ISSN 2347-6893

A comparison and phylogenetic analysis of the pyrenoid ultrastructure of three *Oocystis* species (Oocystaceae, Trebouxiophyceae, Chlorophyta)

Feng Li, Xianghu Huang* and Changling Li
Fisheries College of Guangdong Ocean University, Zhanjiang 524088, P. R. China
277361765@qq.com
*Corresponding author e-mail: hxh166@126.com
ybcl901@126.com

ABSTRACT

The 18S rRNA gene sequences of three *Oocystis* species were determined and subjected to two different phylogenetic analysis algorithms. Phylogenetic analysis indicated that they all belong to Oocystaceae. However, the three strains were not members of a monophyletic cluster. New evidence that the genus *Oocystis* is paraphyletic is provided in this work. The pyrenoid ultrastructure of the three strains was studied using transmission electron microscopy (TEM). Different morphologies of pyrenoids can be distinguished as three types. *Oocystis* sp. had one pyrenoid surrounded by a sheath of starch consisting of four to six starch plates. The pyrenoid matrix was traversed by several tubular thylakoids. *O. nephrocytioides* contained two pyrenoids, with each pyrenoid being homogenous and surrounded by a thick, ring-like starch sheath. The thylakoids extend the length of the chloroplast but never traverse the pyrenoid matrix. No starch sheath pyrenoid has been found in *Oocystis* sp. FACHB 1429, which was traversed by two tubular thylakoids. These results suggest that different morphological features of the pyrenoids, including their associated starch sheath, are species-specific.

Keywords

Oocystis, *Oocystis nephrocytioides*, Oocystaceae, 18S rRNA, phylogeny, ultrastructure, pyrenoids, TEM, taxonomy

Academic Discipline

Microbiology

SUBJECT CLASSIFICATION

QR

Council for Innovative Research

Peer Review Research Publishing System

Journal: Journal of Advances in Biology

Vol. 6, No. 2

editorsjab@gmail.com , editor@cirjab.com

ISSN 2347-6893

INTRODUCTION

Oocystis species are quite common in various surface water bodies, particularly in freshwater ecosystems and predominantly in small lakes and ponds [1]. The genus *Oocysits* was identified and designated by Braun [2] with the initial species being *O. naegelii* A. Braun followed by many descriptions of new species within the genus [3-8]. To date, 39 species have been identified and taxonomically accepted.

For discerning various species, recent studies have used the cytological characters of vegetative cells visible under light microscopy (LM): the number of plastids, the shape of the cell, and the position of the cells within colonies, and the presence or absence of pyrenoids in the chloroplasts of the adult cells [9-12]. Pyrenoid structure is rarely mentioned in species descriptions due to the limited magnification of light microscopy. Berner [13] indicated that the pyrenoid structure is helpful in identifying some species of green algae but generally less useful for distinguishing larger categories. In the genus *Trebouxia*, different arrangements and the forms of thylakoids lamellae within the pyrenoid matrix are species-specific [14]. Similarly, Ikeda and Takeda [15] showed that pyrenoid structure is species-specific and can be a useful marker for the taxonomy of the polyphyletic green algal genus *Chlorella*. However, whether the pyrenoids can be used as a diacritic species feature in *Oocystis* is still under discussion [11, 12, 16]. Therefore, a more detailed examination of pyrenoids in *Oocystis* by electron microscopy to establish the pyrenoid structure as a taxonomic character is warranted.

Molecular methods have become increasingly important in the taxonomic classification of algae. Resulting in some genera and families being split into different lineages [17, 18]. The phylogenetic position of Oocystaceae was first shown by Hepperle et al. [19], which revealed the monophyly of the family. Recently, more species within the Oocystaceae have been studied by molecular phylogenetic analyses, and the paraphyly of the genus *Oocystis* was itself revealed [20-22].

This study investigated the pyrenoid ultrastructure from cultures of three species of *Oocystis*. Three specific types of pyrenoids were distinguished by their different morphologies. We analyzed the phylogeny of three *Oocystis* isolates and discuss the systematic context of Oocystaceae. The cellular ultrastructural details of the pyrenoid in the three strains are described for the first time along with comparisons among others species of *Oocystis* that have been previously published [23-25]. In addition, phylogenetic position for *Oocystis* sp., *O. nephrocytioides*, and *Oocystis* sp. FACHB 1429 within the family Oocystaceae are suggested. New aspects for the taxonomy of *Oocystis* are also discussed.

