bent kidney-shape to oval cells stored in markedly expanded mother cell walls within hyaline mucilage. Moreover, according to Komárek & Fott (1983), *Nephrocytium* is distinguishable from morphologically similar genera in Oocystaceae by its smooth cell wall (different from genus *Juranyiella* Hortobagyi) and presence of pyrenoids (different from genus *Nephrochlamys* Korshikov). According to Wehr *et al.* (2015), *Nephrocytium* is also distinguishable from morphologically similar genera by the presence of mucilage (different from genus *Didymogenes* Schmidle) and the absence of a markedly crescent-shaped cell (which differs from the genera *Tetrallantos* Teiling and *Kirchneriella* Schmidle). According to Bourrelly (1966), *Nephrocytium*

is morphologically distinguishable from the genera it resembles by cells separate from each other (different from genus *Nephrochlamys*), a parietal chloroplast (different from genus *Oonephris* Fott), a lack of gelatinous tract uniting cells (differing from *Tomaculum* Whitford) and no aligned cells along the axis of the colony (different from genus *Elakatothrix* Wille).

Even though *Nephrocytium* is relatively common and is described by many studies, studies on its ultrastructure are scarce, and the phylogenetic position has not yet been determined. In this paper, two *Nephrocytium* species were collected and identified as *Nephrocytium agardhianum* and *Nephrocytium limneticum* according to Komárek & Fott (1983). Ultrastructure observation by transmission electron microscopy (TEM) and phylogenetic analysis based on concatenated 18S rDNA and *rbcL* and *tufA* cpDNA data sets were conducted to assess their taxonomic position.

## Material and methods

### Sampling and cultivation

*Nephrocytium limneticum* (strain LXDQ-20) was obtained from the Gaolan river (31°7'N, 110°49'E), a secondary tributary of the Yangtze River in the Hubei province of China in June 2015, and *Nephrocytium agardhianum* (strain LXDQ-25) was collected from Erhai Lake (25°48'N, 100°13'E) in the Yunnan province of China in March 2016. Single cells were isolated from samples using the serial dilution pipetting technique (Hoshaw & Rosowski 1973) under an inverted microscope (CKX41; Olympus, Tokyo, Japan). Cells were maintained in liquid BG11 medium (Stanier *et al.* 1971) at a constant light source of 30–50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and a temperature of 25 °C. The medium was renewed every 2 weeks until sufficient biomass (>0.5 g fresh mass) was obtained for DNA extraction. Cultures can be obtained from the Freshwater Algae Culture Collection, Institute of Hydrobiology, Chinese Academy of Science (FACHB) under the accession Nos. FACHB–2123 (*Nephrocytium limneticum*) and FACHB–2124 (*Nephrocytium agardhianum*)

### Morphological observation

An Olympus BX53 light microscope with differential interference contrast (DIC) and epifluorescence microscope (EMF) optic (Olympus Corp., Tokyo, Japan) mounted with an Olympus DP80 camera matched with cellSens Standard software was used to observe cell morphology across life cycle stages. Indian ink was used to inspect the gelatinous envelope. For transmission electron microscope (TEM), cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer and then fixed in 1% aqueous  $\text{OsO}_4$  in 0.1 M cacodylate buffer, dehydrated in acetone, embedded in Spurr's resin, and ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds 1963). Zoospore and gamete induction was carried out by flooding and light starvation (Fučíková *et al.* 2013).

### DNA isolation, PCR amplification and sequencing

The algal cells were broken with mini beads in a BeadBeater (3110BX, Biospec Products, Bartlesville, USA). Total DNA was extracted using a Universal DNA Isolation Kit (AxyPrep, Shuzhou, China). Primers and PCR conditions from Xia *et al.* (2013) were used to amplify the 18S rDNA and *rbcL* cpDNA genes, and the methods for amplification of the *tufA* cpDNA gene were described by Famà *et al.* (2002).

### Sequence alignment and phylogenetic reconstruction

Sequences selected from GenBank (URL) were mainly based on previous studies of Sphaeropleales by Fučíková *et al.* (2014). Genus *Mychonastes* was designated to root the tree according to Fučíková *et al.* (2014). The *rbcL* and *tufA* cpDNA genes of strain *Pseudomuriella sp.* Itas 9/21 14-1d were not obtained, nor were *tufA* cpDNA gene sequences for the following taxa: *Botryosphaerella sudetica* (Lemmermann) P.C.Silva, *Characiopodium hindakii* (K.W.Lee & Bold) Floyd & Shin Watanabe, *Mychonastes jurisii* (Hindák) Krienitz, C.Bock, Dadheech & Proschold and *Parapediastrium biradiatum* (Meyen) E.Hegewald. 18S/*rbcL*/*tufA* gene sequences were assembled into contigs and aligned using ClustalX v 2.0 (Larkin *et al.* 2007).

