Molecular phylogeny and morphologic data of strains of the genus *Coelastrella* (Chlorophyta, Scenedesmaceae) from a tropical region in North America (Yucatan Peninsula)

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Botanical Sciences
95 (3): 527-537, 2017

DOI: 10.17129/botsci.1201

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Abstract

Background: Scenedesmaceae family exhibits great morphological variability. High phenotypic plasticity and the presence of cryptic species have resulted in taxonomic re-assignments of Scenedesmaceae members.
Study strains: Strains CORE-1, CORE-2 and CORE-3 were characterized.
Study site: Yucatan Peninsula
Methods: Morphological analyses were executed by optical and scanning electron microscopy. Phylogenetic relationships were examined by ITS-2 and ITS1-5.8S-ITS2 rDNA regions.
Results: Optical and scanning electron microscopy analyses indicated spherical to ellipsoidal cells and autospore formation correspond to members of the family Scenedesmaceae, as well as observable pyrenoid starch plates. Detailed morphology analysis indicated that CORE-1 had visible granulations dispersed on the cell wall, suggesting identity with *Verrucodesmus verrucosus*. However CORE-1 did not show genetic relations with this species, and was instead clustered close to the genus *Coelastrella*. CORE-2 did not show any particular structure or ornamentation, but it did show genetic relations with *Coelastrella* with good support. CORE-3 showed meridional ribs from end to end, one of them forked and well pronounced, and orange cells in older cultures characteristic of Coelastrella specimens. Phylogenetic trees of ITS-2 and ITS1-5.8S-ITS2 rDNA sequences indicated with good support that all strains were related to the genus *Coelastrella* despite their morphologic differences.
Conclusions: This study reports freshwater *Coelastrella* strains from a tropical region in North America (Yucatan Peninsula) for the first time. The results contribute to knowledge of *Coelastrella* species, and the fact that they do not always show structures that are useful for taxonomic assignment, probably as a result of phenotypic plasticity.
Keywords: Microalgae strains, internal transcript spacer-2 (ITS-2), Scenedesmaceae, ITS1-5.8S-ITS2 rDNA analysis, Yucatan peninsula, Tropical region in North America.

Resumen

Antecedentes: La familia Scenedesmaceae exhibe gran variabilidad morfológica La alta plasticidad fenotípica y la presencia de especies crípticas han resultado en reasignaciones taxonómicas de los miembros de la familia Scenedesmaceae.
Cepas bajo estudio: Las cepas CORE-1, CORE-2, CORE-3 fueron caracterizadas.
Sitio de estudio: Península de Yucatán.
Métodos: Los análisis morfológicos fueron realizados por microscopía óptica y electrónica de barrido. Las relaciones filogenéticas fueron examinadas por las regiones ribosomales ITS-2 e ITS1-5.8S-ITS2.
Resultados: Los análisis de microscopía óptica y electrónica de barrido indicaron que las células elipsoidales y la formación de autoesporas corresponden a miembros de la familia Scenedesmaceae, así como el pirenoide y los gránulos de almidón. El análisis detallado de la morfología indicó que CORE-1 presentó granulaciones visibles dispersas en la pared celular, sugiriendo identidad con *Verrucodesmus verrucosus*. Sin embargo, CORE-1 no mostró relaciones genéticas con esta especie, sino que se agrupó cerca del género *Coelastrella*. CORE-2 no mostró ninguna estructura u ornamentación en particular, aunque mostró relaciones genéticas de buen soporte con *Coelastrella*. CORE-3 mostró verrugas o costillas meridionales de polo a polo, uno de ellos bifurcado y bien pronunciado, y en cultivos viejos las células se tornaron de color naranja característico de los especímenes de *Coelastrella*. Los árboles filogenéticos de las secuencias del ADNr ITS-2 e ITS1-5.8S-ITS2, indicaron con buen soporte que todas las cepas se relacionan con el género *Coelastrella* a pesar de sus diferencias morfológicas.
Conclusiones: Este estudio reporta por vez primera, el estudio de cepas de microalgas de agua dulce de *Coelastrella* en la región tropical de América del Norte (Península de Yucatán). Estos resultados contribuyen al conocimiento de las especies de *Coelastrella*, y al hecho de que no siempre muestran estructuras que son útiles para la asignación taxonómica, probablemente como resultado de la plasticidad fenotípica.
Palabras clave: Cepas de microalgas, espaciador del transcrito interno-2 (ITS-2), Scenedesmaceae, análisis del ADNr ITS1-5.8S-ITS2, Península de Yucatan, Región Tropical de Norte América.

The coccoid green algae are one of the most troublesome taxonomic groups (Eliáš *et al*. 2010). Scenedesmaceae is the largest family with 54 genera (Guiry & Guiry 2016), each one displaying great morphological variability, maintained by genetic relationships resulting from autospores (Hegewald 1997), that consequently promote all other mutations (Krienitz & Bock 2012).

Simple morphology, phenotypic plasticity and the presence of cryptic species in the family Scenedesmaceae have contributed to taxonomic complication (Sciuto *et al*. 2015), resulting in constant reassignments (Eliáš *et al*. 2010), such as that which has occurred with various species of the *Coelastrella* subfamily Coelastraceae (Hegewald *et al*. 2010). However, the body of molecular data has supported the transfer of those specimens to other genera; *e.g*., *Coelastrella multistriata* possesses a close relationship with *Scenedesmus vacuolatus*. Despite morphologic differences, genetic similarities based on 18SrDNA supported the transfer of *Coelastrella multistriata* into *Scenedesmus* (Hanagata 1998). This is because the Coelastraceae subfamily with 11 species possesses a close relationship with the Scenedesmaceae family, despite morphologic differences. Coelastraceae show spherical, elliptical and long coenobia (Punčochářová & Kalina 1981), while Scenedesmaceae show flat, straight, and lightly curved coenobia (Guiry & Guiry 2016). Nevertheless, the close genetic relationship suggests that the subfamily Coelastroideae should be treated as the genus *Coelastrella* within Scenedesmaceae together with *Coelastrum*, *Hariotina*, *Asterarcys* and *Dimorphococcus* (Hegewald *et al*. 2010).