MATERIAL and METHODS

Algal culture

In this study, three strains of *Oocystis* were investigated: *Oocystis* sp. isolated from a shrimp aquaculture pond in Zhanjiang, China; *O. nephrocytioides* CCALA 397 (CCALA = Algal Collection, Institute of Hydrobotany, Třeboň, Czech Republic) isolated from a channel of a hatchery in Lake Ohrid, Macedonia; *Oocystis* sp. FACHB 1429 (FACHB = Freshwater Algae Culture Collection at the Institute of Hydrobiology, Wuhan, China), isolated from a sand sedimentation pond in Zhengzhou, China.

Except for *Oocystis* sp. FACHB 1429, which was cultured in BG11 [26] medium, other strains used in this study were cultured in MBB [27] medium at 25°C under a light/dark regime of 12:12 h at a light intensity of about 30-50 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Morphological observations

For TEM, cells of each *Oocystis* species were harvested by centrifugation (3000 rpm), fixed with 3% (w/v) glutaraldehyde in the same culture medium for 2 h at 4°C, and post-fixed with 1% OsO_4 in 0.1 M phosphate buffer for 2 h at 4°C. The fixed materials were dehydrated with a graded acetone series and embedded in Spurr's resin [28]. These sections were stained with uranyl acetate, followed by lead citrate [29] and examined with a JEM-1400 transmission electron microscope.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The polymerase chain reaction (PCR) was performed by using the Taq PCR Master Mix Kit (Sangon, China). The PCR cycling conditions were as follows: 3 min initial denaturation at 95 ℃; 35 cycles of denaturation at 95 ℃ for 1 min, primer annealing at 52 ℃ for 1 min, and extension at 72 ℃ for 1 min; and a final extension of 10 min at 72 ℃. For the amplification of the 18S rRNA gene, either NS1-X + 18L-X primers [30] or Ec18SF + Ec18R primers [22] were used. The PCR product was purified with the E.Z.N.A. Gel Extraction Kit (OMEGA) and sequenced directly or cloned by GenScript (Nanjing) Inc., China. The sequences were assembled with Seqman [31] and were deposited in Genbank under the Accession number KJ713151, KJ522682 and KF928745.

Phylogenetic analyses

The three new 18S rRNA gene sequences were compared with 24 other sequences including *Prasiola mexicana* (Prasiolaceae) as an outgroup taxon (Fig 10). These sequences were obtained from Genbank. The accession numbers of sequences are shown in Fig 10. An alignment of 27 taxa with 1674 base positions were used for the phylogenetic analyses, introns were excluded. The phylogenetic trees were inferred by maximum likelihood (ML) using PhyML version 3.1 [32] and by Bayesian inference (BI) using MrBayes version 3.2 [33]. For ML and BI analyses, a suitable model for the process of DNA substitution was chosen using the Akaike Information Criterion (AIC) with Modeltest version 3.7 [34]. The best model was found to be TrN+I+G. In ML analyses, the base frequencies were A 0.2457, C 0.2313, G 0.2761, T 0.2469; the rate matrix were A-C 1.0000, A-G 2.0141, A-T 1.0000, C-G 1.0000, C-T 5.4674, G-T 1.0000 and the proportion of invariable sites (I = 0.6028) and the gamma distribution shape parameter (G = 0.7491). Bootstrap analysis was performed

with 100 replicates of the dataset of ML to estimate statistical reliability. In BI analysis, two runs with four Monte Carlo Markov chains (MCMC) were carried out for 2 million generations until the average standard deviation of split frequencies between two runs was less than 0.01 (a stationary distribution was assumed). Trees and parameters were sampled every 100 generations. The first 25% of the generations were discarded as burn-in, and the remaining samples were used to construct a Bayesian consensus tree and infer posterior probability.

RESULTS

Transmission electron microscopy

Oocystis have a wide diversity of pyrenoids, in which three different pyrenoid types can be distinguished among the three species.