Maximum Likelihood (ML) analyses were conducted in PAUP 4.0\* 4.0b10 (Swofford 2002) and RAxML 7.0.4 (Stamatakis 2008). The evolutionary models used in ML analyses were selected using jModelTest 2.1.4 (Darriba *et al.* 2012). TIM2+I+G, GTR + I + G and GTR + I + G were designated for the 18S rDNA, *rbcL* cpDNA and *tufA* cpDNA gene sets, respectively. A heuristic search option with random addition of sequences (100 replicates) and the nearest-neighbor interchange branch-swapping algorithm (NNI) were used for tree searching in PAUP; and the GTR substitution model with gamma rate distribution, four discrete rate categories, and starting from a random tree with 100 replicates were implemented in RAxML.

Phylogenetic Bayesian analysis (BI) was performed using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001), and different substitution models of each partition were selected using MrModeltest 2.3 (Nylander 2004) according to the Akaike information criterion (AIC) (Akaike 1974). The best-fit models used in Bayesian analyses were GTR+ $\Gamma$ +I for *rbcL* and *tufA* cpDNA genes, whereas GTR+ $\Gamma$  was the model selected for the 18S rDNA gene. All Bayesian Markov Chain Monte Carlo (MCMC) analyses were run with seven Markov chains (six heated chains, one cold) for 5·10<sup>6</sup> generations, where one tree was kept every 1000 generations. Each analysis reached stationarity (average standard deviation of split frequencies between runs < 0.01) well before the end of the run. A burn-in sample of 1250 trees was removed before calculating the majority rule consensus trees in MrBayes.

## Results

### *Nephrocytium limneticum* (G. M. Smith) G. M. Smith 1933: 503

**Synonyms:**—*Gloeocystopsis limnetica* G. M. Smith 1916, *Nephrocytium agardhianum* var. *szolnokiense* Kiss & Szabó 1975.

**Holotype:**—G. M. Smith 1933: 503. Depicted in Komárek & Fott (1983, pl. 157:3).

#### *Description*

Colonies (rarely unicellular) were microscopic with 2–32 (often 4–8; Figs 1–3) cells embedded in mucilaginous envelope (Fig. 5) and forming spherical to pyramidal to amorphous masses approximately 30–80  $\mu\text{m}$  diameter. Mucilaginous material usually enveloped each group of cells, often in clusters following sporulation (Fig. 3). Cells were kidney-shaped or oval to somewhat curved and were asymmetric with rounded-acuminate to broadly rounded and not tapered apices that were 11–19  $\mu\text{m}$  long and 5–14  $\mu\text{m}$  wide (Figs 1,2,13). Chloroplasts were single-lobed parietal with a single, small pyrenoid when young that gradually fragmented and diffused (Figs 4,13) with a large pyrenoid (or in rare instances, two large pyrenoids) when mature (Figs 5,14). Asexual reproduction was by autospores (Figs 3,6), 2–4–8 per sporangium and released by dissolution, or sometimes rupturing, of the sporangial wall. Mother cell wall fragments were usually dissolved in the colonial mucilage, but occasionally a distinctive remnant was observed. Sexual reproduction and flagellated stages are unknown.

The TEM observation revealed that the cell wall was multilayered, and the surfaces of the daughter cell walls were not smooth (Figs 13–15). Dense and fine ribs were thought to form an irregular network and cover the surface, approximately 0.1–0.2  $\mu\text{m}$  long. The ultrastructure changed with age and was relatively smooth when old or expanded (Fig. 14). The mutually perpendicular cellulose fibrils between adjoining layers, as observed in *Oocystis*, were not visible. The global pyrenoid with a homogenous matrix was situated and surrounded by several thick starch sheathes (Fig. 14). Thylakoids extended the length of the chloroplast and occurred in stacks of four to twelve (Figs 13,14) but never traversed the pyrenoid matrix (Fig. 14). Numerous single lenticular starch grains were visible inside chloroplasts (Fig. 13).

