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A new terrestrial species of *Diplosphaera* (Trebouxiophyceae), *Diplosphaera sundellii*, from a sodic-saline slick in Warren Prairie Natural Area, Arkansas, USA

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Abstract: *Diplosphaera* Bialosuknia emend Vischer (Chlorophyta; Trebouxiophyceae) is a very small green alga commonly found as a photobiont in lichens and free–living in soil. Cells of *Diplosphaera* are spherical or nearly so and often grouped in short chains. There is essentially no morphological variation among the strains of the single recognized species of this genus, *D. chodatii*. In our study of the soil algal communities of Warren Prairie Natural Area in southeast Arkansas, USA, we isolated several strains that matched the morphological characteristics of the genus *Diplosphaera* from the biotic crust of a sodic–saline slick. Phylogenetic analysis of DNA sequences from the ribosomal RNA internal transcribed spacer regions (ITS) and the plastid rbcL gene indicated that the Warren Prairie strains are a new species. This conclusion was also supported by analysis of the secondary structure of the ITS2. All of our results support the description of the new species, *Diplosphaera sundellii*.

Key words: Chlorophyta, *Diplosphaera*, new species, phylogeny, sodic–saline soil, Trebouxiophyceae

Introduction

Diplosphaera Bialosuknia emend Vischer (Chlorophyta; Trebouxiophyceae) is a very small green alga (2–6μm) that is widely distributed in soil (Lewis & Flechtner 2002, as *Stichococcus* sp.; Lewis & Lewis 2005, as *Stichococcus* sp.; Mikhailyuk et al. 2019), subaerial habitats (tree bark) (Medwed et al. 2021), building stones (Hallmann et al. 2013), freshwater (strain isolated by E. Hegewald, Lake Batran in Bali (Pröschold & Darienko 2020), halophilic environments (Sommer et al. 2020), polar habitats (Broady 1982) and as a lichen photobiont (e.g., Thüs et al. 2011, Fontaine et al. 2012, 2013). Cells of *Diplosphaera* are spherical or nearly so and are often grouped in short chains (Ettl & Gärtner 2013). Bialosuknia (1909) was the first to describe the genus *Diplosphaera*. He isolated the alga from the lichen *Lecanora tartarea* collected from the Salève limestones, just south of Geneva, Switzerland. The original diagnosis of the type species, *Diplosphaera chodatii* Bialosuknia, described small, nearly spherical cells with parietal chloroplasts and no pyrenoid. The cells were either solitary or occurred in a pair or even 3–5 individual cells grouped in a chain. Vegetative cell division typically produced irregular, curving chains of cells. Rarely, cells may divide to form 4–celled coenobia or "packets." A second species, *Diplosphaera mucosa*, was described by Broady (1982) and differs from *D. chodatii* by the presence of a mucilaginous layer.

The paucity of definitive morphological features in the genus *Diplosphaera* has made it difficult to describe new species. Pröschold & Darienko (2020) examined several strains of *Diplosphaera* and determined that they could not be separated into different species based on morphology. Moreover, the ITS2 region of the nuclear ribosomal RNA cistron did not vary enough for species delimitation; specifically, there were no compensatory base changes (CBCs) found for the ITS2 among the strains they evaluated. Pröschold & Darienko (2020) therefore reduced the genus to the single species, *D. chodatii*, with one additional variety, *D. chodatii* var. *mucosa* (Broady) Pröschold et Darienko. However, studies of *Diplosphaera* strains from lichens (Thüs et al. 2011; Fontaine et al. 2012, 2013) suggest that there is a large diversity of *Diplosphaera* still awaiting taxonomic evaluation.

Warren Prairie Natural Area (WPNA) in Bradley and Drew counties, Arkansas, USA, comprises a strange mosaic that includes sodic–saline slicks that form flat, crusty depressions in a central area with a zone of lichens and a few rare angiosperms, and an outer zone of cyanobacterial mats with some angiosperms (U.S. Fish and Wildlife Service 1993). The sodic–saline slicks are surrounded by prairie grasses that are flanked by pine

flatwoods, often with an understory of dwarf palmetto (*Sabal minor*). The area is home to several rare plants, including the diminutive angiosperm, *Geocarpon minimum* (Caryophyllaceae), which is tracked as an endangered species. We hypothesized that the soil algal community of the sodic–saline slicks were likely to harbor a unique soil algae community as suggested by the unusual soil chemistry and presence of numerous rare vascular plants. A report by Pittman (U.S. Fish and Wildlife Service, 1993) provides a summary of the soil chemistry and geology of the slicks in WPNA. For this study, we used morphological and molecular techniques to characterize *Diplosphaera* strains that we isolated from a WPNA sodic–saline slick. Based on our results, we determined that our WPNA strains are a new species of *Diplosphaera*.