In *Oocystis* sp., the cell wall is multilayered (Figs 1 and 2). In ultra-thin sections, most of the vegetative cells contain one pyrenoid surrounded by a thick starch sheath (Figs 1 and 2). The starch sheath consists of four to six starch plates (Figs 1 and 2). Several tubular thylakoids penetrate the pyrenoid matrix (Fig 3). Additionally, single lenticular starch grains are visible inside the chloroplast (Figs 1 and 2). The starch grains are not in close association with the pyrenoid.

In *O. nephrocytioides*, multilayer cell walls can be clearly observed (Figs 4 and 5). In ultra-thin sections, most of the vegetative cells contain two pyrenoids (Figs 4 and 5). In each chloroplast, one pyrenoid is homogenous and surrounded by a thick, ring-like starch sheath (Figs 4 and 5). Thylakoids extend the length of the chloroplast, but they never traverse the pyrenoid matrix.

In *Oocystis* sp. FACHB 1429, the cell wall is multilayered (Figs 7 and 8). The pyrenoids have no starch sheath (Figs 7 and 8). Starch is deposited in small grains and concentrated around the pyrenoid (Figs 7 and 8). Extensions of the chloroplast thylakoids penetrate the pyrenoid matrix and form tubules (Figs 8 and 9).

Phylogeny

The phylogenetic trees constructed by ML and BI had similar topologies. As such, only the ML trees are presented here (Fig 10). Both phylogenetic analysis methods yielded very similar results; the monophyly of the family Oocystaceae was substantiated with 96 ML bootstrap support and 0.99 posterior probabilities. The genus *Oocystis* was obviously paraphyletic, as the gene sequence of *Oocystis solitaria* is positioned outside of a clade formed by the remaining members of *Oocystis*. *Oocystis* sp. (KJ713151), *O. nephrocytioides* (KJ522682), and *Oocystis* sp. FACHB 1429 (KF928745) were unambiguously included in the Oocystaceae. *Oocystis* sp. (KJ713151) represented a sister lineage to *O. parva* (JQ315649). *O. heteromucosa* (AF228689) clustered with them and the cluster represented a sister lineage to *O. marssonii* (AF228688). The analysis revealed the close relationship of *O. nephrocytioides* to *E. hubeiensis* (JX018185) with high support (98/0.99 for ML/BI). *Oocystis* sp. FACHB 1429 (KF928745) established a sister to a lineage that contained *Terachlorella alternans* (AF228687), *O. marssonii* (AF228688), *O. heteromucosa* (AF228689), *O. parva* (JQ315800) and *Oocystis* sp. (JQ315800).

DISCUSSION

Both morphological and phylogenetic analysis in the present study indicated that the three strains should be placed in the Oocystaceae. Because pyrenoids divide during chloroplast division [35], only adult cells were used for the comparisons in this study.

The ultrastructure of a pyrenoid, which was traversed by thylakoids, was first shown in a schematic graph of *O. solitaria* [23]. *O. apiculata* pyrenoids are traversed by a single tubular thylakoids, as shown in a TEM-micrograph [24], while recent research shows that pyrenoid ultrastructure in *O. lacustris* was not traversed by thylakoids [25].

The present ultrastructure study further extends our knowledge of pyrenoid structures in *Oocystis*. In the three species, diverse pyrenoid types are revealed by TEM. Pyrenoids of *Oocystis* sp. are traversed by several tubular thylakoids in this study (Fig 3). Pyrenoids of *Oocystis* sp. FACHB 1429 are visibly traversed by two straight tubular thylakoids (Fig 9). In *O. nephrocytioides*, each chloroplast has one pyrenoid with a homogenous matrix. The pyrenoid matrix was not traversed by thylakoids (Fig 6). The three strains also differ in starch sheath structure. Except for *Oocystis* sp. FACHB 1429, which has no starch sheath, the other two strains both have this sheath. However, the starch sheath in *Oocystis* sp. consists of four to six plates and differs from *O. nephrocytioides* which has a closed-ring sheath.