### *Nephrocytium agardhianum* Nägeli 1849: 79, pl. III: C a–h, l–o

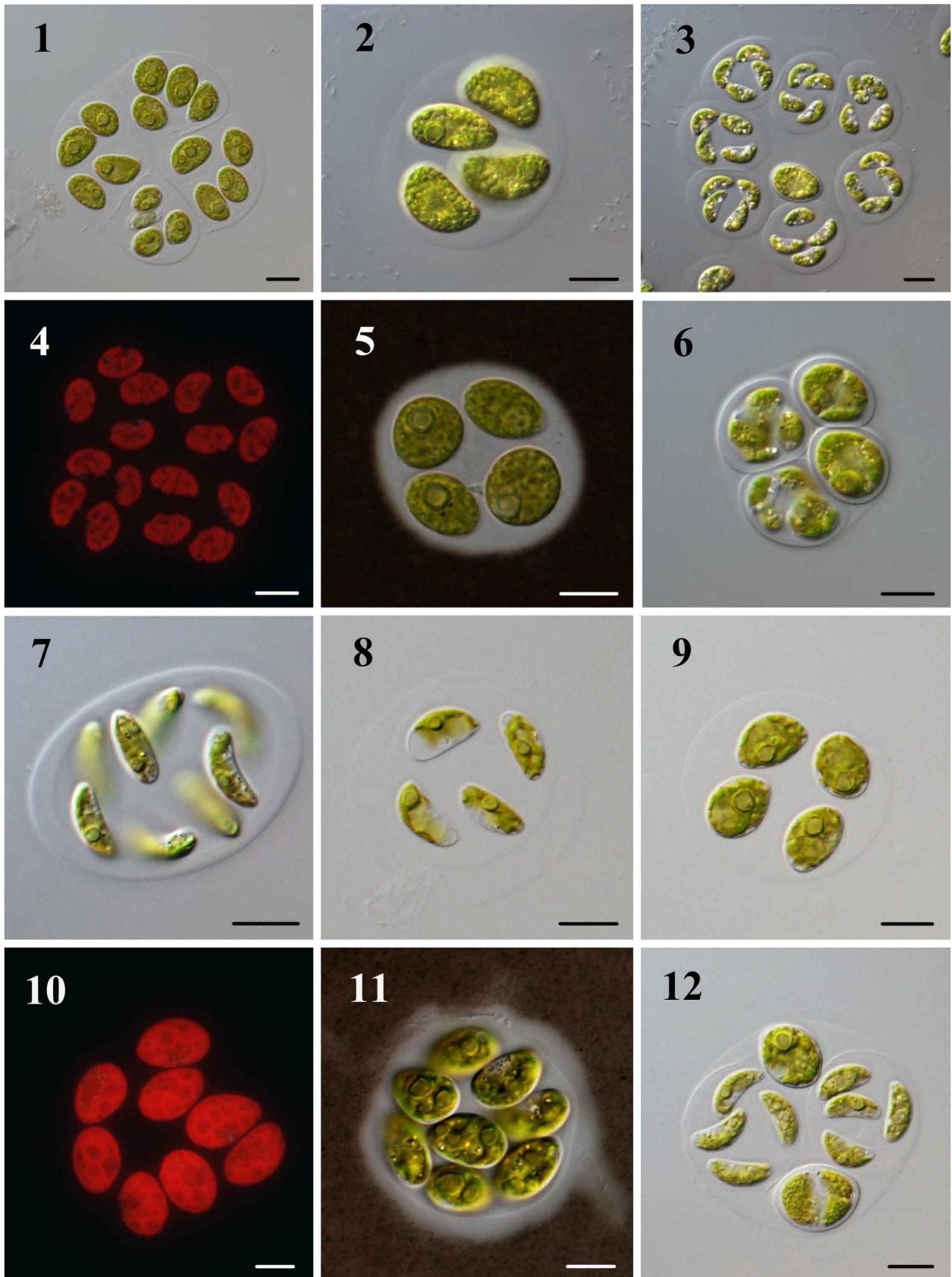
**Synonyms:**—*Nephrocytium naegeli* Grun. in Rabenh. 1868, *Selenococcus farcinalis* Schmidle & Zachar. 1903; incl. var. *minus* Näg. 1849

