Bangladesh J. Plant Taxon. **30**(2): 255-261, 2023 (December) DOI: https://doi.org/10.3329/bjpt.v30i2.70501


# NEW RECORD OF *SCENEDESMUS VACUOLATUS* FROM SOIL IN VOJVODINA, SERBIA

TIMEA HAJNAL JAFARI*, VLADIMIRA ŽUNIĆ, SIMONIDA DJURIĆ AND DRAGANA STAMENOV

*University of Novi Sad, Faculty of Agriculture, Dositeja Obradovića Sq.8, Novi Sad, Serbia*

*Keywords:* Soil algae; *Scenedesmus vacuolatus;* DNA extraction; ITS2.

**Abstract**

Soil algae are photosynthetic microorganisms showing huge differences in morphology depending on the soil type, agricultural practices, and environmental factors. The aim of this research was to compare the results of classical and molecular protocols for microalgal identification. The green microalgae were isolated from acidic arable soil of dystric cambisol type, using BG11 medium and the agar plate method. Firstly, the identification of the microalgae was performed based on its morphological characteristics using light microscopy (LM) and taxonomic monographs and reference books. The isolate was initially annotated on genus level as *Coelastrella* sp. Gene marker (ITS2) was used for molecular identification. According to this protocol, the microalga was identified as *Scenedesmus vacuolatus*. Combination of morphology and DNA-based approach proved to be the most effective for obtaining a consistent species level identification. Moreover, this is the first record of *Scenedesmus vacuolatus* identification from soil in Vojvodina, Serbia.

## Introduction

Microalgae are microscopic organisms (1-900 µm) that can be either prokaryotic or eukaryotic. They can grow and reproduce in freshwater and marine environments, as well as in soil (Nabti *et al*., 2017). It is estimated that out of existing 800.000 microalgae species, around 50.000 are described (Ronga *et al*., 2019), but only a few species are used for different purposes, such as food or animal feed, in the pharmaceutical industry, agriculture, etc (Seman *et al*., 2021). Due to various application potential, microalgae have attracted considerable interest worldwide. The most examined microalgal species originate predominantly from various freshwater environments and belong to the branch of green algae (Chlorophyta) and cyanobacteria (Bumandalai and Tserennadmid, 2019; Hernandez *et al*., 2009).

However, soil algal diversity is also enormous, yet nothing or little is done to uncover their importance in the ecosystems they inhabit. Underestimating the algal diversity in these ecosystems is due to, first of all poor knowledge about their role in soil ecosystem maintenance as well as lack of significant scientific interest by researchers in algal taxonomy and phylogeny (Trbojević and Predojević, 2022).

Though the importance and application of specific soil microalgae are acknowledged by many researchers (Hajnal Jafari *et al*., 2016; Kholssi *et al.*, 2019; Žunić *et al*., 2022), there are no published articles about the diversity and taxonomy of green soil microalgae in Serbia. Generally, studies on the identification of microalgae are done using a classical approach based upon the description of cell and colony structures. This is highly subjective since it requires a microscope of high magnification and a skilled observer. Zou *et al*., (2016) even argued that the morphological

*Corresponding author. E-mail: mikrobiologija@polj.uns.ac.rs

identification of some species is impossible. Moreover, there are morphological adaptation and endemism among many microalgae species which make them difficult to correctly identify (Coleman, 2002). That is why the combination of morphology and DNA-based approach proved to be the most effective for obtaining a consistent species level identification.

Hence, in this study, the aim was to compare the results of classical and molecular identification of soil microalgae using gene marker (ITS2). The identity of the microalgae was initially identified using its morphological characteristics.

## Materials and Methods

### *Isolation and growth conditions*

The green microalgae species was isolated from acidic arable soil (pH 4.31) in Vojvodina, Serbia, using BG11 medium. BG11 medium per one liter of distilled water contains: 1.5 g $NaNO_3$, 0.04 g $K_2HPO_4$, 0.075 g $MgSO_4 \cdot 7H_2O$, 0.036 g $CaCl_2 \cdot 2H_2O$, 0.006 g Citric acid, 0.006 g Ferric ammonium citrate, 0.001 g EDTA, 0.02 g $Na_2CO_3$ and 1ml Trace metal solution A5. The composition of the trace metal mix solution is: 2.86 g $H_3BO_3$, 1.86 g $MnCl_2 \cdot 4H_2O$, 0.22 g $ZnSO_4 \cdot 7H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$, 0.05 g $Co(NO_3)_2 \cdot 6H_2O$. The pH of BG11 medium was adjusted to 7.1 and autoclaved at 121ºC.