Materials and Methods

Strain isolation. Soil samples were collected from two portions of a sodic–saline slick in WPNA (GPS: 33.5825 N; 91.9833 W) on February 17, 2016. Site WPWO is on the south side of the slick (*Geocarpon minimum* is not present). Site WPW is on the north side of the same slick (*Geocarpon minimum* is present). Three soil samples were collected at each site from: 1) the inner slick; 2) mid–crust layer with lichens and a few angiosperms; and 3) the outer layer of algal mats.

For each soil sample, 0.5 grams of soil was mixed with 50 ml of Woods Hole modified (WH+) medium (Fawley et al. 2013), and shaken well. The mixture was allowed to settle for 10 seconds and 100 µl of the supernatant was spread onto a WH+ agar plate. The plates were incubated under cool white fluorescent light until colony growth appeared on the plates. Individual algal colonies were picked from the plates and maintained on agar slants.

An additional soil sample was taken for chemical analysis from the mid–crust region of Site 2. Standard chemical analyses were performed by Ward Laboratories, Kearney, Nebraska, USA (Supplemental Table S1).

Phylogenetic analysis. *Diplosphaera* strains WPW3–1, WPWO2–8, WPWO2–11, WPWO2–17 and WPWO2–24 from WPNA were grown in liquid WH+ medium as described above. DNA was extracted using the technique of Fawley & Fawley (2004), which was modified by the addition of a step to disperse any mucilage that might adhere to the cells. In this method, we harvested the cells by centrifugation, added 200 µl of extraction buffer and agitated the cells with a MiniBeadBeater at high speed for 10 to 15 sec. We pelleted the cells again by centrifugation and added new buffer and proceeded with the normal method of Fawley & Fawley (2004). The 18S rRNA gene was amplified using the Polymerase Chain Reaction (PCR) with the primers 18L–X and NSI–X (Phillips & Fawley 2000) with PCR conditions described in Fawley & Fawley (2004). The internal transcribed spacer region (ITS) was PCR amplified using the primers ITS1 (White et al. 1990) and ITS–BR (Johnson et al. 2007) with annealing temperature at 61°C or Sticho–ITS–F (Fontaine et al. 2012) or ITS5 (Bertini et al. 1999) coupled with ITS4 (White et al. 1990) at 58.5 °C. The plastid rbcL locus was amplified using the primers rbcL 1–20 (Nozaki et al. 1995) and rbcL DesR (Fawley et al. 2011). Sequences were generated directly from the PCR product using the primers used for PCR and the additional primer NS3 (White et al. 1990) for sequencing the 18S gene. Sequencing was performed by Sequetech (Mountain View, CA, USA). Sequence reads were joined using the Staden Package 2.0.0b8 (Bonfield et al. 1995). All sequences were deposited in GenBank with accession numbers OQ296610–2 (18S), OQ298827–31 (ITS) and OQ303887–8 (rbcL).

An ITS alignment was constructed using *Diplosphaera* sequences of Pröschold & Darienko (2020) that included ITS1, 5.8S and ITS2 regions and additional *Diplosphaera* lichen photobionts strains that represented the diversity of photobionts revealed by a BLASTN search. Sequences from *Tetratostichococcus jenerensis* (MT078183) and *Deuterostichococcus epilithicus* (MT078169) were selected as outgroup taxa as they are the closest known relatives of *Diplosphaera* (Pröschold & Darienko 2020). These sequences were aligned with the sequences from the WPNA strains using the LocARNA (Will et al. 2012) server at the Freiburg RNA Tools web site (https://rna.informatik.uni–freiburg.de/). LocARNA produces a multiple sequence alignment based on a consensus secondary structure of the RNA. FastGap (Borchsenius 2009) was also used to produce a version of the alignment with additional binomial characters representing indels. A rbcL alignment was also prepared with MEGA X (Kumar et al. 2018), using sequences taken from a BLAST search in GenBank and aligned using the MUSCLE (Edgar 2004) feature of MEGA X.

