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DNA signaturing derived from the internal transcribed spacer 2 (ITS2): a novel tool for identifying *Desmodesmus* species (Scenedesmaceae, Chlorophyta)

Minh Ly Nguyen [1*], Xuan Cuong Mai [2,3,4], Nhat Ha Chu [1], Dang Mau Trinh [1], Chao–Lin Liu [2] & Chia–Rui Shen [3,4,5]

[1] *The University of Danang – University of Science and Education, Faculty of Biology and Environmental Science, Department of Biotechnology, Danang City 550000, Vietnam; Corresponding author e–mail: nmly@ued.udn.vn*
[2] *Ming Chi University of Technology, Department of Chemical Engineering and Biochemical Technology R&D Center, New Taipei City 24301, Taiwan*
[3] *Chang Gung University, College of Medicine, Department and Graduate Institute of Medical Biotechnology and Laboratory Science, Taoyuan City 333, Taiwan*
[4] *Chang Gung University, College of Medicine, Graduate Institute of Biomedical Sciences, Taoyuan City, 333, Taiwan*
[5] *Lin–Kou Chang Gung Memorial Hospital, Department of Ophthalmology, Taoyuan City 333, Taiwan*

Abstract: The freshwater green algal genus *Desmodesmus* contains many cryptic species that are difficult to identify using morpho–anatomy. This study analyzed 310 ITS2 sequences related to *Desmodesmus* species from GenBank and developed a new tool named DNA signaturing. DNA signaturing is a species identification method using signature sequences specific for each species or strain group within the genus *Desmodesmus*. These signature sequences had 18–33 nucleotides in length. This tool could exactly identify 19 species and 24 strain groups of *Desmodesmus*. In addition, a signature sequence, AGA GGC TTA AAC TGG GAC C, was specific for almost 90% of *Desmodesmus* strains and could represent the genus *Desmodesmus*. A strain MLC1 isolated from freshwater in Da Nang, Vietnam, was identified as *Desmodesus pseudoserratus* by this tool. DNA signaturing tool described here showed the highest efficacy for identifying strains and a remarkable ability to accurately classify cryptic species in the genus *Desmodesmus*.

Key words: Cryptic species, *Desmodesmus*, green microalgae, ITS2, DNA signaturing, signature sequence

Introduction

The genus *Desmodesmus* (Chodat) An, Friedl, et Hegewald (Scenedesmaceae, Chlorophyta), was first published as one of subgenera of *Scenedesmus* by Hegewald, and then elevated to a genus level in 1999 (Hegewald 1978; An et al. 1999; Van Hannen et al. 2000). Currently, forty–six species of *Desmodesmus* have been recorded by the National Center for Biotechnology Information (NCBI) Taxonomy database (https://www.ncbi.nlm.nih.gov/taxonomy).

Desmodesmus species identification has been difficult because of their phenotypic plasticity across growth and development stages, and high morphological similarity in species (Hegewald et al. 2005; Hentschke & Torgan 2010; Lin et al. 2020). Therefore, for in–depth morphological analysis of *Desmodesmus* species, scanning electron microscopy (SEM) was applied to evaluate the ultrastructural features of the cell wall and distinguish species based on spines on the lateral walls and cell polesshort–spined or spineless and the shape of the rosettes or the large warts (An et al. 1999; Hegewald et al. 2005; Vanormelingen et al. 2007; Hentschke & Torgan 2010; Fawley et al. 2011; Bica et al. 2012; Hegewald & Braband 2017). However, this method required access to the electron microscope, which not all research facilities can provide.

The internal transcribed spacer 2 (ITS2) sequence analysis has been a promising candidate for microalgal identification (Fawley & Fawley 2020). *Desmodesmus* was first distinguished from *Scenedemus* based on phylogenetic analysis of ITS2 sequences by An (1999). The ITS2 region was also able to determine several species in *Desmodesmus*, *Scenedesmus*, and *Tetradesmus* (Van Hannen et al. 2000; Johnson et al. 2007; Vanormelingen et al. 2009; Hegewald & Braband 2017). However, the

ITS2 sequence is variable in interspecies and intraspecies. VAN HANNEN et al. (2000) reported the interspecific genetic distance of Scenedesmaceae ranged from 10% to 30% for closely related species and the intraspecific genetic distance ranged from 2% to 10%. This led to a lack of confidence in the species identification by using the ITS2 sequence. Therefore, the ITS2 secondary structure analysis was added to the taxonomy study. In particular, finding the sites of compensatory base changes (CBCs) and hemi–CBCs (hCBCs) in the ITS2 secondary structure could be a useful tool to identify *Desmodesmus* species (MÜLLER et al. 2007; VANORMELINGEN et al. 2007; FAWLEY et al. 2011; HOSHINA 2014; HEGEWALD & BRABAND 2017). A CBC reaches when a paired site in ITS2 secondary structure has both mutated nucleotides, and a hCBC just has one mutated nucleotide in a paired site (MÜLLER et al. 2007; VANORMELINGEN et al. 2007; FAWLEY et al. 2011). While most strains of the same *Desmodesmus* species showed the near–absence of intraspecies CBCs, the number of CBCs diversified from 1 to 11 between the closely related *Desmodesmus* species and could be used to classify species (VANORMELINGEN et al. 2007). Nevertheless, the use of this method involved multiple stages of structural prediction, structural homology, CBCs sites analysis, and then comparison with the recorded species (SCHULTZ & WOLF 2007; HEGEWALD & BRABAND 2017). Furthermore, about 21 *Desmodesmus* species were analyzed for CBCs, but the data obtained were not conjugated into a complete data set for species identification (VANORMELINGEN et al. 2007; FAWLEY et al. 2011; HEGEWALD & BRABAND 2017). Therefore, CBC data in *Desmodesmus* has still limited and not integrated yet. *Desmodesmus* species identification is still an open matter.