Microalgae isolation started with using agar plate method. Microalgae were grown in Petri dishes on solid BG11 medium under aseptic conditions at room temperature (24±2°C) and day: night photoperiod 14:10. Every 14 days, one colony was selected and transferred to fresh agar plates. Elimination of contaminants and pure culture was obtained by the agar streaking method and continued growth upon sub-culturing.

### *Cell morphology and identification*

Taxonomic identification was performed on the basis of cell morphology. Morphological observations were done using a binocular light research microscope at 400× and 1000× magnification (Motic, BA210). Identification was based on taxonomic monographs and reference books for terrestrial algae and cyanobacteria (Komárek and Fott, 1983; Komárek and Anagnostidis, 2005; Bellinger and Sigee, 2010)

### *Molecular identification*

Total genomic DNA was isolated from fresh algal biomass using the EURx DNA extraction protocol for environmental samples - soil (GeneMATRIX Soil DNA Purification Kit™). 50 ml of fresh algal biomass was centrifuged, and the pellet was used according to the protocol.

### *PCR amplification protocol*

ITS2 region was attempted to PCR amplification using the ITS2 primers according to Hadi *et al*., (2016) and Liu *et al.* (2014): ITS-2_f, 5'-AGGAGAAGTCGTAACAAGGT-3' (Tm=56.9°C) and ITS-2_r, 5'-TCCTCCGCTTATTGATATGC (Tm=61.5°C). Amplicon size varies (nucleotides span) 600-1000 bp, depending on algal strain. The 25 µL PCR reaction mix was composed of 10 µL of ultrapure water, 12.5 µL of MasterMix, 0.25 µL of each primer ITS2 forward and reverse. At the end, 2 µL of DNA template was added in the PCR reaction mixture in Eppendorf.

The PCR reactions were performed in the PCR thermocycler TC-412 Thermal Cycler (United Kingdom) using a program designed for the amplification of ITS regions. The program starts with initial denaturation at 96°C, 40 cycles (96ºC - 1 min. of template denaturation, 52ºC - 1 min. primer annealing, and 72ºC – 1 min. for extension) and final extension at 72ºC for 5 min and hold at 4ºC.

***Gene visualization***

10 µL of each PCR sample were mixed with 3µL 6x DNA Loading Dye (Thermo Fisher Scientific Baltics UAB, Lithuania) and loaded onto 1.5% agarose gel and electrophoresed using 1x TBE (Tris Borate EDTA) buffer (Sigma Aldrich Co., USA). Gene Ruler 100 bp DNA Ladder (Thermo Fisher Scientific Baltics UAB, Lithuania) was loaded onto the first and/or last lane. Agarose gel and electrophoresis was run at 100 W for 45 min. After that, gel was added to 1% ethidiumbromide solution (250ml $dH_2O$ + 20 µL ethidiumbromide) for 30 min. Bands were visualized with a UV lamp in BlueCube 300 (SERVA Electrophoresis GmbH, Germany). Genomic DNA was sent out for sequencing to Macrogen© (Seoul, South Korea) and the result was compared with sequences available on the NCBI website database through the BLAST bioinformatics tool.

**Results and Discussion**

Based on the morphology obtained using light microscopy (LM), the taxonomy was further performed using reference books for terrestrial algae and cyanobacteria. The isolated strain was annotated as a member of the genus *Coelastrella*. It is a single celled, non motile green algae with a round to coccoid shape of cell, size 4-12 µm (young and adult). Inside the cell, a cup-shaped chloroplast as well as a single pyrenoid can be easily observed (Fig. 1). The size and shape of microalgal cells depend on maturity, cell growth phase and origin (Darienko *et al*., 2019). Equal-sized autospores and autosporangia (mother cell) with autospores inside the cell were also present. The release of autospores was obtained by the rupture of the mother cell wall. Traditionally, taxonomic classification and subsequent identification depend on the morphological description of cell and colony features. This could generate mistakes in the taxonomic identification (Gour *et al.*, 2016). Krienitz *et al*., (2003) also stated that the classical approaches using morphological characters for the circumscription of coccoid green algae do not adequately reflect the phylogenetic relationships. Though it is important to examine the culture and take images for any future record and publication, one should be aware of relying only on LM images because many different algae species may look alike. Having that in mind, the next step was using a DNA sequence for species-level identification.