Maximum likelihood analyses of the alignments used IQ–Tree v 2.2 (Minh et al. 2020) with standard bootstrap analysis (500 iterations). Several analyses of the ITS region were performed to evaluate the effects of partitioning, model selection and inclusion of indel and 5.8S rDNA data. The GTR+F+R4 model of substitution was used as the standard substitution model, in accordance with Abadi et al. (2019), who suggested that a parameter–rich GTR model will produce results that are equivalent to the results of analyses using the models selected by various model selections programs (e.g., jModelTest 2 (Darriba et al. 2012; ModelFinder (Kalyaanamoorthy et al. 2017)).

The initial analysis of ITS data included the entire ITS1, 5.8S rDNA and ITS2 data set using the GTR+F+R4 model. Another analysis was performed with the ITS1 and ITS2 as separate partitions, as implemented in IQ–Tree 2 (Chernomor et al. 2016) and excluding the 5.8S data. ModelFinder was used to assess the best–fit models of substitution, which were TN+F+R4 for ITS1 and TPM3+F+R4 for ITS2. A third analysis used the complete ITS1, 5.8S rDNA and ITS2 data set with concatenated presence/absence indel data from FastGap included as a separate partition. The rbcL data set was used for two maximum likelihood analyses, the first analysis with IQ–Tree v2.2 using the GTR+F+R4 model and the second analysis using the TIM+F+G4 model selected by ModelFinder. All results were evaluated with standard bootstrap analysis with 500 replicates. Phylogenetic trees were visualized with iTOL (Letunic & Bork 2021) and finished using Inkscape (Inkscape.org) and Paint.net (getpaint.net).

ITS2 secondary structure was generated using Mfold (Zuker 2003) and visualized with Forna (Kerpedjiev et al. 2015) incorporated in the ViennaRNA Web Services (Gruber et al. 2008). Final annotations were made using Inkscape and Paint.net.

Light Microscopy. For differential interference contrast microscopy, we used a Nikon E600 microscope (Nikon, Melville, New York, USA) equipped with 60× and 100× Plan Apochromat objectives (numerical aperture 1.4 or 1.45). Digital images and measurements were captured with an Olympus SC180 camera system with CellSens imaging software (Olympus America, Center Valley, Pennsylvania, USA).

Results

Soil chemistry analysis of the sodic–saline slick in WPNA indicated a pH of 7.3. The dominant exchange cations were sodium (326 ppm), magnesium (91 ppm), calcium (314 ppm) and potassium (41 ppm). Complete results of the soil analysis are given in Supplemental Table S1. Values are all consistent with the characteristics of sodic–saline soils and other studies of WPNA slicks (U.S. Fish and Wildlife Service, 1993).

The partial 18S rDNA sequences generated from strains WPW3–1, WPWO2–8 and WPWO2–24 were all identical, with a maximum of 1818 bases, including a 446 base putative group I intron. The sequence of our strains (including intron and with intron removed) was used for a BLASTN search, with the highest similarity found for a *Stichococcus* sp., strain MBIC 10465 (now NBRC 102782; AB183601) with the source given as sediment, Pacific Ocean, Okinawa, and an uncultured 18S clone (EF591011) from a benthic sample of highly acidic Rio Tinto in Spain (Aguilera et al. 2007). These sequences were included in the *Diplosphaera* clade by Hodač et al. (2016). The 18S rDNA of both of these sequences differed from the WPNA sequence by a single substitution, but they also lacked the intron found in the Warren Prairie strains. The authentic reference sequences most similar to our sequence were all *Diplosphaera chodatii* (strains SAG 2.82, SAG 9.82, SAG 11.88 and SAG 49.86), which differed from WPWO2–8 by 2 substitutions and 1 indel. These strains also did not have the intron. *Diplosphaera chodatii* strain SAG 48.86 18S rDNA differed from the WPNA sequence by 3 substitutions with no indels, but SAG 48.86 includes an intron similar to that of the WPNA strains. The 18S sequence of another authentic *D. chodatii* strain, SAG 2049, differed from our sequence by 4 substitutions with no indels. This strain did not have the intron. The 18S rDNA of the single species of the genus sister to *Diplosphaera*, *Tetratostichococcus jenerensis*, differs from our sequence by 7 substitutions. These results indicate that our strains are placed in the genus *Diplosphaera*.