In this study, we aimed to develop a new tool for *Desmodesmus* identification. We hypothesized that the appearance of CBCs and hCBCs on the ITS2 secondary structure was a consequence of characteristic nucleotides differences in the DNA sequence. Each helix of ITS2 secondary structure was in the range of 20–80 nucleotides in length (VAN HANNEN et al. 2002; FAWLEY et al. 2011; HEGEWALD & BRABAND 2017). Consequently, the presence of CBCs on the same helix demonstrated the existence of a short DNA sequence that contains these CBCs and could be species–specific. Therefore, we analyzed multiple ITS2 sequences to find short species–specific nucleotide sequences for each *Desmodesmus* species. This technique named "DNA signaturing" contained these signature nucleotide sequences. This novel tool is useful to drive the process of *Desmodesmus* identification more simply and accurately. In addition, to increase DNA signaturing's reliability, this method also inherited the results of *Desmodesmus* identification based on the morphological analyses of *Desmodesmus* strains as well as their ITS2 secondary structure analysis from previous studies.

MATERIALS AND METHODS

Sampling, isolation, and culturing. The microalgal sample in freshwater was collected at coordinates of 16°05'40.5" N, 108°07'52.7" E in Da Nang city, Vietnam. The strain was isolated based on the method of SINGH et al. (2015) and cultured in BG 11 medium (RIPPKA 1992) with a 16:8 hour light/dark cycle at 25 °C and illumination intensity of 1700 lux. Morphological characteristics of these strains were observed under a Zeiss Axio Lab1 light microscope (Suzhou Co., Ltd). They were imaged, described, and compared against the microalgae taxonomic literature (HENTSCHKE & TORGAN 2010; BELLINGER & SIGEE 2015) and the database of Algaebase.org (https://www.algaebase.org/).

DNA extraction, PCR amplification, and DNA sequencing. Total DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method (PHILLIPS et al. 2001) with several modifications.

The ITS2 region was amplified through PCR using Aeris Thermal Cycler ESCO, Singapore. The used primer pairs were ITS1 (5' TCC GTA GGT GGA CCT GCGG 3') and ITS4 (5' TCC TCC GCT TAT TGA TATG 3') (HENRION et al. 1992). The reaction mixture contained 1X Dream Taq Buffer, 1 Unit of DreamTaq DNA polymerase (Thermo Fisher Scientific), 100–120 ng of total DNA, 1 µM of each primer, yielding a final volume of 20 µl by double–distilled water (ddH_2O). The PCR product was purified and sequenced by the Sanger method at Apical Scientific (Malaysia).

ITS2 sequence collection and alignment. The ITS2 sequences of *Desmodesmus* were collected from GenBank (https://www.ncbi.nlm.nih.gov/) and the ITS2 databases (http://its2.bioapps.biozentrum.uni–wuerzburg.de/). A total of 266 sequences containing the ITS2 region of *Desmodesmus* species, 19 of *Scenedesmus* species, and 24 of *Tetradesmus* species were collected (Supplemental Information). *D. denticulatus* (Lagerheim) S.S.An, T.Friedl et E.Hegewald was proven that it was different from the ingroup *Desmodesmus* taxa (JOHNSON et al. 2007; VANORMELINGEN et al. 2007; FAWLEY et al. 2011). Therefore, ITS2 sequences from four strains of *D. denticulatus* were used as the outgroup.

The sequences were then aligned using ClustalW integrated into MEGA 7 software to precisely determine and analyzed the ITS2 region of the obtained sequences (THOMPSON et al. 1994; KUMAR et al. 2016). The pairwise alignment and multiple alignment were optimized with the parameter of gap opening penalty and gap extension penalty, which are 15 and 6.66, respectively. A alignment file containing all aligned sequences was uploaded on a publicly accessible repository, figshare (https://figshare.com/) with the digital object identifier (DOI): https://doi.org/10.6084/m9.figshare.19295426.