**Holotype:**—Nägeli 1849: 79, pl. III: C a–h, l–o. Depicted in Komárek & Fott (1983, pl. 157:1).

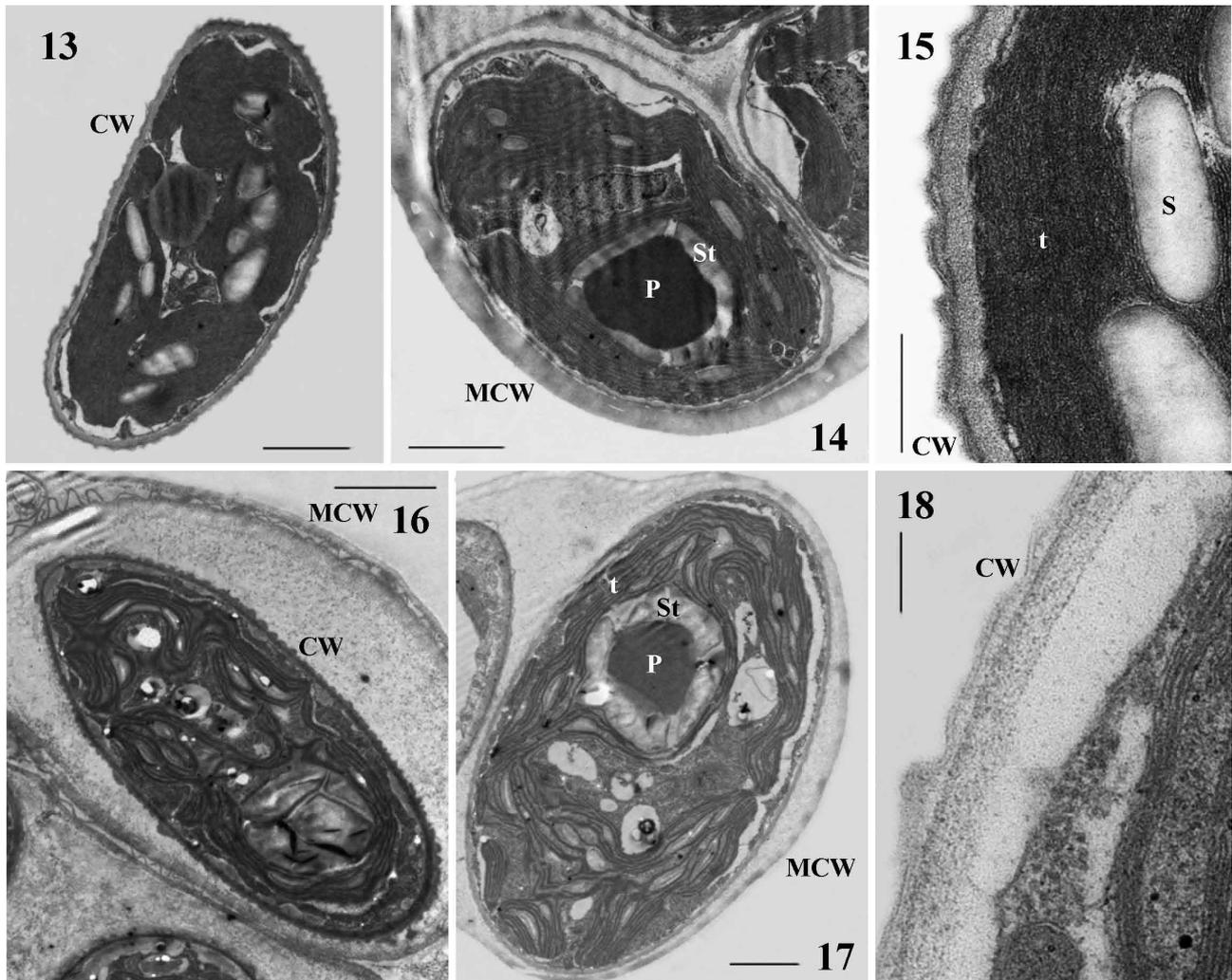
#### *Description*

Colonies were found to be somewhat asymmetrically oval with 8–16 cells helically embedded (Fig. 7). Cells were cylindrical to oval, obviously bent and asymmetric (Fig. 7). Cell size was 12–25  $\mu\text{m}$  long and 6–13  $\mu\text{m}$  wide. The mucilage envelope beside the mother cell wall was more or less present (Fig. 11). Chloroplasts were single-lobed parietal with a single pyrenoid when young and gradually diffused and filled the whole cell when mature (Figs 8,10). Asexual reproduction was by 4–8 autospores (Fig. 12) that were released by dissolution of the expanded mother cell wall. Sexual reproduction and flagellated stages are unknown. When maintained in cultured dishes, colonies were mostly round with fewer cells (mostly 4) (Figs 8,9), and the mature cells were kidney-shaped to oval, with rounded-acuminate to broadly rounded apices (Fig. 9).