Sequencing the complete ribosomal ITS region (including the 5.8s rDNA) of the ribosomal RNA cistron and the plastid rbcL locus resulted in identical sequences for all WPNA strains. Phylogenetic analysis of the ITS sequences with *Tetratostichococcus jenerensis* and *Deuterostichococcus epilithicus* as outgroup and employing the GTR+F +R4 substitution model is shown in Fig. 1. Partitioning the ITS1 and ITS2, eliminating the 5.8 S rDNA region of the alignment and using substitution models selected by ModelFinder did not alter the topology of the tree, except for a slight repositioning of UTEX 1177, which was never grouped with other strains with bootstrap support. Bootstrap support was always within 4 percentage points of the values in Fig. 1. Another analysis with indel data included as a separate partition also did not

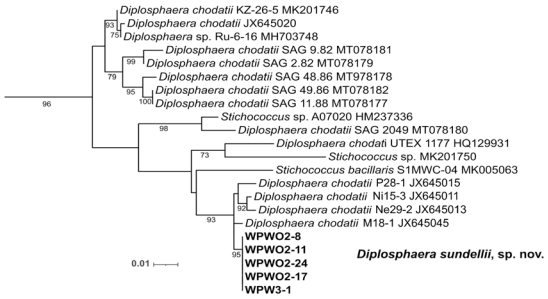


Fig. 1. Results of Maximum–Likelihood phylogenetic analysis of the nuclear ribosomal ITS1–5.8S–ITS2 region using the GTR+ F+R4 model. The outgroup taxa, *Tetratostichococcus jenerensis* (MT078183) and *Deuterostichococcus epilithicus* (MT078169) are not shown due to long branches. Bootstrap values greater than 70 are indicated.

vary from Fig. 1 by more than 4 percentage points in bootstrap support values. The WPNA strains were allied with *D. chodatii* and the full ITS sequences of the WPNA strains differed from reference *D. chodatii* sequences by 23 to 31 substitutions in our alignment. Four *Diplosphaera* strains (JX645011, JX645013, JX645015 and JX645045) are representative of a large group of sequences in GenBank (Supplemental Table S2) that form a well–supported lineage with our WPNA strains. The four strains differ in ITS sequences from the sequence of the WPNA strains by only 4 to 6 substitutions and required at most one or two single nucleotide gaps to align. The strains included in the analysis were all photobionts of the saxicolous aquatic lichen, *Dermatocarpon luridum* var. *luridum* (Fontaine et al. 2012).

The analysis of plastid rbcL sequence data (Fig. 2) revealed a tree structure similar to that found using nuclear ITS sequence data. Analysis with the GTR+ F+R4 and the TIM+F+G4 models both produced the same tree, with bootstrap support values all within 4 percentage points. Our WPNA *Diplosphaera* strains were part of a lineage that is separate from the *D. chodatii* strains, with bootstrap support. This lineage included only one other *Diplosphaera* strain (JN573860), although the bootstrap support for this grouping was low (73%). A separate lineage comprised 3 strains (JN573833, JN573847 and JN573824), all photobionts from lichen species in the family Verrucariaceae (Thüs et al. 2011). The *D. chodatii* strain SAG 2049 was also separate from the extensive lineage that included the type strain of *D. chodatii*, SAG 49.86. Additional GenBank sequences from photobionts in lichens of the Verrucariaceae were very similar to the type strain of *D. chodatii* and formed a well–supported lineage that also included the *D. chodatii* strains SAG 11.88, SAG 49.86 (type strain of *D. chodatii*) and SAG 2.82. The partial rbcL sequence from our WPNA strains differed from the type strain of *D. chodatii* by 23 substitutions.

Differences in the ITS2 secondary structure have been shown to provide strong evidence for

delineation of new species, particularly compensatory base changes (CBCs) present in the helices (COLEMAN 2003). The complete secondary structures of the ITS2 for the WPNA strains and *D. chodatii* strain SAG 49.86 are shown in Supplemental Fig. S1. The secondary structure includes the normal 4 helices. There are two major differences revealed by the secondary structure. There are a large number of indels (12) between the two strains, including two 2 bp indels that are paired in the stem region of helix 2, indicated in Fig. 3. There is also a compensatory base change in helix 4 (Fig. 3); however, this CBC is only present when comparing SAG 11.88, SAG 49.86 and SAG 48.86 to the WPNA strains. For other strains of *D. chodatii* this region is a hemi–CBC when compared to the WPNA strain secondary structure.