Our TEM-investigation show that the multilayered cell wall is in conformity with previously published morphological data [24, 36-39] which revealed that the cell walls in Oocystaceae are composed of several layers. Recently, phylogenetic analysis has been performed on some species of *Oocystis*, and the fact that the genus *Oocystis* is paraphyletic has been confirmed [19-21]. Our results, however, have indicated that the three strains used in our study represent two different lineages (Fig 10). This finding is in agreement with the morphological observations, which revealed notable differences in the ultrastructure of the pyrenoid. This strongly suggests that *Oocystis* is not monophyletic. Xia et al. [22] reported that the mother cell wall of *E. hubeiensis* gradually changed into a colorless mucilage, corresponding with that of *O. nephrocytioides*. In addition, the structures of the pyrenoids in the two strains are reportedly similar. This is further confirmed by our analysis that *O. nephrocytioides* demonstrastes the closest relationship to *E. hubeiensis* (JX018185) with high support (98/0.99 for ML/BI).

As proposed by Krienitz [40] for *Ankistrodesmus*, when traditional morphological criteria fail to distinguish real monophyletic groups, we could potentially establish the “large” genera for whole clusters. Hepperle et al. [19] stated that a redefinition of the currently paraphyletic genus *Oocystis* is necessary. In the current study, *Oocystis* sp. and *Oocystis* sp.

FACHB 1429 are more closely related to the *O. marssonii* and *O. heteromucosa* clade than to *O. solitaria*. However, we are unable to determine the real *Oocystis* lineage, because the ultrastructure of pyrenoids and the molecular data in other *Oocystis* species, especially in *O. naegelii* A. Braun 1855, were not available during this study. Based on the similarity of the morphology of the pyrenoids and the phylogenetic position, *O. marssonii* and *O. heteromucosa* appeared to be similar to *Oocystis* sp. in pyrenoid structure. It is suggested that *O. nephrocytioides* should be reclassified to the genus *Ecballocystis*.

CONCLUSIONS

Different morphological features of the pyrenoids, including the associated starch sheath, are species-specific. Therefore, the structure of the pyrenoid matrix and its starch sheath can be used as diacritic species features in *Oocystis*. This is very important for redefining and revealing the phylogenetic position of the genus *Oocystis*.

ACKNOWLEDGMENTS

This research was financially supported by Science and Technology Planning Project of Guangdong Province, China (No. 2010B060200011) and the Fund Project for Transformation of Agricultural Science and Technology Achievements from Guangdong Province, China (2012NL047). Dr. Thomas Dreschel provided editorial review on the final version of thus manuscript.

REFERENCES

[1] C. Hoek.1995. Algae: an introduction to phycology.

[2] A. Braun. 1855. Algarum unicellularium genera nova et minus cognita: praemissis observationibus de algis unicellularibus in genere.

[3] N. Wille. 1879. Ferskvandsalger fra Novaja Semlja samle de af Dr. Kjellman paa Nordenskiölds expedition 1875.

[4] W. West. 1893. Notes on Scotch fresh-water algae. Jour. of Bot. vol. 31, 97-104,

[5] R. Chodat. 1897. Algues pélagiques nouvelles. Bull. Herb. Boissier. vol. 5, 119-120,

[6] J. W. Snow. 1903. The plankton algae of Lake Erie, with special reference to the Chlorophyceae, US Government Printing Office.

[7] B. Fott and I. Čado. 1966. Oocystis nephrocytioides sp. Nov. Phycologia. vol. 6, 47-50.

[8] R. D. Groover and H. C. Bold. 1968. Phycological Notes I. Oocystis polymorpha sp. Nov. The Southwestern Naturalist. vol., 129-135.

[9] R. Smith and H. Bold. 1966. Phycological studies VI, Investigations of the algal genera Eremosphaera and Oocystis. The University of Texas Publication. vol. 6612, 1-121.

[10] H. Řeháková and B. Fott. 1969. Die Variabilität der Arten der Gattung Oocystis A. Braun. Studies in phycology. vol. 145-198.

[11] D. M. John, B. A. Whitton and A. J. Brook. 2002. The freshwater algal flora of the British Isles: An identification guide to freshwater and terrestrial algae.

[12] M. Stoyneva, C. Cocquyt, G. Gärtner and W. Vyverman. 2007. Oocystis lacustris Chod (Chlorophyta, Trebouxiophyceae) in Lake Tanganyika (Africa). Linzer Biologischen Beiträge. vol. 39, 571-632.

[13] T. Berner. 1993. Ultrastructure of microalgae.