Phylogenetic analysis. The ITS2 sequences were analyzed using the neighbor–joining (NJ) clustering method and evolutionary distances were computed using the p–distance method (SAITOU & NEI 1987; NEI & KUMAR 1993). Unrooted NJ phylogeny from ITS2 sequences was built to classify the collected ITS2 sequences with high sequence similarity into groups (Fig. S1). The rooted NJ phylogenic trees from ITS2 sequences were applied for phylogenetic analysis (Fig. S2). The Maximum Likelihood (ML) phylogenetic tree was inferred using 50 representative ITS2 sequences to better understand the process of sequence evolution (TAMURA & NEI 1993; YANG & RANNALA 2012). The Tamura–Nei model was used

because it could be applied for samples with characteristics such as variation among sites and heterogeneity and assume equality substitution rates among sites as well as substitution patterns among lineages (Tamura & Nei 1993). Initial tree(s) for the heuristic search were obtained automatically by applying NJ and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with a superior log–likelihood value (Tamura & Nei 1993). Bootstrapping with 1,000 replicates was applied to estimate the confidence of the topology of both phylogenetic trees (Felsenstein 1985). All the above programs were integrated and analyzed in MEGA 7 (Kumar et al. 2016). The phylogenetic tree was observed and noted with iTOL (Letunic & Bork 2021).

Taxon names on phylogenies included species names from GenBank, strain ID, accession number (AC no.), and the group name to facilitate the analysis process. Group names are based on the first symbol of the species name (e.g. Group A for strains of *D. armatus*) or the first two letters of the species name (e.g. Group AS for strains of *D. asymmetricus*) (Fig. S1 and Fig. S2).

***Desmodesmus* strains information analysis.** The ITS2 sequences belonging to the same clade in unrooted NJ phylogeny were rearranged adjacent in the alignment file. Unrooted NJ tree showed two types of clades, (1) the clade containing the strains of one *Desmodesmus* species and (2) the clade containing the strains of multiple *Desmodesmus* species. Some *Desmodesmus* species have several synonyms due to the complex taxonomic history, the latter was analyzed and identified to clarify the information of the strains by the NCBI Taxonomy database or derived from the collections such as the culture collection of algae and Protozoa (CCAP) (https://www.ccap.ac.uk/), culture collection of algae at the University of Texas at Austin (UTEX) (https://utex.org), or culture collection of algae at the University of Göttingen (SAG) (http://sagdb.uni–goettingen.de/index.php). In addition, the ITS2 secondary structure of each strain in this clade was also predicted and evaluated by the ITS2 database to increase the confidence in their relationships.

DNA signaturing analysis. In the event of a clade containing the strains of one *Desmodesmus* species, the short DNA sequences containing 18 to 33 nucleotides to ensure specificity for species were discovered throughout the entire length of the ITS2 DNA sequences. These short DNA sequences containing at least one CBC or hCBC from referenced articles (Van Hannen et al. 2002; Vanormelingen et al. 2007; Fawley et al. 2011; Hegewald & Braband 2017) were then analyzed for similarity with sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi). If the results obtained from BLAST just appear the analyzed species, the short DNA sequences could be recognized as signature sequences and determined their position on ITS2 secondary structures using the ITS2 database, and then recorded in Table S1. The ITS2 sequences of species without CBC data were assessed holistically and selected visually for signature sequences.

In the case of a clade containing multiple strains of different species, the signature sequences were searched visually to represent the strain group. Each species or strain group was sought for as many signature sequences as possible and listed in the Table S1.

Results

Microalgal isolation

An isolate MLC1 was assignable to *Desmodesmus* based on external features. This isolate consisted of 2 cells fused at the flanks, with a length of 11 ± 1 μm and a width of 4.5 ± 0.5 μm each, elliptical in shape, rounded at one end and tapering at the other with a thick cell wall composed of many layers. The ITS2 region of isolate MLC1 was sequenced and submitted to GenBank with the AC no. MZ412709.

Signature sequence finding

Most of the signature sequences were identified at different positions ranging from 30 to 70, 110–150, and 200–240 in the ITS2 sequences of *Desmodesmus*. These regions correspond to helices I, II, and III on the ITS2 secondary structures.

Each signature sequence in DNA signaturing was checked specificity by BLAST. In some cases, the BLAST results showed different species from *Scenedesmus* and *Tetradesmus*. The ITS secondary structure and taxonomic history were then evaluated for these *Scenedesmus* and *Tetradesmus* species. If their information satisfied the following conditions: having a similar secondary structure or having a synonym for the analyzed species or being analyzed from other studies but have not been updated on online data, they would be added into *Desmodesmus* data. Thus, we added 19 ITS2 sequences of *Scenedesmus* species, and 24 of ITS2 sequences of *Tetradesmus* species on *Desmodesmus* data (Supplemental Information).

Nineteen *Desmodesmus* species and twenty–four strain groups have been identified by DNA signaturing (Table S1). In addition, three species with only one ITS2 sequence including *D. baconii*, *D. insignis*, and *D. serratoides* were found. Two strains *D. communis* strain CCMA–UFSCar–030 and *D. abundans* strain BZ– 3–3 did not belong to any strain group (Supplemental Information).