Light microscopy indicated that the WPNA *Diplosphaera* strains are solitary or in pairs in liquid culture; however, on agar they can form chains and irregular "packages" of cells, but not regular coenobia (Figs 4–9). In liquid culture, small cells are nearly spherical (Figs 4–5), but become more ellipsoidal as they increase in size. Cell size ranged from 2.5 µm to 3.5 µm in width, and 3.0 µm to 7.0 µm in length. Larger cells were occasionally observed (Fig. 4). A thin cell wall without any presence of mucilage was observed. The chloroplasts were parietal with 1 cup–shaped chloroplast in young cells, and 2 elliptical chloroplasts in larger ellipsoidal cells, probably in preparation for cell division (Fig. 5). Lipid bodies were rarely observed in small cells but were frequently encountered in larger ellipsoidal cells (Fig. 4). Reproduction is by fission with two spherical cells produced (Fig.5). This morphology was fairly consistent across the WPNA *Diplosphaera* strains that we examined in liquid culture. However, strain WPW3–1 cells were slightly longer (up to 7.0 µm) compared to the other strains (up to 5.2 µm).

In older cultures grown on agarized medium, cells form larger cells with thick cell walls that form irregular packets or rarely short chains (Figs 6–7). These cells have numerous inclusions (Figs 6–7) that were identified as lipid bodies in *D. chodatii* by Vischer (1960). Upon transfer to new agarized medium, these enlarged cells undergo a process of sporulation to produce smaller cells similar to those grown in liquid medium (Figs 8–9). Three days after the transfer of a three–month old culture of strain WPW3–1 to new agarized medium, most large cells (Fig. 8) have divided within the confines of the thick mother cell wall to produce two smaller ellipsoidal cells. After 4 days on the new medium (Fig. 9), the cells have divided again, resulting in cells similar in size and shape to cells grown in liquid medium. The old, thickened mother cell walls of the sporangium are clearly visible (Figs 8–9).