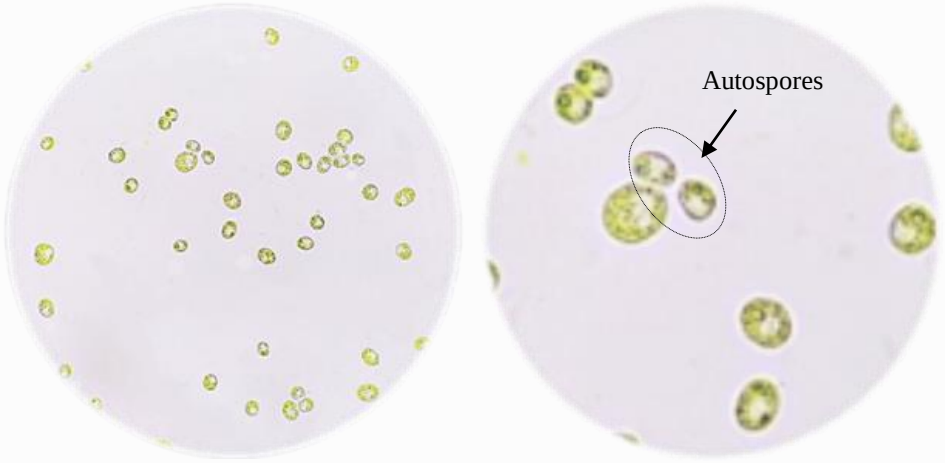


Fig. 1. Morphology of the soil microalgae *Coelastrella* sp.
(magnification 40× and 1000×)

Considered as one of the frequently used molecular markers for distinguishing between eukaryotic species, the internal transcribed spacer 2 (ITS2) is a gene used in several applications among which molecular phylogenetic analyses can be included. The primary sequence of ITS2 region is highly conserved within species, but is highly divergent between species (Hoshina, 2014). Fawley and Fawley (2020) stated that for algae, and especially for green algae, the ITS2 region is an important region for species identification. Fig. 2 shows the band of sample 63s amplified using ITS2 rDNA primers.

A total of 715 base pair sequences was obtained which was submitted to Genbank for BLAST search. The sequence got labeled as *Scenedesmus vacuolatus* 63 (Accession number: OP808227). The level of identity between the submitted sequence and a published sequence was 99.8%.

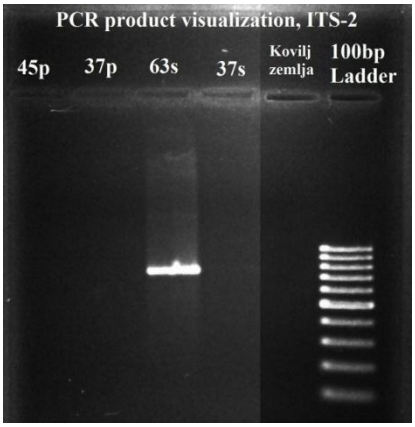


Fig. 2. PCR amplification results of *Scenedesmus vacuolatus* (63s) ITS-2 region

The phylogenetic tree (Fig. 3) shows the relationship of *Scenedesmus vacuolatus* 63 to other strains of microalgae based on their ITS2 sequences. The closest sequences were *Scenedesmus vacuolatus* S12, *Coelastrella vacuolata* FACHB-3315 and *Coelastrella vacuolata* FACHB-3314 with over 99% similarity. The other nearest group includes the strains *S. vacuolatus* SAG211-8c, *S. vacuolatus* SAG211-8e and *S. vacuolatus* UTEX252. The most distant branch is *Desmodesmus regularis* SAG24.95 (AM228925). The classification of *Scenedesmus vacuolatus* is given below:

**Domain: Eukarya; Phylum: Chlorophyta; Class: Chlorophyceae; Order: Sphaeropleales Family: Scenedesmaceae; Genus: *Scenedesmus*; Species: *S. vacuolatus*** (Shihira & Krauss) E. Kessler, M. Schäfer, C. Hümmer, A. Kloboucek & V. Huss

The molecular identification (BLAST search) showed that the studied strain had a different genus name compared to the one annotated based on morphology. However, that is not something unexpected in algal systematics. The demarcation of genera and species in the family Scenedesmaceae is rather difficult. Numerous phylogenetic studies revealed the existence of complex relationships among algal species since morphological and molecular identification often resulted in different outcomes (Lakshmana Senthil *et al*., 2019). *Chlorella fusca* var. *vacuolata*, *Graesiella vacuolata* and *Coelastrella vacuolata* (Kalina and Puncocharova, 1987; Hegewald and Hanagata, 2002) are the nomenclatural or homotypic synonyms for *Scenedesmus vacuolatus* that can be found in the literature covering algae identification and taxonomy (Darienko *et al*., 2010;

Shetty *et al.*, 2021). Basically, our morphology based identification matched the DNA bases, even though the genus names differed. This result is consistent with the initial idea that a combined classical and molecular method of identification has to be applied in order to perform an accurate genus/species level identification.