Almost ninety percent (278/310) of the *Desmodesmus* strains contained the sequence AGA GGC TTA AAC TGG GACC belonging to helix III on the ITS2 secondary structure. This short sequence was then analyzed by BLAST and the results showed only the *Desmodesmus* species and the *Scenedesmus* or *Tetradesmus* species mentioned above. Therefore, this short sequence was considered to be the signature sequence for *Desmodesmus* species.

DNA signaturing application

The full ITS2 sequences of isolate MLC1 were analyzed by BLAST, and as the result, *D. pseudoserratus* was the highest similar species with a rate of 93.17%. The results achieved from the ITS2 database in searching for homologous secondary structural models showed that the isolate MLC1 was similar to *D. santosii*, *D. perdix*, and *D. serratus*. These results matched

their genetic relationship in the phylogenetic trees, but this isolate MLC1 could not be identified with the exact species. Using the DNA signaturing method isolates MLC1 was identified as *D. pseudoserratus* immediately (Table S1).

Phylogenetic analysis

Three *Desmodesmus* species clusters were remarked on phylogenic trees (Fig. 1 and Fig. S2). The *Demosdesmus* clusters including *D. armatus* and related taxa (cluster 1); *D. communis* and related taxa (cluster 2); and *D. serratus* and related taxa (cluster 3), were commanded by a representative species with the most ITS2 sequences in each cluster.

Although *D. denticulatus* strains were identified as an outgroup, their position was inside other *Desmodesmus* species and divided them into two branches. A branch contained *D. serratus* and related taxa (branch 1) which were owned by monophyly and the remain comprised two other *Desmodesmus* clusters (branch 2). The branch length on the ML and NJ trees showed the genetically distance for species (Fig. 1 and Fig. S2). Therefore, there was the high genetic variation between two *Desmodesmus* branches as well as between each *Desmodesmus* branch and *D. denticulatus*. Therefore, it was possible that other Scenedesmaceae species may appear inside two *Desmodesmus* branches on the phylogeny.

DISCUSSION

The lack of species identification characters and morpho–anatomical variation within *Desmodesmus* species makes it difficult to identify species. In this study, we developed a DNA signaturing tool that makes the identification of *Desmodesmus* species simpler. The ITS2 sequence was analyzed to find the signature sequences that represent each species or strain group.

This novel method was based on the CBCs and hCBCs positions of *Desmodesmus* species from previous studies to find signature sequences (VANORMELINGEN et al. 2007; FAWLEY et al. 2011; HOSHINA 2014; HEGEWALD & BRABAND 2017). For instance, HEGEWALD & BRABAND (2017) used SEM and ITS2 secondary structures to thoroughly analyze and identify *D. communis* cryptic species, including *D. communis, D. rectangularis*, *D. protuberans, D. protuberans* var. *communioides*; *D. schmidtii*; *D. pseudocommunis*; and *D. pseudoprotuberans*. We have applied the ITS2 database for analyzing the ITS2 sequences of these species and only the ITS2 secondary structural model of *D. communis* was obtained. This showed the identification of these species was challenging. We then consulted the results from HEGEWALD & BRABAND (2017) which showed that *D. rectangularis* differed from *D. communis* by only one nucleotide at position 30 in the ITS2 secondary structure of *D. communis*. Therefore, an 18–nucleotides sequence containing the above distinct nucleotide was selected and analyzed by BLAST. This short sequence not only distinguished *D. rectangularis* from *D. communis* but also other *Desmodesmsus* species. Similarly, *D. pseudocommunis* differed by two different nucleotides at positions 24 and 27 on the ITS2 secondary structure of *D. communis* (HEGEWALD & BRABAND, 2017). Therefore, the short sequence containing these two positions was analyzed by BLAST and showed the specific to *D. pseudocommunis*. In addition, *D. serrtus* and related taxa were evaluated to achieve high morphological similarities, so they have been carefully analyzed by electron microscopy as well as their ITS2 secondary