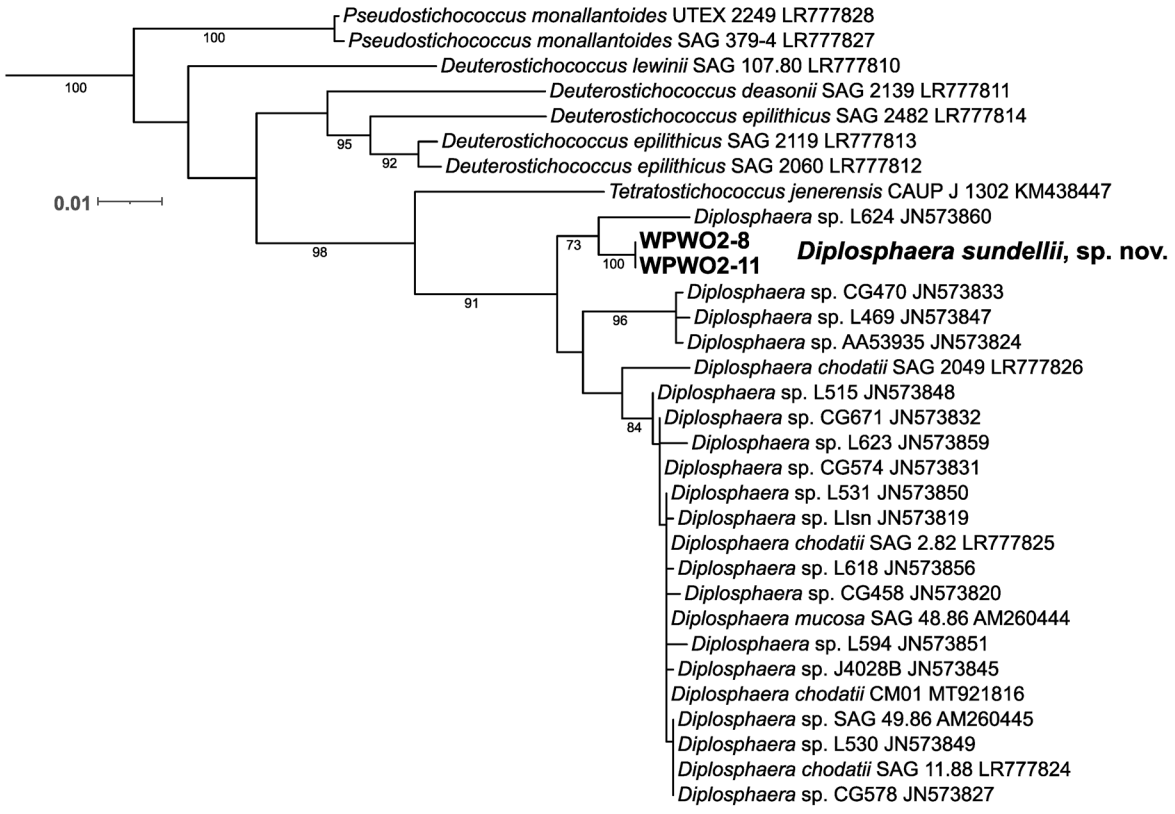


Fig. 2. Results of Maximum–Likelihood phylogenetic analysis of a partial plastid rbcL DNA sequence data set using the GTR+ F+R4 model. The tree was rooted using two sequences from *Desmococcus olivaceus* (LR777807, LR777808) as outgroup (not shown). Bootstrap values greater than 70 are indicated.

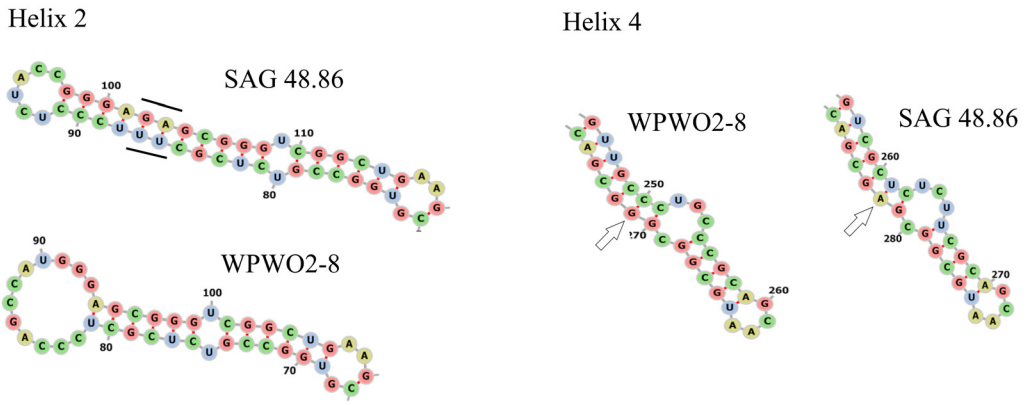


Fig. 3. Main features of the ITS2 secondary structures for *Diplosphaera chodatii* (type strain SAG 49.86, MT078182) and *D. sundellii*, sp. nov. (type strain WPWO2–8). The region on helix 2 that includes two paired insertions in SAG 49.86 compared to *D. sundellii* is shown on the left and the single CBC present in helix 4 is indicated. Full secondary structures are presented in Supplementary Fig. S1.

Description of new taxon

***Diplosphaera sundellii* K.N. Thermozier, M.W. Fawley et K.P. Fawley sp. nov. (Figs 4-9)**

Description: Cells solitary or in pairs in liquid culture or chains and irregular "packages" of cells on agar. In liquid culture, cells are nearly spherical to ellipsoidal, 2.5–3.5 µm (width) × 3.0–7.0 µm (length). Larger cells occasionally observed. Cell wall thin without mucilage. Chloroplast parietal with 1 cup–shaped chloroplast in young cells. Two elliptical chloroplasts in larger cells. Lipid bodies rarely observed in small cells but frequently encountered in larger ellipsoidal cells. Reproduction by fission with two spherical cells produced. In older cultures grown on agar, cells form irregular packets or rarely short chains of large cells (up to 6.1 × 9.8 µm) with thick cell walls and numerous lipid bodies. The enlarged cells undergo sporulation and produce smaller cells when transferred to new agarized medium.