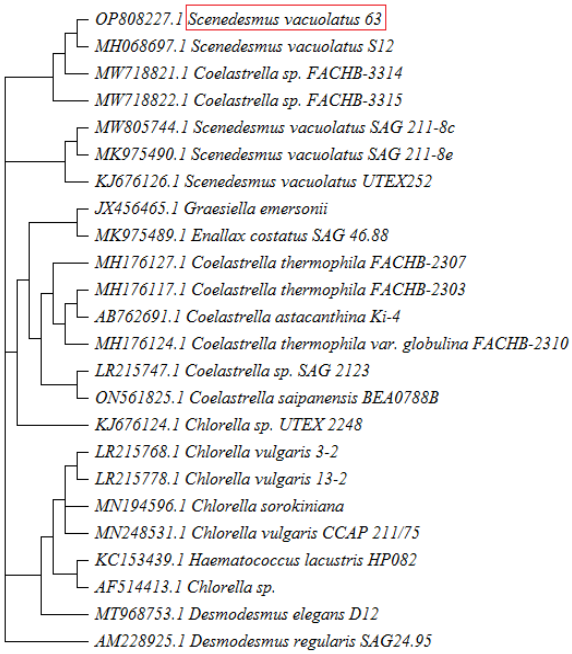


Fig. 3. Maximum likelihood phylogenetic tree based on ITS2 sequences of *Scenedesmus vacuolatus* 63 gained by bootstrap analysis in 1000 repetition

According to the literature survey, there is no report regarding the diversity of green soil microalgae in Serbia. In this context, this is the first record of *Scenedesmus vacuolatus* from the Vojvodina region in Serbia.

**Acknowledgements**

This work was supported by the Ministry of Science, Technological Development and Innovation, Republic of Serbia, Contract No. 451-03-47/2023-01/ 200117. Timea Hajnal Jafari designed the experiments, interpreted the results and wrote the paper. Vladimira Žunić worked on laboratory research. Dragana Stamenov was involved in experiments and manuscript preparation. Statistical data processing was done by Simonida Djurić.

**References**

Bellinger, E.G. and Sigee, D.C. 2010. Freshwater Algae: Identification and Use as Bioindicators. John Wiley & Sons, Ltd. ISBN 978-0-470-05814-5.

Bumandalai, O. and Tserennadmid, R. 2019. Effect of *Chlorella vulgaris* as a biofertilizer on germination of tomato and cucumber seeds. Inter. J. Aquat. Biol. **7**(2): 95-99.

Coleman, A.W. 2002. Microbial eukaryote species. Sci. **297**: 337.

Darienko, T., Rad, M.C., Campbell, C. and Pröschold, T. 2019. Are there any true marine *Chlorella* species? Molecular phylogenetic assessment and ecology of marine *Chlorella*-like organisms, including a description of Droopiellagen. nov. Systemat. Biodiver. **17**(8): 811–829.

Darienko, T., Gustavs, L., Mudimu, O., Rad, M.C., Schumann, R., Karsten, U., Friedl, T. and Pröschold, T. 2010. *Chloroidium*, a common terrestrial coccoid green alga previously assigned to *Chlorella* (Trebouxiophyceae, Chlorophyta). European J. Phycol. **45**(1): 79-95.

Fawley, M.W. and Fawley, K.P. 2020. Identification of eukaryotic microalgal strains. J. Appl. Phycol. **32**: 2699–2709.

Gour, R.S., Chawla, A., Singh, H., Chauhan, R.S. and Kant, A. 2016. Characterization and screening of native *Scenedesmus* sp. isolates suitable for biofuel feedstock. PLoS ONE **11**(5):e0155321.

Hadi, S.I.I.A., Santana, H., Brunale, P.P.M., Gomes, T.G., Oliveira, M.D., Matthiensen, A., Oliveira, M.E.C., Silva, F.C.P. and Brasil, B.S.A.F. 2016. DNA barcoding green microalgae isolated from neotropical inland waters. PLoS ONE **11**: 1-18.

Hajnal-Jafari, T., Djuric, S. and Stamenov, D. 2016. Influence of green algae *Chlorella vulgaris* on initial growth of different agricultural crops. Zbornik Matice Srpske za Prirodne Nauke **130**(130): 29-33.