[14] T. Friedl. 1989. Comparative ultrastructure of pyrenoids inTrebouxia (Microthamniales, Chlorophyta). Plant systematics and evolution. vol. 164, 145-159.

[15] T. Ikeda and H. Takeda. 1995. Species – specific differences of pyrenoids in Chlorella (Chlorophyta). Journal of Phycology. vol. 31, 813-818.

[16] F. Hindák. 1988. Studies on the Chlorococcal Algae (Chlorophyceae). IV.

[17] T. Friedl and C. O'Kelly. 2002. Phylogenetic relationships of green algae assigned to the genus Planophila (Chlorophyta): evidence from 18S rDNA sequence data and ultrastructure. European Journal of Phycology. vol. 37, 373-384.

[18] F. Leliaert, D. R. Smith, H. Moreau, M. D. Herron, H. Verbruggen, C. F. Delwiche and O. De Clerck. 2012. Phylogeny and molecular evolution of the green algae. Critical Reviews in Plant Sciences. vol. 31, 1-46.

[19] D. Hepperle, E. Hegewald and L. Krienitz. 2000. Phylogenetic position of the Oocystaceae (Chlorophyta). Journal of Phycology. vol. 36, 590-595.

[20] M. Pažoutová, P. Škaloud and K. Nemjová. 2010. Phylogenetic position of Ooplanctella planoconvexa, gen. et comb. nova and Echinocoleum elegans (Oocystaceae, Trebouxiophyceae, Chlorophyta). Fottea. vol. 10, 75-82.

[21] L. Krienitz and C. Bock. 2011. Elongatocystis ecballocystiformis gen. et comb. nov., and some reflections on systematics of Oocystaceae (Trebouxiophyceae, Chlorophyta). Fottea. vol. 11, 271-278.

[22] S. Xia, H. Zhu, Y. Y. Cheng, G. X. Liu and Z. Y. Hu. 2013. Phylogenetic position of Ecballocystis and Ecballocystopsis (Chlorophyta). Fottea.

[23] E. Schnepf, W. Koch and G. Deichgräber. 1966. Zur Cytologie und taxonomischen Einordnung von Glaucocystis. Archiv für Mikrobiologie. vol. 55, 149-174.

[24] D. G. Robinson and R. White. 1972. The fine structure of Oocystis apiculata W. West with particular reference to the wall. British Phycological Journal. vol. 7, 109-118.

[25] M. P. Stoyneva, E. Ingolic, G. Gaertner and W. Vyverman. 2009. The pyrenoid ultrastructure in Oocystis lacustris CHODAT (Chlorophyta, Trebouxiophyceae). Fottea. vol. 9, 149-154.

[26] R. Stanier, R. Kunisawa, M. Mandel and G. Cohen-Bazire. 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriological reviews. vol. 35, 171.

[27] H. C. Bold. 1949. The morphology of Chlamydomonas chlamydogama, sp. Nov. Bulletin of the Torrey Botanical Club. vol. 101-108.

[28] A. R. Spurr. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of ultrastructure research. vol. 26, 31-43.

[29] E. S. Reynolds. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. The Journal of cell biology. vol. 17, 208-212.

[30] K. A. Phillips and M. W. Fawley. 2000. Diversity of coccoid algae in shallow lakes during winter. Phycologia. vol. 39, 498-506.

[31] S. R. Swindell and T. N. Plasterer. 1997. SeqMan, vol. of Sequence data analysis guidebook.

[32] S. Guindon and O. Gascuel. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic biology. vol. 52, 696-704.

[33] J. P. Huelsenbeck and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics. vol. 17, 754-755.

[34] D. Posada and T. R. Buckley. 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Systematic biology. vol. 53, 793-808.

[35] H. Sluiman and G. Lokhorst. 1988. The ultrastructure of cellular division (autosporogenesis) in the coccoid green alga, Trebouxia aggregata, revealed by rapid freeze fixation and freeze substitution. Protoplasma. vol. 144, 149-159.

[36] H. Sachs, I. Grimm and D. Robinson. 1976. Structure, synthesis and orientation of microfibrils. I. Architecture and development of the wall of Oocystis solitaria. Cytobiologie.