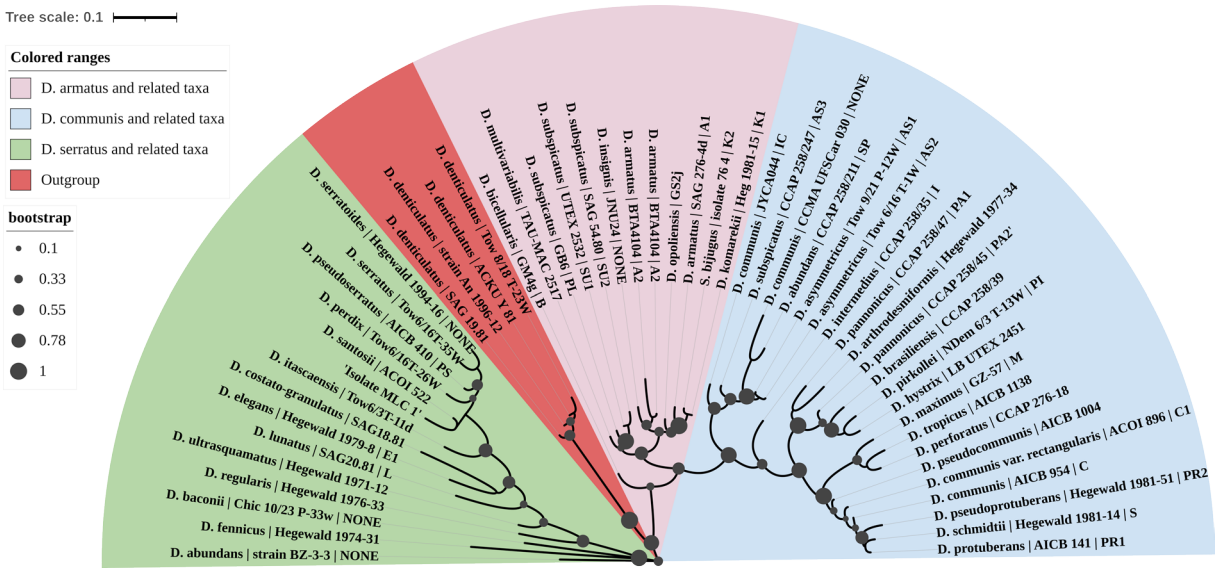

Fig. 1. Maximum likelihood phylogeny of fifty representative Desmodesmus species based on the Tamura–Nei model with bootstrap values from 1000 replicates.

structure. Indeed, differences in ultrastructural features of the cell wall were noted, and the helix regions which vary between these species were helix I and helix III (Vanormelingen et al. 2007; Fawley et al. 2011). Hence, we focused on these two helices to search for the signature nucleotide sequences of *D. serrtus* and related taxa (Table S1). Conclusively, the DNA signaturing tool inherited the reliability of species identification from previous methods (Vanormelingen et al. 2007; Fawley et al. 2011; Hegewald & Braband, 2017).

Isolate MLC1 in this study was identified in many ways including basic morphological assessment by light microscopy, searching for sequence homology on BLAST, and searching for homologous secondary structure on the ITS2 database. However, it was not possible to determine the exact species level. MLC1 was then identified as *D. pseudoserrtus* by DNA signaturing.

Unrooted NJ phylogeny was used to divide *Desmodesmus* strains into groups. However, *Desmodesmus* species data were not synchronized on online databases, leading to complications in analyzing the phylogenetic tree of *Desmodesmus* species. Several strains in some species had large genetic divergence and belong to different clades (i.e. *D. communis* strains, *D. abundans*, *D. pannonicus*...) (Fig. S2). Thus, we evaluated the information of these strains by the algal collection websites and found their homology modeling of ITS2 secondary structure by ITS2 database. It was an important step as it concerned the reliability and effectiveness of DNA signaturing. Particularly, group A1 including *D. armatus* strains, *D. schnepfii* strain CCAP 258/73, and *D. pannonicus* strains was renamed *D. armatus* I because their ITS2 secondary structural modeling was 100% similar to *D. armatus* strain GS2o (Supplementary information). In addition, *Scenedesmus ecornis* strain UTEX LB 1359 was a synonym of *D. bicellularis* or *Scenedesmus longus* was a synonym of *D. komarekii* derived from the UTEX website. Furthermore, we detected twenty–three *T. deserticola* strains from Zou et al. (2016) and one *T. deserticola* strain from Alam et al. (2019), which possess a 100% structural similarity of ITS2 sequence to *D. subspicatus* strain UTEX 2532 (Supplementary information). This is considered a prime example of the complexity of the data circulating on GenBank.

The phylogenetic tree structures in this study were homogeneous with previous studies (An et al. 1999; Van Hannen et al. 2002; Buchheim et al. 2011; Fawley et al. 2011; Hegewald et al. 2013; Hegewald & Braband 2017). *D. denticulatus* was considered to be genetically distinct from the rest of *Desmodesmus* species and proposed as an outgroup (Johnson et al. 2007; Vanormelingen et al. 2007; Fawley et al. 2011). When analyzing large numbers of *Desmodesmus* species, the observed large genetic variation between them was recorded. *Desmodesmus* branch 1 consiting of *D. serratus* and related taxa was genetically distinct from *Desmodesmus* branch 2 including two other *Desmodesmus* clusters. The occurrence of *D. denticulatus* between two *Desmodesmus* branches represented other Scenedesmaceae species (Fig. 1 and Fig. S2). This was reinforced and remarked by the results of Buchheim et al. (2011) which showed the appearance of *Tetradesmus*, *Scenedesmus* and *Enallax* species inside two *Desmodesmus* branches. The genetic relationship of *Desmodesmus* species is still a matter of clarification.