Coelastrella, named by Chodat (1922), shows distribution in Korean rivers (Song & Singh 2014), Australian soil (Tschaikner *et al*. 2007, Tschaikner *et al*. 2008), rock surfaces (Abe *et al*. 2004), Bulgarian soil (Uzonov *et al*. 2008), and an alpine zone in New Zealand (Gopalakrishnan *et al*. 2014). It was recently reported for the first time in freshwater in North America (Neofotis *et al*. 2016), but prior to this study it had not been reported in a tropical region of North America (Yucatan Peninsula). Its habitats range from aerial to terrestrial with relative humidity (40 %) or without (Song & Singh 2014), as well as at high altitudes (Gopalakrishnan *et al*. 2014).

Coelastrella shows morphologic traits similar to *Scotiellopsis* (Punčochářová & Kalina 1981). Although both belong to the Coelastroideae subfamily (Guiry & Guiry 2016), they are characterized by spherical to elliptical coenobia with meridional ribs on the cell surface, but they differ in number and polar thickening (Hanagata 2001). These structures are generally not visible under light microscopy, because there are also coenobia that lack cell wall ridges (Hegewald *et al*. 2010). In contrast, electron microscopy has aided elucidation based on these structures, although sometimes the cell wall might be artificially wrinkled during sample preparation (Kaufnerová & Eliáš 2013). Cell wall sculptures observed in *Coelastrella* by electron microscopy can be helpful for their identification (Tschaikner *et al*. 2007) meanwhile molecular identification will become a universal tool in botanical studies (Manoylov 2014). In this regard, ITS-2 sequencing has been successfully used to verify the taxonomy of closely related species (Ruhl *et al*. 2010), while ITS1-5.8S-ITS2 has been used to successfully confirm the identification of freshwater green microalgae strains (Timmins *et al*. 2009, Hadi *et al*. 2016). The ITS1-5.8S-ITS2 region from virtually all Viridiplantae can be amplified with a single set of universal primers (White *et al*. 1990), despite these markers being of high variability (Hall *et al*. 2010). The delimitation in *Coelastrella* needs more attention (Kaufnerová & Eliáš 2013). In fact, it has been suggested that morphological diversity raises the question of whether these algae are a natural assemblage or an artificial group (Hegewald *et al*. 2010). In this study, three strains from shallow waters of Yucatan were examined using optical and electron microscopy for ultrastructure analysis and using molecular data based on ITS-2 and ITS1-5.8S-ITS2 phylogenetic studies to gain more insights into their taxonomic status.

Material and methods

Microalgal strains. Three strains labeled CORE-1, CORE-2 and CORE-3 were studied. All of them were provided by the Marine and Freshwater Microalgae Collection of the Yucatan Peninsula (FICOYUC) at the Alfredo Barrera Marin Herbarium of the Universidad Autónoma de Yucatán in Mérida, Yucatán, México. All microalgae were first maintained in Bristol medium at 27 °C, 12:12 light–dark, with a light intensity of 100 μmol/m^2/s. Growth curves for each microalga were carried out in 50 ml Erlenmeyer flasks containing 10 ml fresh TAP medium (Gorman

& Levine, 1965). They were incubated at 27 ± 2 °C, 120 rpm (Newbrunswick Scientific, Excella E10), and a light intensity of 100 μmol/m^2/s with a 16:8 (light/dark) photoperiod. Samples were taken every day (500 μL) and cell concentration was determined using a hemocytometer (Andersen 2005).

Morphological analysis. Morphology features observed through optical microscopy (Nikon, Eclipse E200) were recorded, placing special emphasis on cell wall structures as a means of taxonomic identification by SEM micrographs. Lugol's solution (Jalmek SY050-13) was used for pyrenoid staining with the following procedure: 50 μL of Lugol's solution was mixed in 200 μL of microalgae culture. Subsequently, one drop (10 μL) was taken and visualized under light microscopy. Starch granules were observed from pyrenoid staining (Kumar & Singh 1971, Throndsen 1978).

Scanning electron microscopy (SEM). For the SEM analysis, 200 mg of microalgal cells in stationary phase were used, because the growth stops and almost the same cell size is observable in all cells (Berge *et al*. 2012). They were recovered and centrifuged (Hettich, Mikro 200R) at 9,727 ×g for 20 min. The supernatant was discarded and the pellet was transferred to a nylon membrane (GE Healthcare Biosciences, RPN203B) and kept in desiccators under vacuum for 24 h in 2.5 % glutaraldehyde (Polysciences, 00376) at 4 °C. This procedure was repeated three times to guarantee cell fixation. Fixation agent was then removed by centrifugation at 9,727 ×g for 20 min. The pellet was subsequently washed six times in 0.2 M phosphate buffers solution (pH 7.1). Sequential dehydration was performed in an ethanol series (30, 40, 55, 70, 85 and 100 %) at 4 °C. Individual samples were placed in microporous specimen capsules (C1178, Sigma) to be dried using Critical Point Dryer (Samdri 795). The samples were then mounted on specimen mounts for scanning electron microscopes and gold coated (Desk-II Cold sputter/ETCH Unit). SEM was performed (JEOL JSM6360 LV) (Kaufnerová & Eliá? 2013).

Genetic identification. At the stationary growth phase, the microalgae cells were harvested by centrifugation (OrtoAlresa, Digicen 21R) (30 ml) and washed three times in bidistilled sterile water. The cells (*c.a*. 200 mg) were stored at -20 °C, until use. For DNA extraction, we followed the protocol modifications of Youssef *et al*. (2015).