Hegewald, E. and Hanagata, N. 2002. Validation of the new combinations of *Coelastrella* and *Neodesmus* and the description of the new subfamily Desmodesmoideae of the Scenedesmaceae (Chlorophyta). Archiv fuer Hydrobiologie Supplement **143** [Algological Studies *105*]: 7-9.

Hernandez, J.P., De-Bashan, L.E., Rodriguez, D.J., Rodriguez, Y. and Bashan, Y. 2009. Growth promotion of the freshwater microalga *Chlorella vulgaris* by the nitrogen-fixing, plant growth-promoting bacterium *Bacillus pumilus* from arid zone soils. European J. Soil Biol. **45**(1): 88-93.

Hoshina, R. 2014. DNA analyses of a private collection of microbial green algae contribute to a better understanding of microbial diversity. BMC Res Notes **7:** 592.

Kalina, T. and Puncocharova, M. 1987. Taxonomy of the subfamily Scotiellocystoideae Fott 1976 (Chlorellaceae, Chlorophyceae). Archiv fur Hydrobiologie. Stuttgart (Supplement 73) Algol. Stud. **45:** 473-521.

Kholssi, R., Marks, E.A.N., Miñón, J., Montero, O., Debdoubi, A.C. and Rad, 2019. Biofertilizing effect of *Chlorella sorokiniana* suspensions on wheat growth. J. Plant Growth Regul. **38:** 644-649.

Komárek, J. and Anagnostidis, K. 2005. Cyanoprokaryota 2 (*Oscillatoriales*). – *In:* Büdel, B., Krienitz, L., Gärtner, G. & Schagerl, M. (eds) Elsevier/Spektrum, Heidelberg: Süßwasserflora von Mitteleuropa **19/2**: 759.

Komárek, J. and Fott, B. 1983. Chlorococcales. – In: Huber-Pestalozzi, G. (ed.) Schweizerbart, Stuttgart: Das Phytoplankton des Süßwassers **7:** 1043.

Krienitz, L., Hegewald, E., Hepperle, D. and Wolf, M. 2003. The systematics of coccoid green algae: 18S rRNA gene sequence data versus morphology. Biologia **58**: 437–446.

Lakshmana, S., Suja, C.P., Anantharaman, P., Kannan, K. and Kathiresan, S. 2019. First record of *Coelastrella vacuolata* (Chlorophyta: Scenedesmaceae) in Tuticorin coast, Gulf of Mannar. Indian Journal of Geo Marine Sciences **48**(12): 1860-1863.

Liu, J., Gerken, H. and Li, Y. 2014. Single-tube colony PCR for DNA amplification and transformant screening of oleaginous microalgae. J. Appl. Phycol. **26**: 1719–1726.

Nabti, E., Jha, B. and Hartmann, A. 2017. Impact of seaweeds on agricultural crop production as biofertiliser. Inter. J. Environ. Sci. Technol. **14**: 1119-1134.

Ronga, D., Biazzi, E., Parati, K., Carminati, D., Carminati, E. and Tava, A. 2019. Microalgal Biostimulants and Biofertilisers in Crop Productions. Agronomy **9**(192): 1-22.

Seman, V., Hajnal J.T., Djurić S. and Stamenov, D. 2021. The capacity of soil microalgae to improve germination and initial growth of white radish and kohlrabi. Contemporary Agriculture **70**(1-2): 36-40.

Shetty, P., Farkas, A., Pap, B., Hupp, B., Ordog, V., Bíro, T., Varga, T. and Maroti, G. 2021. Comparative and phylogenomic analysis of nuclear and organelle genes in cryptic *Coelastrella vacuolata* MACC-549 green algae. Algal Res. **58**: 102380.

Trbojević, I. and Predojević, D. 2022. Algae in shallow and small water bodies of Serbia: a frame for species and habitat protection. In: Pešić, V., Milošević, Đ., Miliša, M. (eds) Small water bodies of the western balkans. Springer water, Springer, Cham, Switzerland, pp. 147-188.

Zou, S., Fei, C., Wang, C., Gao, Z., Bao, Y., He, M. and Wang, C. 2016. How DNA barcoding can be more effective in microalgae identification: a case of cryptic diversity revelation in *Scenedesmus* (Chlorophyceae). Scient. Rep. **6**: 36822.

Žunić, V., Hajnal J.T., Grabić, J., Djurić, S. and Stamenov, D. 2022. Hydroponic systems: exploring the balance between co-cultivation of *Chlorella vulgaris* and Swiss chard (*Beta vulgaris* L. subsp. *cicla*). J. Appl. Phycol. **34**: 903–913.

*(Manuscript received on 05 July 2023; revised on 02 December 2023)*