It has been proven that *Desmodesmus* isolates with the high similarity of ITS2 sequences showed exceedingly slight morphological differences (Johnson et al. 2007; Vanormelingen et al. 2007; Fawley et al. 2011; Hadi et al. 2016; Hegewald & Braband 2017). Three *Desmodesmus* clusters included species that were shown to be morphologically similar and were analyzed thoroughly by SEM (Vanormelingen et al. 2007; Hentschke & Torgan 2010; Fawley et al. 2011; Hegewald & Braband 2017). Besides, two *Desmodesmus* branches could be seperated according to the basis of morphological features. Specifically, the species of *Desmodesmus* branch 1 are spineless species, while the species of *Desmodesmus* branch 2 have spines but vary in length, shape, and size (Hentschke & Torgan 2010; Fawley et al. 2011; Hegewald & Braband 2017). Therefore, the species belonging to the same *Desmodesmus* cluster had more similar morphological characteristics. Accordingly, different morphological features among these three *Desmodesmus* clusters could be observed under light microscopy to narrow the species range when identifying novel *Desmodesmus* isolates.

DNA signaturing has been demonstrated to be a powerful tool in the species identification of microalgae. In the next step, we will perform two parallel works: (1) expand the scope of signature sequence finding to all Scenedesmaceae species and (2) develop DNA signaturing into microalgae identification software online for easy practical application.

References

Abdulsamad, J. K.; Varghese, S. A. & Thajudeen, J. (2021): Cost effective cultivation and biomass production of green microalga *Desmodesmus subspicatus* MB. 23 in NPK fertilizer medium. – Journal of Microbiology, Biotechnology and Food Sciences 2021: 599–604.

Akgul, F.; Kizilkaya, I.T.; Akgul, R. & Erdugan, H. (2017): Morphological and molecular characterization of *Scenedesmus*–like species from Ergene river basin (Thrace, Turkey). – Turkish Journal of Fisheries and Aquatic Sciences 17: 609–619.

Alam, M.M.; Mumtaz, A.S.; Russell, M.; Grogger, M.; Veverka, D. & Hallenbeck, P.C. (2019): Isolation and characterization of microalgae from diverse Pakistani habitats: Exploring third–generation biofuel potential. – Energies 12: 2660.

An, S. S.; Friedl, T. & Hegewald, E. (1999): Phylogenetic relationships of *Scenedesmus* and *Scenedesmus*-like coccoid green algae as inferred from ITS-2 rDNA sequence comparisons. – Plant Biology 1: 418–428.

Angelaalincy, M.; Senthilkumar, N.; Karpagam, R.; Kumar, G. G.; Ashokkumar, B. & Varalakshmi, P. (2017): Enhanced extracellular polysaccharide production and self–sustainable electricity generation for PAMFCs by *Scenedesmus* sp. SB1. – ACS Omega 2: 3754–3765.

Bellinger, E.G. & Sigee, D.C. (2015): Freshwater algae: identification, enumeration and use as bioindicators, 2nd edition. – 304 pp., John Wiley & Sons.

Bica, A.; Barbu–Tudoran, L.; Druga, B.; Coman, C.; Nicoara, A.; Szöke Nagy, T. & Dragoş, N. (2012): *Desmodesmus communis* (Chlorophyta) from Romanian freshwaters: coenobial morphology and molecular taxonomy based on the ITS2 of new isolates. – Ann RSCB 17: 16–28.

Buchheim, M.A.; Keller, A., Koetschan, C.; Förster, F.; Merget, B. & Wolf, M. (2011): Internal transcribed spacer 2 (nu ITS2 rRNA) sequence–structure phylogenetics: towards an automated reconstruction of the green algal tree of life. – PloS One 6: e16931.

Chanu, N.K.; Mandal, M.K. & Chaurasia, N. (2021): Correlating the influence of biochemical parameters in environment with pesticide tolerance of non–target algae. – Biologia 76: 307–319.

Dragoş, N.; Chiriac, C.; Porav, S.; Szőke–Nagy, T.; Coman, C.; Török, L. & Hegedűs, A. (2019): *Desmodesmus tropicus* (Chlorophyta) in the Danube Delta–reassessing the phylogeny of the series Maximi. – European Journal of Phycology 54: 300–314.

Fawley, M.W.; Fawley, K.P. & Hegewald, E. (2011): Taxonomy of *Desmodesmus serratus* (Chlorophyceae, Chlorophyta) and related taxa on the basis of morphological and DNA sequence data. – Phycologia 50: 23–56.

Fawley, M.W.; Fawley, K.P. & Hegewald, E. (2013): *Desmodesmus baconii* (Chlorophyta), a new species with double rows of arcuate spines. – Phycologia 52: 565–572.

Fawley, M.W. & Fawley, K.P. (2020): Identification of eukaryotic microalgal strains. – Journal of Applied Phycology 32: 2699–2709.

Felsenstein, J. (1985): Confidence limits on phylogenies: An approach using the bootstrap. – Evolution 39: 783–791.

Guruvaiah, M.; Narra, M.; Shah, E.; James, J. & Kurchania, A. (2015): Utilization of dairy wastewater for pollutants removal and high lipid biomass production by a newly isolated microalgal strains chloromonas playfairii and *Desmodesmus opoliensis*. – International Journal of Applied Sciences and Biotechnology 3: 699–707.