When observed with TEM, the surface of daughter cell walls were found to not be smooth (Figs 16–18) and were sculptured by an irregular network of dense and fine ribs 0.1–0.2  $\mu\text{m}$  long, which were not obviously on the mother cell wall (Figs 16,17). The *Oocystis*-like ultrastructure of the cell wall was not observed. Thylakoids were observed in stacks of two to five, and none penetrated the pyrenoid matrix, which was surrounded by several starch sheathes (Figs 16,17).



**FIGURES 1–12.** Light microscopy of *Nephrocytium limneticum* cultured (1–6), and *Nephrocytium agardhianum* in the field (7), and cultured (8–12). 1. Young cells in colony. 2. Old cells in colony. 3. Autospores in sporangium. 4. Autofluorescence showing the shape of chloroplasts. 5. Negative stain by ink showing the mucilage envelope. 6. Sporangia in colony. 7 Colony in the field habit. 8. Young cells in colony. 9. Old cells in colony. 10. Autofluorescence showing the shape of chloroplasts. 11. Negative stain by ink showing the mucilage envelope. 12. Autospores in a sporangium. Scale bar 10  $\mu\text{m}$ .



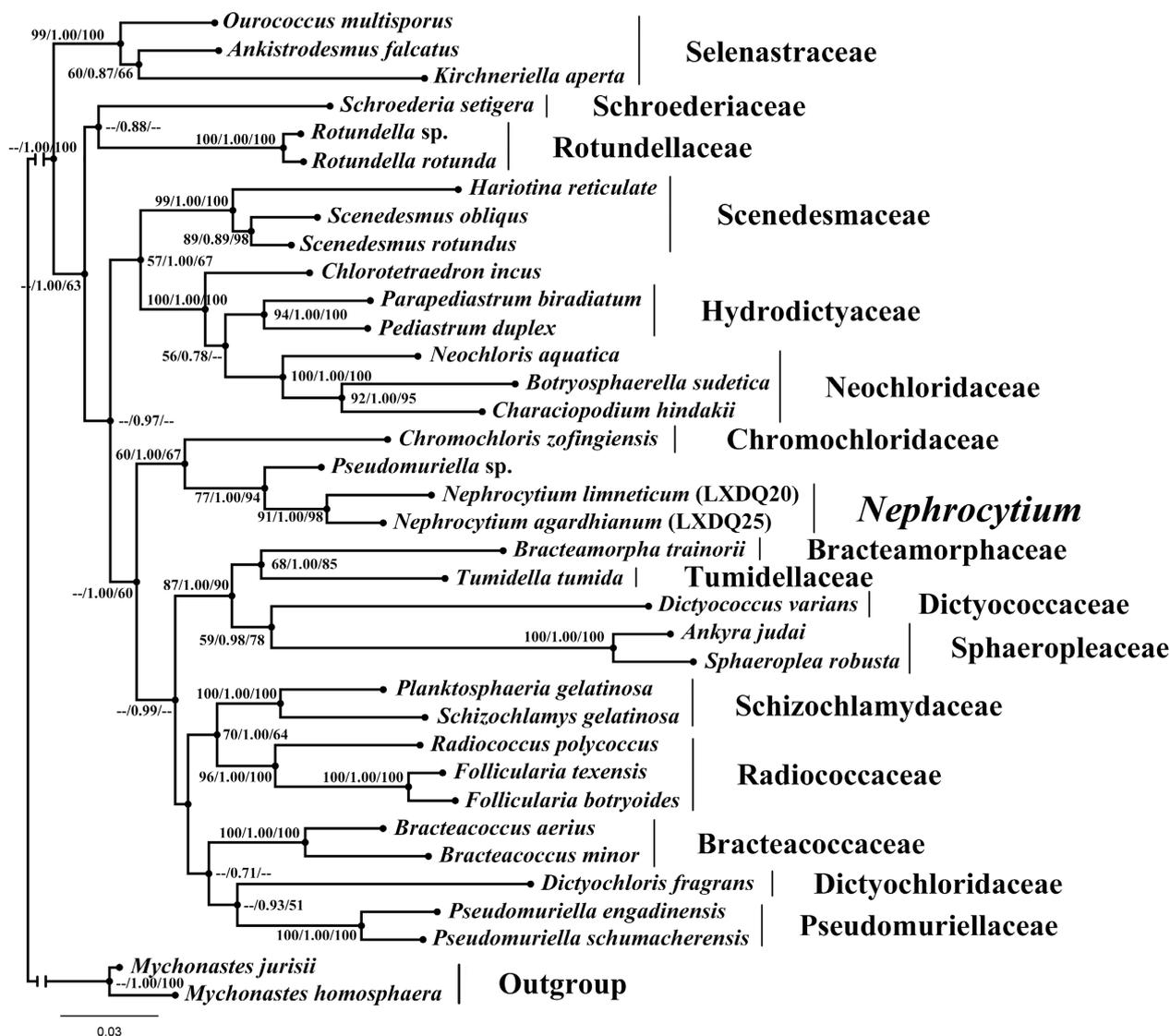
**FIGURES 13–18.** Transmission electron microscopy of *Nephrocytium limneticum* (13–15) and *Nephrocytium agardhianum* (16–18). 13,15. Longitudinal section of a cell, showing the undulated surface of cell wall. 14,17. Cells within the mother cell wall, showing the presence of the pyrenoid and the smooth mother cell wall. 15,18. Details of cell wall. (CW=cell wall, MCW=mother cell wall, P=pyrenoid, St=starch sheath, S=starch grains, t=thylakoids). Scale bar 2  $\mu\text{m}$  (13–14, 16–17), 0.5  $\mu\text{m}$  (15), 0.2  $\mu\text{m}$  (18).