Holotype: Permanent slide of strain WPWO2–8, deposited in the Arkansas State University Herbarium (STAR), State University, Arkansas, USA as accession STAR037512.

Type locality: Warren Prairie Natural Area, Bradley County, Arkansas, USA; 33.5825˚ N, 91.9833˚ W.

Habitat: Soil surface; biotic crust at edge of sodic–saline slick.

Etymology: "*sundellii*" is an epithet that honors Dr. Eric Sundell, Professor Emeritus of Biology at The University of Arkansas at Monticello, for his botanical research in Warren Prairie Natural Area.

Reference strain: WPWO2–8 culture maintained at The University of the Ozarks, Clarksville, AR, USA and deposited in the CCMP collection of the National Center for Marine Algae and Microbiota, East Boothbay, Maine, USA as CCMP3705.

Material analyzed: WPNA strains: WPW3–1, WPWO2–8, WPWO2–11, WPWO2–17, and WPWO2–24.

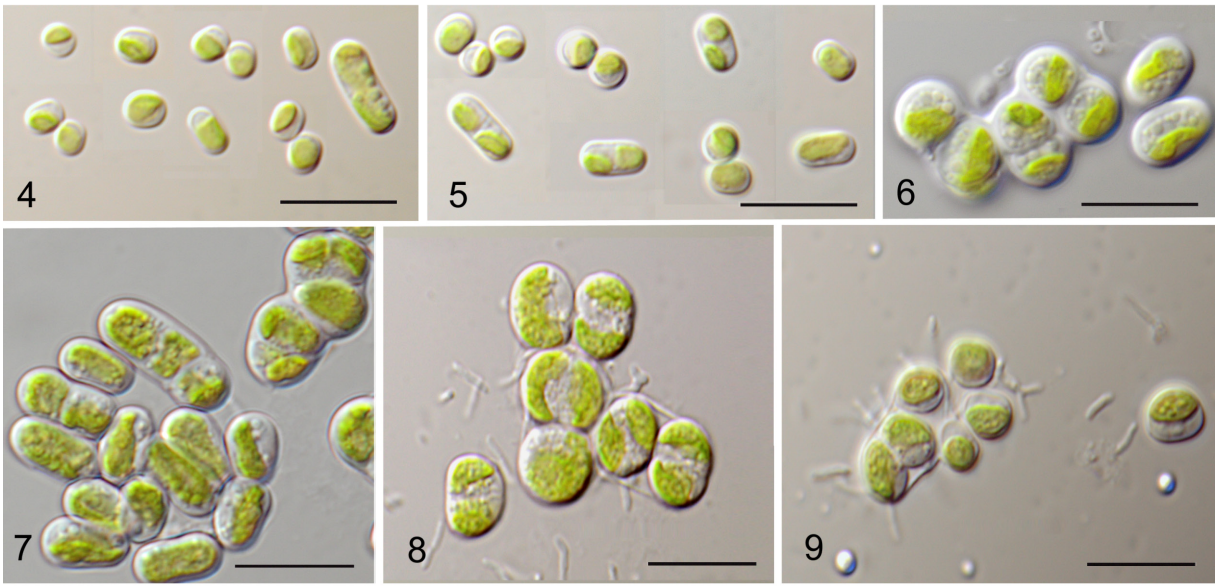
GenBank accession numbers: OQ296610 (nuclear18S rDNA), OQ2988231 (nuclear ribosomal ITS) and OQ303887 (plastid rbcL).

Discussion

The morphology of the new species *Diplosphaera sundellii* from WPNA is consistent with the features of the genus *Diplosphaera* as described by Bialosuknia (1909) and emended by Vischer (1960). These features include vegetative cell division by fission with the production of small spherical cells that frequently remain in pairs. There is also a distinct morphological difference between cells grown in liquid versus agar. Finally, *D. sundellii* produces sporangia that release small aplanospores when grown on agar. This process is identical to the sporulation in the emended description of the type species, *D. chodatii*, by Vischer (1960). Overall, the morphological features of *D. sundellii* are similar to *D. chodatii* with the exception of the production of elongated cells by a strain of the new species. However, Pröschold & Darienko (2020) showed that different strains of *D. chodatii* can vary somewhat in the production of elongated cells, so this is likely a somewhat plastic feature of the genus.