ITS-2 and ITS1-5.8S-ITS2 amplification. ITS-2 and ITS1-5.8S-ITS2 amplifications were implemented using the forward and reverse primers proposed by Van Hannen *et al*. (2002) and Timmins *et al*. (2009) respectively. Both assays were carried out with 10 μL of reaction mixture containing 1x PCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs (Bioline, DM109H), 1 mM primer, 1 U *Taq* DNA polymerase recombinant (Invitrogen 11,615-010) and 10 ng of DNA. PCR conditions for ITS-2 included 1 cycle of 1 min at 94 ºC for initial denaturation, followed by 35 cycles of 1 min at 94 ºC, 1 min at 55 ºC, 1.5 min at 72 ºC, and finally 7 min at 72 ºC. Conditions for ITS1-5.8S-ITS2 amplifications were 1 cycle of 5 min at 95 ºC for initial denaturation, followed by 30 cycles of 40 s at 95 ºC, 40 s at 56 ºC, 40 s at 72 ºC, and finally 10 min at 72 ºC. Amplicons were checked by electrophoresis on 1 % agarose gel in 1 x TAE buffer. The amplification products (fragments of approximately 314 pb from ITS-2 and 714 bp from ITS1-5.8S-ITS2), were purified using NucleoSpin® Gel and the PCR Clean-up commercial kit (Macherey-Nagel, Düren, Germany) to eliminate primers dimers according to the manufacturer's instructions. Direct sequencing of the amplification products was carried out by a two-directional (reverse and forward) procedure (Clemson Sequencing Service).

The PCR products from ITS-2 sequencing showed double peaks in the electropherogram generated as an output of direct sequencing. They were therefore inserted in pGEM-T Easy Vector System (A1360, Promega) and cloned in *Escherichia coli* (DH5α) cells. The plasmids were purified with QIAprep Spin Miniprep Kit (27104, QIAGEN). The sequencing was subject to both forward and reverse sides using M13 primer.

ITS-2 and ITS1-5.8S-ITS2 sequence analysis. The ITS-2 (314 pb) and ITS1-5.8S-ITS2 sequences (714pb: 27pb of 18S rDNA; 213pb of ITS-1; 181pb of 5.8S rDNA and 280 pb of ITS-2) were

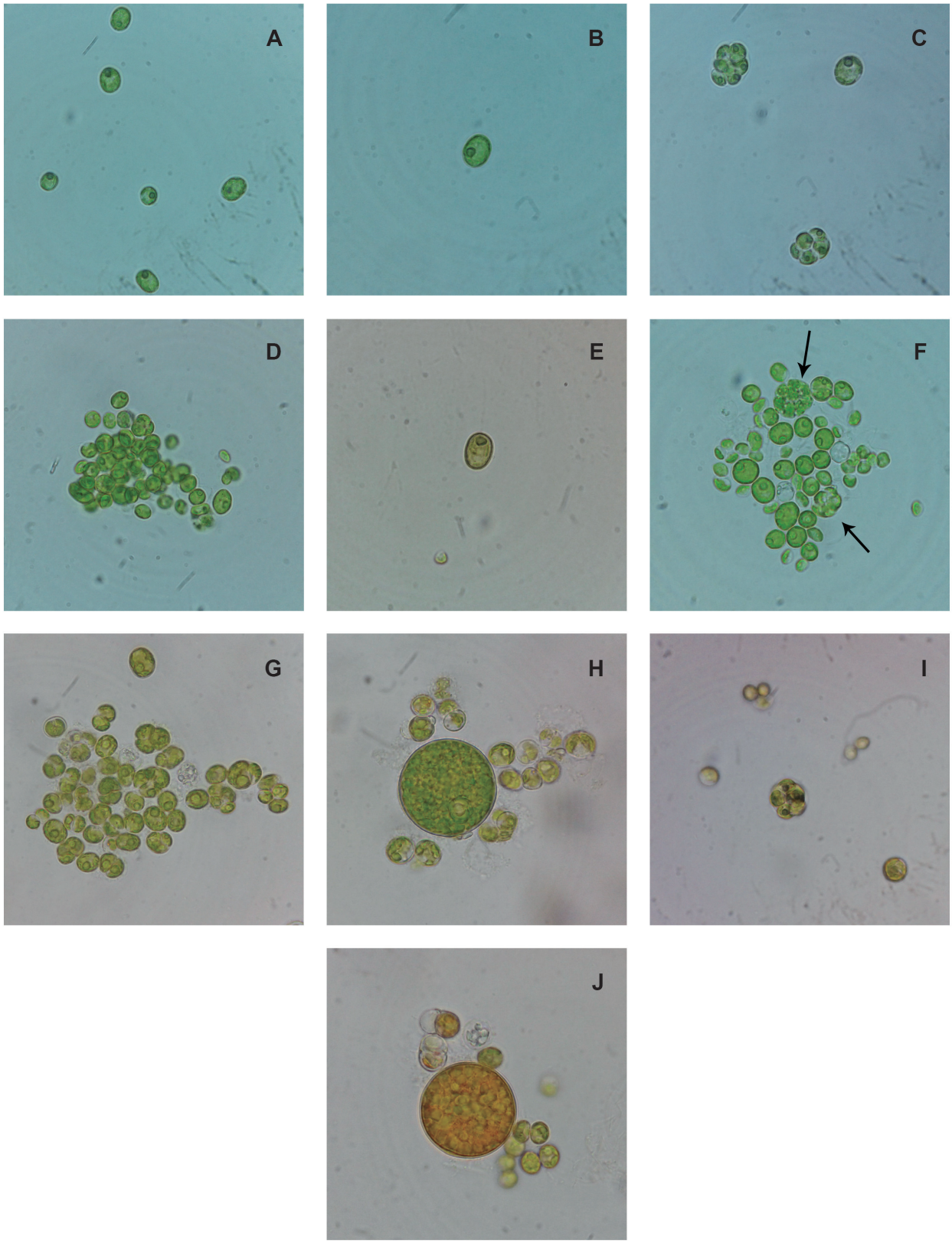

Figure 1. Vegetative cells of microalgae strains. **A-C**. CORE-1. **A**. unicellular organization of the cells. **B**. pyrenoid visible. **C**. autospores with pyrenoids. **D-F**. CORE-2. **D**. colonial organization. **E**. pyrenoid staining with starch cells. **F**. autospores in sporangium indicated by arrows. **G-I**. CORE-3. **G**. colonial organization. **H**. Visible starch sheaths of the pyrenoid. **I**. autospores in sporangium. **J**. orange appearance of the cells.

subjected to a BLAST search to determine their identities and assess their homologies and similarities to those in GenBank. Sequences with E-value closer to 0.00, 100 % coverage and identity 96 % > with each entry were aligned using CLUSTALX (EMBL, Heidelberg, Germany) and manually adjusted by MEGA version 2.1. Molecular phylogenetic analyses by the maximum likelihood method based on the Kimura 2-parameter model (Kimura 1980) were conducted using MEGA version 2.1 (Kumar *et al*. 2016). One thousand replica samplings were analyzed for percent bootstrap values in a neighbor-joining tree. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (2 categories (+G, parameter = 0.0500)). Evolutionary analyses were conducted in MEGA 7 (Kumar *et al*. 2016). Reference sequences retrieved from GenBank of the ITS1-5.8S-ITS2 sequences were KX940913, KX940914, KX940915 for CORE-1, CORE-2 and CORE-3 respectively.