#### Molecular phylogenetics

We obtained 6 gene sequences and combined these with 92 sequences obtained from GenBank, for a total of 36 included taxa. *Nephrocytium agardhianum* differed from *N. limneticum* by 42 of 1623 positions of the 18S rDNA gene with 97% similarity by BLAST searches. Similarly, the species differed at 51 positions of *rbcL* cpDNA (95% similarity), and 62 bases of *tufA* cpDNA were different (93% similarity). Sequences obtained herein were submitted to GenBank under the accession numbers KY094106–KY094111.

The concatenated data set (18S rDNA, *rbcL* cpDNA and *tufA* cpDNA) contained 3263 characters. The aligned 18S rDNA data set totaled 1515 characters. Among these, 434 sites (28.6%) were variable and 277 sites (18.3%) were parsimony informative. The aligned *rbcL* cpDNA data set totaled 1041 characters and 409 sites (39.3%) were variable and 343 sites (32.9%) were parsimony informative. The aligned *tufA* cpDNA data set totaled 707 characters with 363 variable sites (51.3%), 310 of which were parsimony informative (43.8%).

The phylogenetic trees constructed by ML and BI had similar topologies and only one of them is presented. The 18S/*rbcL*/*tufA* combined genes tree (Fig. 19) resolved our two newly isolated strains as a well supported new clade in Sphaeropleales. An uncertain *Pseudomuriella* sp. (AY195974) and genus *Chromochloris* were found to be the closest relatives to *Nephrocytium* with good nodal support.



**FIGURE 19.** Phylogenetic tree inferred using concatenated genes of 18S rDNA, *rbcl* and *tufA* cpDNA, along with sequences of additional taxa of Sphaeropleales. Bootstrap support from maximum likelihood (ML, constructed by PAUP), Bayesian inference (BI) posterior probabilities and bootstrap support from maximum likelihood (ML, constructed by RAxML) are presented on the nodes, in that order. Values above 0.5 for BI and 50 for ML are shown.