Hadi, S.I.; Santana, H.; Brunale, P.P.; Gomes, T.G.; Oliveira, M.D.; Matthiensen, A. & Brasil, B.S. (2016): DNA barcoding green microalgae isolated from neotropical inland waters. – PloS One 11: e0149284.

Hegewald, E. & Braband, A. (2017): A taxonomic revision of *Desmodesmus* serie *Desmodesmus* (Sphaeropleales, Scenedesmaceae). – Fottea 17: 191–208.

Hegewald, E. (1978): Eine neue Unterteilung der Gattung Scenedesmus Meyen. – Nova Hedwigia: 343–376.

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 13: 149–164.

Hegewald, E.; Coesel, P.F. & Hegewald, P. (2002): A phytoplankton collection from Bali, with the description of a new *Desmodesmus* species (Chlorophyta, Scenedesmaceae). – Algological Studies/Archiv für Hydrobiologie, Supplement Volumes: 51–78.

Hegewald, E.; Schmidt, A.; Braband, A. & Tsarenko, P. (2005): Revision of the *Desmodesmus* (Sphaeropleales, Scenedesmaceae) species with lateral spines. 2. The multi–spined to spineless taxa. – Algological Studies/Archiv für Hydrobiologie, Supplement Volumes: 1–38.

Henrion, B.; Tacon, F.L. & Martin, F. (1992): Rapid identification of genetic variation of ectomycorrhizal fungi by amplification of ribosomal RNA genes. – New Phytologist 122: 289–298.

Hentschke, G.S. & Torgan, L.C. (2010): *Desmodesmus* and *Scenedesmus* (Scendesmaceae, Sphaeropleales, Chlorophyceae) in aquatic environments from the Coastal Plain of Rio Grande do Sul, Brazil. – Rodriguésia 61: 585–601.

Hoshina, R. & Imamura, N. (2004): Phylogenetic position of endosymbiotic green algae in *Paramecium bursaria* Ehrenberg from Japan. – Plant Biology 6: 447–453.

Hoshina, R. (2014): DNA analyses of a private collection of microbial green algae contribute to a better understanding of microbial diversity. – BMC Research Notes 7: 1–15.

Insumran, Y. (2016): Promising lipid source for biodiesel production from *Desmodesmus* sp. RMUO8 (Sphaeropleales: Scenedesmaceae). – Prawarun Agricultural Journal 13: 96–104.

Jeon, S.L. & Hegewald, E. (2006): A revision of the species *Desmodesmus perforatus* and *D. tropicus* (Scenedesmaceae, Chlorophyceae, Chlorophyta). – Phycologia 45: 567–584.

Johnson, J.L.; Fawley, M.W. & Fawley, K.P. (2007): The diversity of *Scenedesmus* and *Desmodesmus* (Chlorophyceae) in Itasca state park, Minnesota, USA. – Phycologia 46: 214–229.

Kaplan–Levy, R.N.; Alster–Gloukhovski, A.; Benyamini, Y. & Zohary, T. (2016): Lake Kinneret phytoplankton: integrating classical and molecular taxonomy. – Hydrobiologia 764: 283–302.

Kaur, S.; Sarkar, M.; Srivastava, R.B.; Gogoi, H.K. & Kalita, M.C. (2012): Fatty acid profiling and molecular characterization of some freshwater microalgae from India with potential for biodiesel production. – New Biotechnology 29: 332–344.

Kessler, E.; Schäfer, M.; Hümmer, C.; Kloboucek, A. & Huss, V.A.R. (1997): Physiological, biochemical, and molecular characters for the taxonomy of the subgenera of *Scenedesmus* (Chlorococcales, Chlorophyta). – Botanica Acta 110: 244–250.

Koike, K.; Akai, N.; Liao, L. M.; Ikeda, S. & Yoshimatsu, S. (2013): Chlorophycean parasite on a marine fish, *Sillago japonica* (Japanese sillago). – Parasitology international 62: 586–589.

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.

Letunic, I. & Bork, P. (2021): Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. – Nucleic acids research 49(W1): W293–W296.

Li, A.; Zhang, L.; Zhao, Z.Y.; Ma, S.S.; Wang, M. & Liu, P.H. (2016): Prescreening, identification and harvesting of microalgae with antibacterial activity. – Biologia 71: 1111–1118.

Lin, W.J.; Ho, H.C.; Chu, S.C.; & Chou, J.Y. (2020): Effects of auxin derivatives on phenotypic plasticity and stress tolerance in five species of the green alga *Desmodesmus* (Chlorophyceae, Chlorophyta). – PeerJ 8: e8623.

Lortou, U. & Gkelis, S. (2019): Polyphasic taxonomy of green algae strains isolated from Mediterranean freshwaters. – Journal of Biological Research–Thessaloniki 26: 1–12.