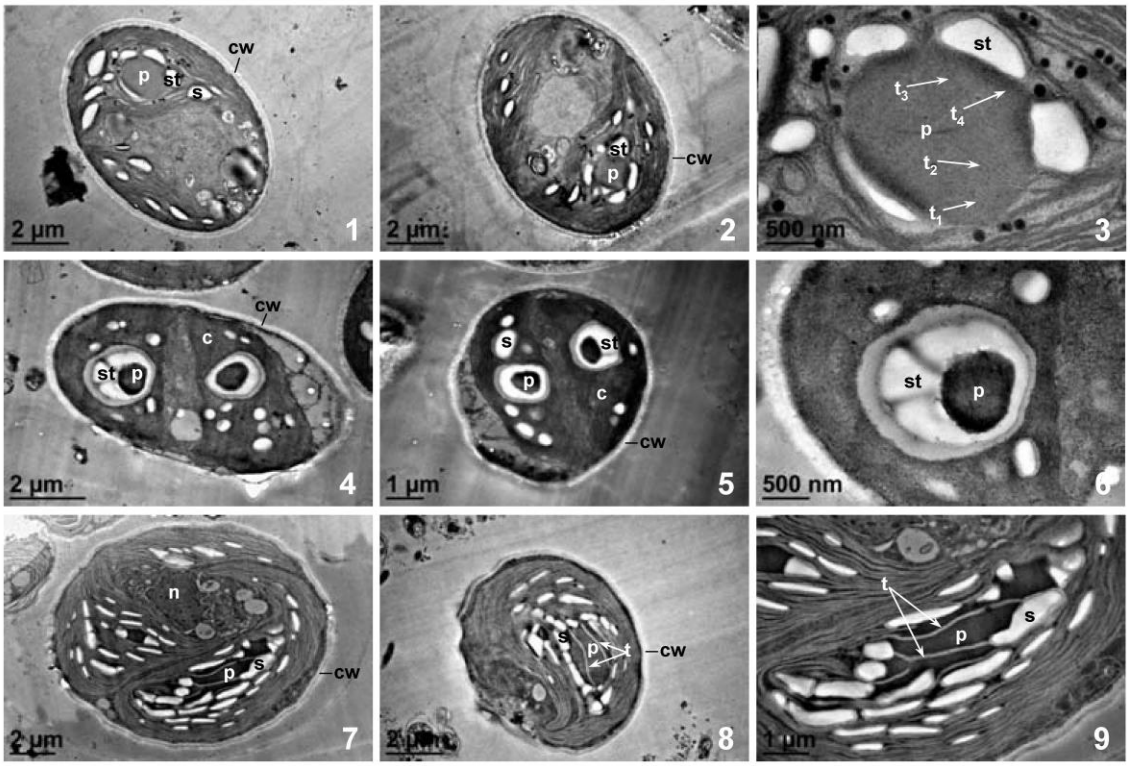
[37] H. Quader, D. Robinson and R. Van Kempen. 1983. Cell wall development in Oocystis solitaria in the presence of polysaccharide binding dyes. Planta. vol. 157, 317-323.

[38] H. Quader. 1986. Cellulose microfibril orientation in Oocystis solitaria: proof that microtubules control the alignment of the terminal complexes. Journal of cell science. vol. 83, 223-234.

[39] T. Fujino and T. Itoh. 1994. Architecture of the cell wall of a green alga, Oocystis apiculata. Protoplasma. vol. 180, 39-48.

[40] L. Krienitz, I. Ustinova, T. Friedl and V. A. Huss. 2001. Traditional generic concepts versus 18S rRNA gene phylogeny in the green algal family Selenastraceae (Chlorophyceae, Chlorophyta). Journal of Phycology. vol. 37, 852-865.

ISSN 2347-6893



Figures 1-9. Electron micrographs of 3 *Oocystis* species. Figures 1-2. Vegetative cell of *Oocystis* sp. Figure 3. Details of the pyrenoid in *Oocystis* sp. (cw = cell wall, p = pyrenoid, st = starch sheath, s = starch grains, t = thylakoids in the pyrenoid matrix). Figures 4-5. Vegetative cell of *Oocystis nephrocytioides*. Figure 6. Details of the pyrenoid in *Oocystis nephrocytioides*. (c = chloroplast, cw = cell wall, p = pyrenoid, st = starch sheath, s = starch grains). Figures 7-8. Vegetative cell of *Oocystis* sp. FACHB 1429. Figure 9. Details of the pyrenoid in *Oocystis* sp. FACHB 1429 (cw = cell wall, n = nucleus, p = pyrenoid, st = starch sheath, s = starch grains, t = thylakoids). Scale bar 500 nm (9, 12), 1 µm (11, 15), 2 µm (7, 8, 10, 13, 14).