Results

Morphological analysis. The morphology observed by light microscopy (LM) was complemented by scanning electron microscopy (SEM) micrographs. CORE-1 showed an ovoid to ellipsoidal cell shape and rough cell wall. Asexual reproduction was visible by autosporulation. Each sporangium was composed of 2-6 young autospores with visible pyrenoid (Figure 1 C). The cell arrangement in sporulation showed cells in two superposed lines. The cell measurements were 7.01±0.28 µm in length and 4.56±0.31 µm in width with some granulations of 159 nm dispersed on the cell wall (Figure 2 A-B: indicated by arrows). The pyrenoid was defined by two or three divisions of starch granules.

CORE- 2 showed spherical and ellipsoidal cell shapes with one pole lightly developed (Figure 1 D-F). The cell was of 8.4±1.2 µm in length by 6.5±0.91 µm in width (Figure 2 C-D). The cell wall was smooth and 2-8 autospores were observed per sporangium (Figure 1 F: indicated by arrows). The pyrenoid was observed after Lugol staining, showing mostly continuous starch granules.

CORE-3 showed ovoid cells of 10.0 ± 0.23 µm in length and 6.7 ± 0.14 µm in width. A smooth cell wall was observed via LM (Figure 1; G-I), while ribs in a meridional distribution were observed via SEM (Figure 2; E-F). One of the ends was observed to be forked and well pronounced. Autospore formation (2-8 autospores) was observed. A visible pyrenoid composed of two or three divisions of starch plates was observed, sometimes observed to be continuous without divisions and with the vacuole visible. One interesting characteristic was the orange coloration of cells in older cultures (Figure 1; J).

Genetic analysis. The sequences of each of the three strains were used as a "query" in nucleotide Blast tools (NCBI website) to recover the ten best matches for each input sequence, based on percentage of sequence coverage, E-value and percentage of identity. The sequences selected were used for ITS-2 and ITS1-5.8S-ITS2 rDNA phylogenetic tree construction. These displayed clusters with bootstrap values above 50 %. The length of each branch denoted evolutionary distance.

The phylogenetic tree resulting from ITS-2 sequences (Figure 3) presented a high support. CORE-1 was clustered with good bootstrap support, near to *Asterarcys* sp. MS3 and *Coelastrella* sp. M60. CORE-2 was well supported in its monophyly with *Coelastrella* sp. accessions (KU507, KU502). Meanwhile, CORE-3 was clustered with high support, but evolutionarily separate from other strains, and in a node dominated by the genus *Coelastrella*, together with *Chlamydomonas moewusii* and other related species, such as *Haematococcus pluvialis* and *Imbribryum alpinum*.

Using ITS1-5.8S-ITS2 sequences, the phylogenetic relationships were very similar and resolved at 50 % bootstrap values (Figure 4). CORE-1 forms a node with *Scenedesmus rotundus* CFR 1-06/FW, *Asterarcys* sp. MS3, and *Coelastrella* sp. CORE-2 was placed close to the same *Coelastrella* accessions, as in the ITS-2 phylogenetic tree. Meanwhile CORE-3 was paraphyletic with high support and clustered with others accessions of *Coelastrella* genera. This clustering confirms in a certain way, the relationship of all strains with *Coelastrella* species. It is important to mention that the phylogenetic tree constructed by both ribosomal regions analyzed showed