Luo, W.; Proeschold, T.; Bock, C. & Krienitz, L. (2010): Generic concept in *Chlorella*-related coccoid green algae (Chlorophyta, Trebouxiophyceae). – Plant Biology 12: 545–553.

Müller, T., Philippi, N., Dandekar, T., Schultz, J., & Wolf, M. (2007). Distinguishing species. – RNA 13: 1469–1472.

Nei, M. & Kumar, S. (2000): Molecular evolution and phylogenetics. – 348 pp., Oxford University Press, Oxford.

Phillips, N.; Smith, C.M. & Morden, C.W. (2001): An effective DNA extraction protocol for brown algae. – Phycological Research 49: 97–102.

Rippka, R. (1992): Pasteur culture collection of cyanobacterial strains in axenic culture. – Catalogue and taxonomic handbook. Catalogue of strains 1992/1993: 1–103.

Rocha, R.P.; Machado, M.; Vaz, M.G.M.V.; Vinson, C.C.; Leite, M.; Richard, R.; Mendes, L.B.B.; Araujo, W.L.; Caldana, C.; Martins, M.A.; Williams, T.C.R. & Nunes–Nesi, A. (2017): Exploring the metabolic and physiological diversity of native microalgal strains (Chlorophyta) isolated from tropical freshwater reservoirs. – Algal Research 28: 139–150.

Saitou, N. & Nei, M. (1987): The neighbor–joining method: a new method for reconstructing phylogenetic trees. – Molecular Biology and Evolution 4: 406–425.

Schultz, J. & Wolf, M. (2009): ITS2 sequence–structure analysis in phylogenetics: a how–to manual for molecular systematics. – Molecular Phylogenetics and Evolution 52: 520–523.

Singh, P.; Gupta, S.K.; Guldhe, A.; Rawat, I. & Bux, F. (2015): Microalgae isolation and basic culturing techniques. – In: Kim, S.K. (ed.): Handbook of marine microalgae. – pp. 43–54, Academic Press, Elsevier.

Tamura, K. & Nei, M. (1993): Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. – Molecular Biology and Evolution 10: 512–526.

Thompson, J.D.; Higgins, D.G. & Gibson, T.J. (1994): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position–specific gap penalties and weight matrix choice. – Nucleic Acids Research 22: 4673–4680.

Van Hannen, E.J.; Fink, 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.

Vanormelingen, P.; Hegewald, E.; Braband, A.; Kitschke, M.; Friedl, T.; Sabbe, K. & Vyverman, W. (2007): The systematics of a small spineless *Desmodesmus* species, *D. costato-granulatus* (Sphaeropleales, Chlorophyceae), based on its2 rDNA sequence analyses and cell wall morphology. – Journal of Phycology 43: 378–396.

Vanormelingen, P.; Vyverman, W.; De Bock, D.; Van Der Gucht, K. & Meester, L.D. (2009): Local genetic adaptation to grazing pressure of the green alga *Desmodesmus armatus* in a strongly connected pond system. – Limnology and Oceanography 54: 503–511.

Vieira, H.H.; Bagatini, I.L.; Guinart, C.M. & Vieira, A.A.H. (2016): tufA gene as molecular marker for freshwater Chlorophyceae. – Algae 31: 155–165.

Wang, L.; Wang, L.; Manzi, H.P.; Yang, Q.; Guo, Z.; Zheng, Y.; Liu, X. & Salama, E.S. (2022): Isolation and screening of *Tetradesmus dimorphus* and *Desmodesmus asymmetricus* from natural habitats in Northwestern China for clean fuel production and N, P removal. – Biomass Conversion and Biorefinery 12: 1503–1512.

Yang, Z. & Rannala, B. (2012): Molecular phylogenetics: principles and practice. – Nature Reviews Genetics 13: 303–314.

Zhou, Z.; Shen, D.; Gao, B.; Huang, L.; Li, A. & Zhang, C. (2016): Cultivation of a starch–rich microalga *Desmodesmus insignis* in dairy wastewater and removal of nitrogen and phosphorus. – Plant Science Journal 34: 446–459.

Zou, S.; Fei, C.; Wang, C.; Gao, Z.; Bao, Y.; He, M. & Wang, C. (2016): How DNA barcoding can be more effective in microalgae identification: a case of cryptic diversity revelation in *Scenedesmus* (Chlorophyceae). – Scientific Reports 6: 1–13.

Supplementary material

The following supplementary material is available for this article:

Table S1. DNA signaturing of *Desmodesmus*
Fig S1. Unrooted neighbor–joining phylogeny (with bootstrap values from 1000 replicates) using ITS2 sequences of *Desmodesmus* species.
Fig S2. Rooted neighbor–joining phylogeny (with bootstrap values from 1000 replicates) using ITS2 sequences of *Desmodesmus* species.
Supplementary Information. The list of collected strains belongs to *Desmodesmus* with strains ID and accession number from Genbank. Recent identification and groups species were determined by DNA signaturing and unrooted neighbor–joining phylogenetic tree.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

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