ISSN 2347-6893

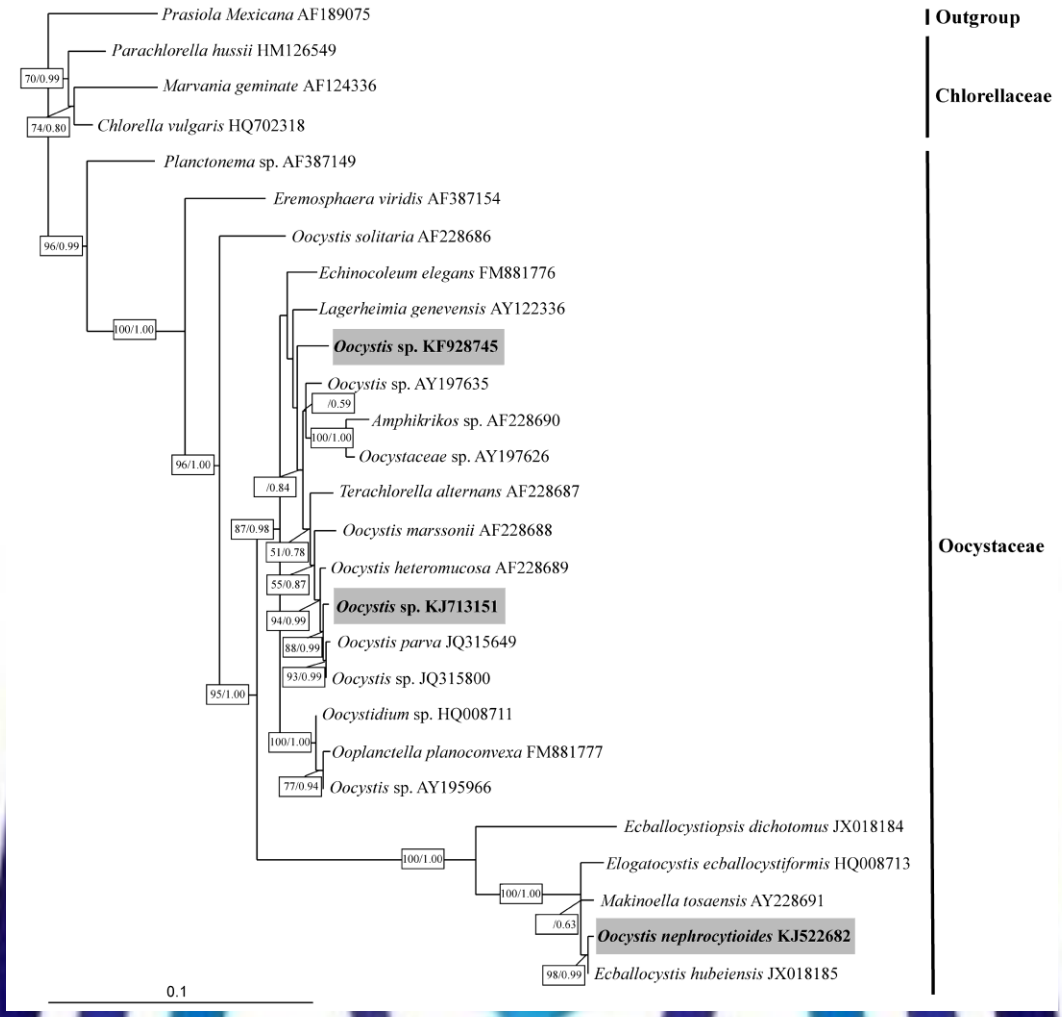


Figure 10. Phylogenetic tree of 18S rRNA sequences of members of Oocystaceae and relatives. Bootstrap support from maximum likelihood (ML) and Bayesian inference (BI) posterior probabilities are presented on the nodes. Values above 50 for ML and 0.50 for BI are shown. The sequences obtained in this study are shaded gray.