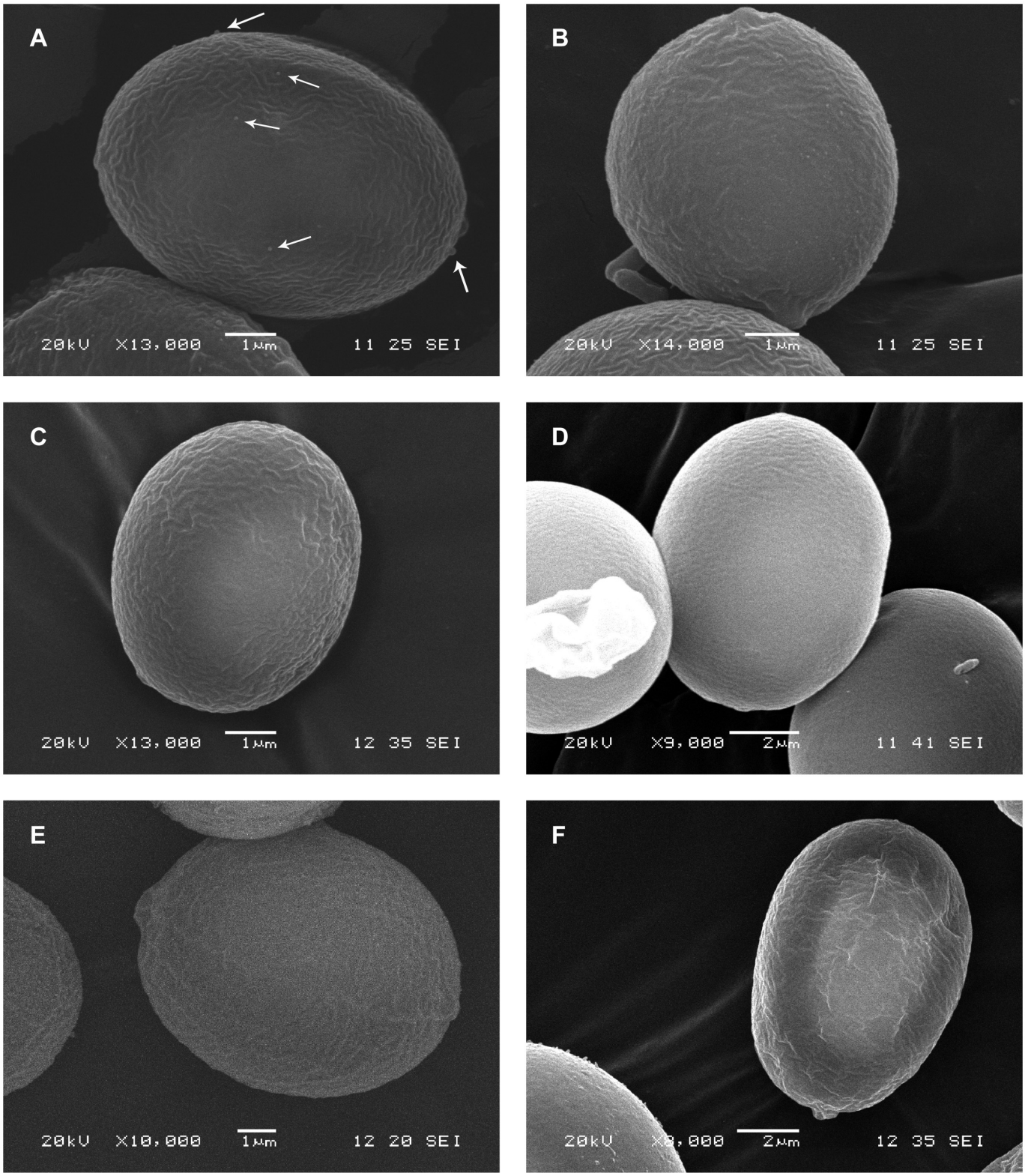

Figure 2. A-F. SEM of the cells of the study. CORE-1. **A**. cell wall appearance (granulations indicated by rows). **B**. well pronounced apex. CORE-2. **C**. vegetative cells. **D**. smooth appearance of the cell. CORE-3. **E**. meridional ribs. **F**. forked and well pronounced apex.

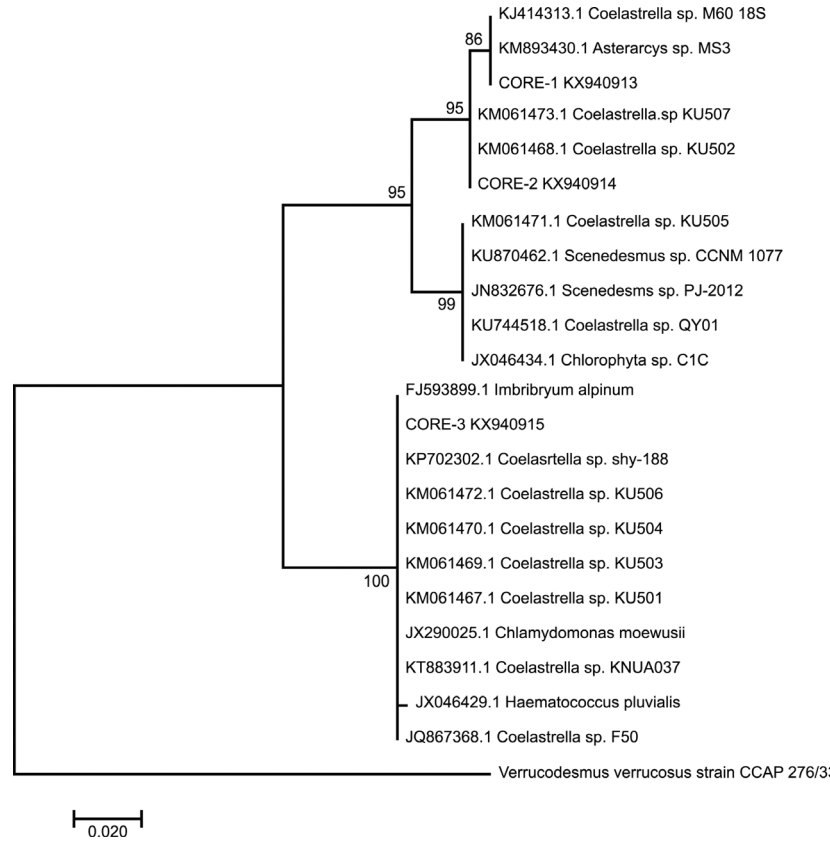

Figure 3. Phylogenetic tree inferred from ITS2 rDNA sequences constructed with the neighbor-joining method. The number of the branches indicated bootstrap values (1,000 replicates).