## Discussion

*Nephrocytium* was morphologically distinguished by its bent kidney-shape to oval cells stored in markedly expanded mother cell walls (Komárek & Fott 1983). Cell shape, arrangement of cells in colony, apical shape, gelatinous colony shape and incrustations over the surface of the gelatinous colony were considered important criteria (Komárek & Fott 1983). Among the 8 described *Nephrocytium* species, strain LXDQ-25 resembled the type species *Nephrocytium agardhianum* with the feature of asymmetrically cylindrical to oval bent cell shape and an asymmetrically oval or kidney-shaped gelatinous colony. Strain LXDQ-20 shared a similar morphology with *Nephrocytium limneticum* because of the kidney-shaped cell shape with not obvious tapered end and a globose or broadly ellipsoidal gelatinous colony (Komárek & Fott 1983). Apart from the cell shape and gelatinous colony shape, colony scale and ultrastructure were found to differ between these two species. *Nephrocytium limneticum* was much more prone to form composite colonies embedded some 4-cell colonies, which often formed clusters following sporulation, whereas *N. agardhianum* commonly formed single 4-cell colonies without connection. Multiple pyrenoids were easily observed among mature cells of *N. agardhianum*, but not in *N. limneticum* (Figs 5,11) while *N. limneticum* had more stacks of thylakoids than *N. agardhianum* did (Figs 14,17).

Previous morphological studies placed *Nephrocytium* in Oocystaceae due to its multilayered cell wall, propagation only via autospores, and daughter cells stored in markedly expanded mother cell walls for a long time (Komárek & Fott 1983). However, this was not supported by our research. Oocystaceae was revealed to have a characteristic cell wall substructure that was composed of several cellulose layers with perpendicular fibril orientations (Robinson & Preston 1972; Quader & Robinson 1981). Komárek (1979) distinguished this family from other coccoid green algae using this feature. Hepperle *et al.* (2000) affirmed this feature in accordance with the molecular data and suggested it as a diacritic criterion to differentiate the Oocystaceae from other trebouxioephycean lineages. However, *Nephrocytium* was not believed to possess this *Oocystis*-like ultrastructure by Štenclová (2013) and our TEM observation confirmed this, implying that previous hypotheses of the taxonomic position are not supported, and therefore *Nephrocytium* ought not be included in Oocystaceae.

Phylogenetic research of genus *Nephrocytium* performed by Štenclová (2013) using 18S rDNA suggested a taxonomic position in Sphaeropleales as a sister with the Selenastraceae. However, this proposed phylogenetic relationship was not supported by high bootstrap support values or high Bayesian posterior probabilities. Tippery *et al.* (2012) considered that single genes might lead to anomalous placements for some taxa within Chlorophyceae. A lack of phylogenetic signal could result from the use of a single gene, and a need for phylogenetic analyses utilizing multiple genes was recommended. Therefore, a combined data analysis was used in this study. Results showed a well-supported clade containing genus *Nephrocytium*, an unknown species of *Pseudomuriella*, and genus *Chromochloris* Kol & Chodat (1934), which implied a close phylogenetic relationship between *Nephrocytium* and *Chromochloris* (Chromochloridaceae).

Genus *Chromochloris* experienced a series of taxonomic transfers, but was resurrected by Fučíková & Lewis (2012). The type species *Chromochloris zofingiensis* (Dönn) Fučíková & Lewis (2012) has been well-studied as a model organism for secondary carotenoid production. The development of the chloroplast was considered the most significant feature of *C. zofingiensis*. Kalina (1987) stated that the initial single chloroplast augmented and then divided into numerous polygonal plates that were well visible in adult vegetative cells. In the old cells, the chloroplast structure was hardly discernible as a hollow sphere with distinct granulation. Rather, it was similar to genus *Nephrocytium*. On the other hand, an SEM and a TEM observation by Kalina (1987) revealed an irregular network of dense, fine ribs on the cell wall surface of *C. zofingiensis*, which was also similar with *Nephrocytium*. Cell wall structure can yield significant characters for taxonomy of coccoid green algae (Kalina 1987). The featured ultrastructure of the cell wall might be considered an important diagnostic criterion in the *Nephrocytium* and *Chromochloris* clade. Similarities in the development of chloroplast morphology, the cell wall ultrastructure and results of our multigene phylogeny imply a close taxonomic relationship between *Nephrocytium* and *Chromochloris*. *Chromochloris* belonged to Chromochloridaceae according to multi-locus analyses (Fučíková *et al.* 2014). Even though there is a close relationship between *Nephrocytium* and *Chromochloris*, an obvious morphological distinction may hint at a different family level affiliation. Additional taxa between them might have not been discovered. More taxon sampling is needed for further confirmation.

## Acknowledgments

This project was supported by National Natural Science Foundation of China (No.31670202) and the Special Foundment of Science and Technology Basic Work of China (grant no. 2014FY120200).

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