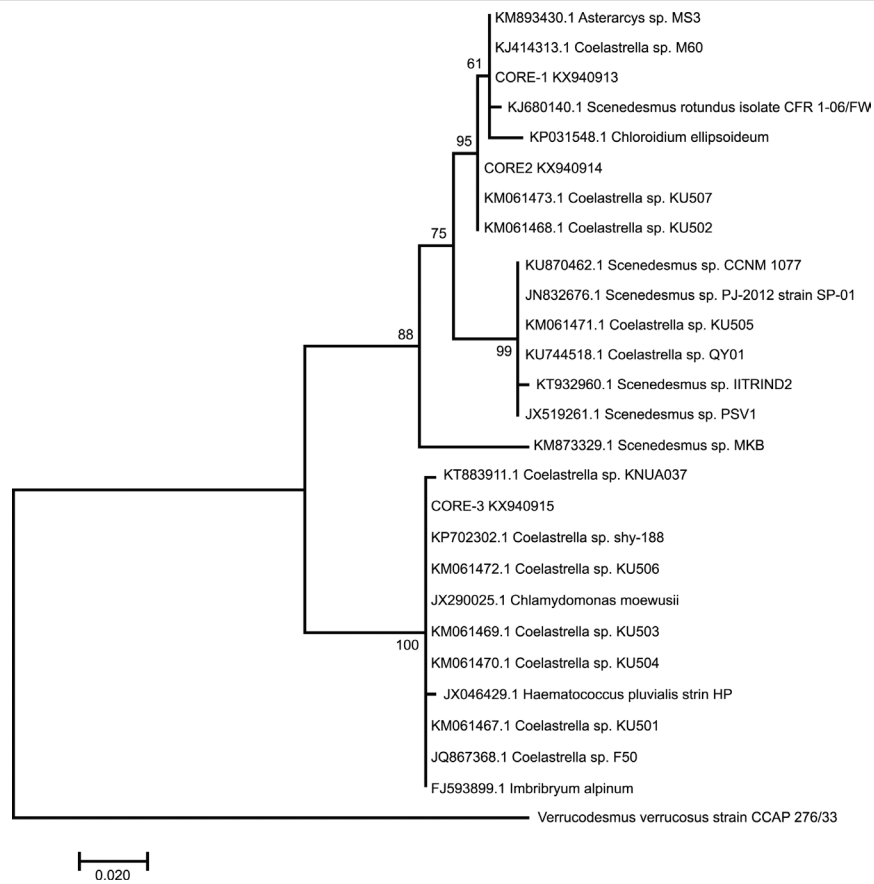

Figure 4. Phylogenetic tree inferred from ITS1-5.8S-ITS2 rDNA sequences constructed with the neighbor-joining method. The number of the branches indicated bootstrap values (1,000 replicates).

well supported nodes. Therefore, the genetic relationship of the strains under study must be considered for taxonomic assignment.

Verrucodesmus verrucosus was included to contrast taxonomically with CORE-1, but it was clustered as an external outgroup in both phylogenetic trees.

Discussion

Morphologic analysis indicated that all strains belong to the family Scenedesmaceae. The autospore formation characteristic of Scenedesmaceae was visible in all strains under study, observed on light microscopy (Figure 1). SEM analysis of CORE-1 showed visible granulations dispersed over the cell wall surface of some cells and scarce in others (Figure 2; A-B). The granulations are a taxonomic character with identity to *Scenedesmus verrucosus* (Y.V. Roll) (Hegewald *et al*. 2013, Ramos *et al*. 2015), a basionym of *Verrucodesmus verrucosus* (Y.V. Roll) from their separation by rRNA gene sequences (ITS1/5.8S/ITS2) (Hegewald *et al*. 2013). Two lines of 6 intercalated cells were observed (Figure 1; C). *Verrucodesmus verrucosus* exhibits the arrangement of cells in two series of 4 or 8 cells as a taxonomic trait (Ramos *et al*. 2015). Thus, based on morphological analysis, CORE-1 belongs to *V. verrucosus*.

CORE-2 is rather simple and does not show any structure on the cell wall that could aid with its identification.

In CORE-3, meridional ribs were lightly evident, corresponding to the genus *Coelastrella* (Chodat 1922). *Coelastrella* belongs to the Scenedesmaceae (Hegewald & Hanagata 1999). This species is aerial to terrestrial with humidity (40 %) or without (Song & Singh 2014), and at high altitudes (Gopalakrishnan *et al*. 2014). It shows distribution in different regions (Abe *et al*. 2004, Tschaikner *et al*. 2007, Tschaikner *et al*. 2008, Uzonov *et al*. 2008, Song & Singh 2014), and was recently reported in North America (Neofotis *et al*. 2016). However prior to this study *Coelastrella* had not been reported in tropical regions of North America (Yucatan Peninsula).

CORE-3 showed meridional ribs characteristic of *Coelastrella* (Uzonov *et al*. 2008), and should therefore be considered *Coelastrella* sp. In CORE-3, an orange coloration was also observed in older cultures under light microscopy, consistent with *Coelastrella* specimens, which show a red (Guiry &Guiry 2016) or orange coloration (Hu *et al*. 2013) in matured cultures, similar to that of *Coelastrella striolata* var. *multistriata* (Trenkwalder) dependent on a low nitrogen concentration in the culture medium (Abe *et al*. 2004). The autospore number of CORE-3 (2-8) was different to *Coelastrella* members (Chodat 1922), which show 2-18 autospores released from mother cells (Guiry & Guiry 2016).

The rDNA sequences analyzed provided more insights into the taxonomic status. This is probably due to the increased number of taxa verified in algal gene libraries (Manoylov 2014). Phylogenetic analysis of the genus *Coelastrella* possesses higher molecular diversity, as suggested by our results via both ITS-2 and ITS1–5.8S–ITS2 sequence analysis and those of Kaufnerová and Eliáš (2013). Interestingly, through the ITS-2 or ITS1–5.8S–ITS2 phylogenetic trees, the positions of clades in the tree were resolved based on the high support.

The ITS-2 sequenced region has helped to discriminate at the species level, and clarified the relationships of *Coelastrella* with other genera of the family Scenedesmaceae, such as *Hariotina*, *Asterarcys*, *Coelastrum* and *Dimorphococcus* (Hegewald *et al*. 2010).

Contrary to the morphological analysis that showed identity with *V. verrucosus*, CORE-1 was nested within *Coelastrella* and *Asterarcys* by ITS-2 sequences and with the genera *Coelastrella* and *Scenedesmus* by ITS1–5.8S–ITS2 sequences, consistent with clustering by 18S rDNA sequences (Kaufnerová & Eliáš 2013) and ITS-2 region (Hewegald *et al*. 2010). Initially the Coelastraceae subfamily includes *Asterarcys*, a very different morphologic genus but one which has been nested between taxa nominally added to *Coelastrella*. A similar situation occurs with *Scenedesmus rotundus* (Hegewald *et al*. 2013) or *Scenedesmus rubescens*, which maintain close relationships with the genus *Coelastrella* (Chodat 1922), probably due to minor differences in the 18S rDNA sequence (Kaufnerová & Eliáš 2013), which were evidenced by the genetic markers analyzed in this study.

Morphology observed in CORE-1 indicated identity with *Verrucodesmus verrucosus*. However, molecular traits from two taxonomic markers indicated no genetic relation with *V. ver-*

rucosus. Its position in the phylogenetic tree is very far away, and it can even be considered an external outgroup. Thus, it is not possible to conclude that CORE-1 belongs to *V. verrucosus*. On the contrary, there is a strong possibility that it is genetically closest to *Coelastrella* and distant to *V. verrucosus*.

CORE-2 forms a monophyletic clade with *Coelastrella* accessions reported as hydrogen producers (Pongpadung *et al*. 2015). Considering the absence of structures on the cell wall, it was not possible suggest taxonomic identity. However, based on molecular analysis CORE-2 should be considered to belong to *Coelastrella*.

The taxonomic identity of CORE-1 and CORE-2 was based on phylogenetic evidence. This has already been performed for members of *Coelastrella, e.g*. *Coelastrella multistriata* possesses a close relationship with *Scenedesmus vacuolatus*. Despite morphologic differences, genetic similarities based on 18SrDNA supported transfer of *Coelastrella multistriata* into *Scenedesmus* (Hanagata 1998).

CORE-3 formed one clade with *Coelastrella* and other very different genera with low support: *Haematococcus pluvialis*, *Chlamydomonas moewusii* (CCAP 11/64A) and *Imbribryum alpinum*. The only relation between CORE-3 and *Haematococcus* is the coloration of cultures due to astaxanthin production capability, because this genus is not related to the family Scenedesmaceae. On the other hand, it has been suggested that *Chlamydomonas moewusii* (CCAP 11/64A) was misidentified because it is morphologically different from *Coelastrella* (Kaufnerová and Eliáš, 2013). *Imbribryum alpinum* is also very different from the other species. The morphology and molecular characters of CORE-3 were consistent with *Coelastrella*, and the genetic results indicate that it probably belongs to *Coelastrella*.

Based on the results obtained, it is possible to suggest that *Coelastrella* members do not always show morphological traits congruent with *Coelastrella* descriptions. This is probably due to high susceptibly to phenotypic plasticity, which interferes with taxonomic assignments based only in morphology. The sequencing of molecular markers employed in this study was used for taxonomic assignment, supporting the assertion by Manoylov (2014) that "molecular identification will become a universal tool in biological studies". In this regard, this report contributes to knowledge of *Coelastrella* species and provides the first description of three of them found in a tropical region of North America in the Yucatan Peninsula.

Conclusions

The three strains under study showed morphologic characteristics of the family Scenedesmaceae. The morphologic and molecular analysis of CORE-3 was close to the taxonomic descriptions of *Coelastrella*. Morphologic and genetic analysis of CORE-1 and CORE-2 rendered different results. While the morphologic traits of CORE-1 were quite similar to *Verrucodesmus verrucosus*, genetic analysis indicated a relationship with *Coelastrella* species, and did not support morphologic identity with *V. verrucosus*. CORE-2 did not show taxonomic traits because of the absence of structures. However, genetic analysis indicated that this strain belongs to *Coelastrella*.

Therefore, based on the high support of the nodes of the phylogenetic analysis by ITS-2 and ITS1-5.8S-ITS-2 sequences, all strains under study belong to the genus *Coelastrella*. Further sequencing analysis with 18S rDNA could contribute to supporting the phylogenetic status of CORE-1 and CORE-2 within *Coelastrella*.

Acknowledgement

The authors gratefully acknowledge the financial support granted by the Consejo Nacional de Ciencia y Tecnología (CONACyT, Mexico) through project No. 166371 and the graduate grant for the first author. We acknowledge Paola Marfil Lara' help with editing the figures.

Literature cited

Abe K, Takizawa H, Kimura S, Hirano M. 2004. Characteristics of chlorophyll formation of the aerial microalga *Coelastrella striolata* var. *multistriata* and its application for environmental biomonitoring. *Journal of Bioscience and Bioengineering* **98**: 34-39. DOI:10.1016/S1389-1723(04)70239-X

Andersen RA. 2005. Algal Culturing Techniques. Burlington: ElsevierAcademic Press.
Berge T, Daugbjerg N, Hansen PJ. 2012. Isolation and cultivation of microalgae select for low growth rate and tolerance to high pH. *Harmful Algae* **20**: 101-110. DOI:10.1016/j.hal.2012.08.006
Chodat R. 1921. Matériaux pour l'histoire des algues de la Suisse. *Bulletin de la Société Botanique de Geneve* **13**: 66-114.
Elíâs M, Němcová Y, Škaloud P, Neustupa J, Kaufnerová V, Šejnohová L. 2010. *Hylodesmus singaporensis* gen. et sp. nov., a new autosporic subaerial green alga (Scenedesmaceae, Chlorophyta) from Singapore. *International Journal of Systematic and Evolutionary Microbiology* **60**: 1224-1235. DOI:10.1099/ijs.0.012963-0
Gopalakrishnan KK, Novis PM, Visnovsky G. 2014. Alpine Scenedesmaceae from New Zealand: new taxonomy. *New Zealand Journal of Botany* **52**: 84-99. DOI:10.1080/0028825X.2013.859628
Gorman DS, Levine RP. 1965. Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences USA* **54**: 1665-1669.
Guiry MD, Guiry GM. 2016. AlgaeBase. World-wide electronic publication. National University of Ireland, Galway.
Hadi SIIA, Santana H, Brunale PPM, Gomes TG, Oliveira MD, Matthiensen A, Oliveira MEC, Silva FCP, Brasil BSAF. 2016. DNA Barcoding Green Microalgae Isolated from Neotropical Inland Waters. *PLoS ONE* **11**: e0149284. DOI:10.1371/journal.pone.0149284
Hall JD, Fučíková K, Chien L., Lewis LA, Karol KG. 2010. An assessment of proposed DNA barcodes in freshwater green algae. *Cryptogamie, Algologie* **31**: 529-555.
Hanagata N. 2001. New species of *Coelastrella* and *Scenedesmus* (Chlorophyceae, Chlorophyta). *Journal of Japanese Botany* **76**: 129-136.
Hanagata N. 1998. Phylogeny of the subfamily Scotiellocystoideae (Chlorophyceae, Chlorophyta) and related taxa inferred from 18S ribosomal RNA gene sequence data. *Journal of Phycology* **34**: 1049-1054. DOI: 10.1046/j.1529-8817.1998.341049.x
Hegewald E. 1997. Taxonomy and Phylogeny of *Scenedesmus*. *Algae* **12**: 235-246.
Hegewald E, Hanagata N. 2000. Phylogenetic studies on Scenedesmaceae (Chlorophyta). *Algological Studies/Archiv für Hydrobiologie* **100** (supplement):29-49.
Hegewald E, Wolf M, Keller A, Friedl T, Krienitz L. 2010. ITS2 sequence-structure phylogeny in the Scenedesmaceae with special reference to *Coelastrum* (Chlorophyta, Chlorophyceae), including the new genera *Comasiella* and *Pectinodesmus*. *Phycologia* **49**: 325-335. DOI:10.2216/09-61.1
Hegewald E, Bock C, Krienitz L. 2013. A phylogenetic study on Scenedesmaceae with the description of a new species of *Pectinodesmus* and the new genera *Verrucodesmus* and *Chodatodesmus* (Chlorophyta, Chlorophyceae). *Fottea, Olomouc* **13**: 149-164. DOI: 10.5507/fot.2013.013
Hu C-W., Chuang L-T., Yu P-C., Chen C-NN. 2013. Pigment production by a new thermotolerant microalga *Coelastrella* sp. F50. *Food Chemistry* **138**: 2071-2078. DOI:10.1016/j.foodchem.2012.11.133
Kaufnerová V., Eliáš M. 2013. The demise of the genus *Scotiellopsis* Vinatzer (Chlorophyta). *Nova Hedwigia* **97**: 415-428. DOI:10.1127/0029-5035/2013/0116
Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111-120. DOI:10.1007/BF01731581
Krienitz L, Bock C. 2012. Present state of the systematics of planktonic coccoid green algae of inland waters. *Hydrobiologia* **698**: 295-326. DOI 10.1007/s10750-012-1079-z
Kumar HD, Singh HN. 1971. A textbook on algae. London: Macmillan Education UK.
Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**: 1870-1874. DOI:10.1093/molbev/msw054
Manoylov KM. 2014. Taxonomic identification of algae (morphological and molecular): species concepts, methodologies, and their implications for ecological bioassessment. *Journal of Phycology* **50**: 409-424. DOI:10.1111/jpy.12183
Song MA, Lee O-M. 2014. A study of newly recorded genera and species of aerial algae in the order Chlorococcales (Chlorophyta) from the Hongcheon-river, Korea. *Journal of Ecology and Environment* **37**: 315-325. DOI:10.5141/ecoenv.2014.034
Neofotis P, Huang A, Sury K, Chang W, Joseph F, Gabr A, Twary S, Qiu W, Holguin O, Polle JEW. 2016. Characterization and classification of highly productive microalgae strains discovered for biofuel and bioproduct generation. *Algal Research* **15**: 164-178. DOI:10.1016/j.algal.2016.01.007
Pongpadung P, Liu J, Yokthongwattana K, Techapinyawat S, Juntawong N. 2015. Screening for hydrogen-producing strains of green microalgae in phosphorus or sulphur deprived medium under nitrogen limitation. *ScienceAsia* **41**: 97-107. DOI: 10.2306/scienceasia1513-1874.2015.41.097
Punčochářová M, Kalina T. 1981. Taxonomy of the genus *Scotiellopsis* Vinatzer (Chlorococcales, Chlorophyta). *Algological Studies/Archiv für Hydrobiologie* **27** (supplement):119-147.

Received:
January 12th, 2017

Accepted:
February 9th, 2017

Ramos GJ.P, Bicudo CE de M, Moura CW do N. 2015. Scenedesmaceae (Chlorophyta, Chlorophyceae) de duas áreas do Pantanal dos Marimbus (Baiano e Remanso), Chapada Diamantina, Estado da Bahia, Brasil. *Hoehnea* **42**: 549-566. DOI:10.1590/2236-8906-03/2015

Ruhl MW, Wolf M, Jenkins TM 2010. Compensatory base changes illuminate morphologically difficult taxonomy. *Molecular Phylogenetics and Evolution* **54**: 664-669. DOI:10.1016/j.ympev.2009.07.036

Sciuto K, Lewis LA, Verleyen E, Moro I, La Rocca N. 2015. *Chodatodesmus australis* sp. nov. (Scenedesmaceae, Chlorophyta) from Antarctica, with the emended description of the genus *Chodatodesmus*, and circumscription of *Flechtneria rotunda* gen. et sp. nov. *Journal of Phycology* **51**: 1172-1188. DOI: 10.1111/jpy.12355.

Throndsen J. 1978. The dilution culture method. *In*: Sournia A (ed). *Phytoplankton Manual*. Paris:UNESCO, Monographs on Oceanographic Methodology 6, 218-224.

Timmins M, Thomas-Hall SR, Darling A, Zhang E, Hankamer B, Marx UC, Schenk PM. 2009. Phylogenetic and molecular analysis of hydrogen-producing green algae. *Journal of Experimental Botany* **60**: 1691-1702. DOI:10.1093/jxb/erp052

Tschaikner A, Gärtner G, Kofler W. 2008. *Coelastrella aeroterrestrica* sp. nov. (Chlorophyta, Scenedesmoideae) a new, obviously often overlooked aeroterrestrial species. *Algological Studies* **128**: 11-20. DOI:10.1127/1864-1318/2008/0128-0011

Tschaikner A, Ingolić E, Stoyneva MP, Gärtner G. 2007. Autosporulation in the soil alga *Coelastrella terrestris* (Chlorophyta, Scenedesmaceae, Scenedesmoideae). *Phytologia balcanica* **13**: 29-34.

Uzonov BA, Stoyneva MP, Gärtner G, Kofler W. 2008. First record of *Coelastrella species* (Chlorophyta: Scenedesmaceae) in Bulgaria. *Berichte des Naturwissenschaftlichen-Medizinischen Verein Innsbruck* **95**: 27-34.

Van Hannen E, FinkGodhe P, Lurling M. 2002. A revised secondary structure model for the internal transcribed spacer 2 of the green algae *Scenedesmus* and *Desmodesmus* and its implication for the phylogeny of these algae. *European Journal of Phycology* **37**: 203-208. DOI:10.1017/S096702620200361X

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds.) *PCR protocols: a guide to methods and applications PCR protocols: a guide to methods and applications*. San Diego: Academic Press, pp. 18.

Youssef M, Valdez-Ojeda R, Ku-Cauich JR, Escobedo-GraciaMedrano RM. 2015. Enhanced Protocol for Isolation of Plant Genomic DNA. *Journal of Agriculture and Environmental Sciences* **4**: 172-180. DOI:10.15640/jaes.v4n2a20