Although the 18S rDNA sequence of *D. sundellii* supports the placement of our new species in the genus *Diplosphaera*, it provides little taxonomic evidence at the species level. Therefore, we examined both the ribosomal ITS region and the plastid rbcL locus.

The ITS region, including ITS1, 5.8S and ITS2, differed from the type strain of *D. chodatii* by 23 substitutions. This large difference in ITS regions suggests that the WPNA strains represent a new species. Phylogenetic analysis of published *Diplosphaera* complete ITS regions and the WPNA strains (Fig. 1) support the monophyly of the genus and show that the WPNA strains are in a well–supported lineage that also includes strains isolated from the lichen, *Dermatocarpon luridum.* Other features of the ITS tree are similar to the results of Pröschold & Darienko (2020), except that there are longer branches in our tree, perhaps because we used the complete ITS region of *Diplosphaera* strains and closely related outgroup taxa. In contrast, Pröschold & Darienko (2020) used a combined analysis of ITS2



Figs 4–9. Light microscopy of *D. sundellii*, sp. nov. strains: (4) strain WPWO2–8 grown in liquid medium, young culture; (5) strain WPW3–1 in liquid medium, young culture; (6) WPWO2–8 grown on agarized medium, old culture; (7–9) series showing the formation of aplanospores in strain WPW3–1; (7) old culture of WPW3–1 on agar; (8) cells 3 days after transfer to new agar medium, showing the early stage of aplanospore formation; (9) cells 4 days after transfer to new medium showing release of small aplanospores.

and 18S data of the *Stichococcus* group. Alignment of ITS data can be very difficult over multiple genera and it is likely that species–level information was lost in their more comprehensive analysis. Our results also suggest that other *D. chodatii* strains, such as SAG 2049 and UTEX 1177, represent additional new species of *Diplosphaera*.

Phylogenetic analysis of a 940 base pair portion of published *Diplosphaera* rbcL sequences and our WPNA strains also indicate monophyly of the genus. The WPNA strains are in a well–supported lineage that also includes strain L624 isolated from the lichen, *Agonimia repleta* in the family Verrucariaceae. The strain MPN178 (JN573842) was isolated from the lichen, *Dermatocarpon luridum* and the rbcL sequence is nearly identical (not shown) to that of the L624 strain (THÜS et al. 2011). This strain was not included in our analysis because the sequence was short. Three other strains, CG470 isolated from *Endocarpon pusillum*, L469 from *Agonimia tristicula* and AA53935 from *Staurothele frustulenta*, form a well–supported lineage separate from the larger group of *D. chodatii* strains including the type species (SAG 49.86) and *D. chodatii* var. *mucosa* (SAG 48.86). Our results are completely consistent with those of THÜS et al. (2011) and support the erection of the new species, *D. sundellii*. Moreover, this analysis also indicates that more work needs to be done to characterize other potential new species of *Diplosphaera*.

Phylogenetic analysis of both data sets utilized the GTR+F+R4 model of substitution and these results were compared to the results obtained with models selected by ModelFinder in IQ–Tree2. No substantive differences in tree topology or bootstrap support were observed for both loci. These results support the findings of ABADI et al. (2019) that the use of a parameter rich GTR model is a good alternative to model selection by existing software. In addition, we determined that there was no need to partition the ITS1 and ITS2 regions and eliminate the 5.8S region from our analysis, nor was there any benefit from adding indel data generated by FastGap.

The ITS2 secondary structure for *D. sundellii* also supports the new species (Fig. 3). Although a comparison of the secondary structure of *D. sundellii* to the type strain for *D. chodatii* indicates a CBC, this CBC is present as a hemi CBC in other strains of *D. chodatii*. However, the presence of two 2–base indels in the regions that form helix 2 is a very significant difference between the two secondary structures.

Our basic philosophy for naming new species requires multiple lines of evidence including the use of multiple genes or DNA regions for a robust species description (FAWLEY et al. 2011). Together, the nuclear 18S rDNA, nuclear rDNA ITS regions and plastid rbcL sequence analyses, as well as the ITS2 secondary structure, provide very strong evidence for the new species *D. sundellii*. This is despite the fact that there are essentially no morphological differences between *D. sundellii* and *D. chodatii.* In addition, the phylogenetic analyses of ITS and rbcL sequence data from GenBank strongly suggest that more species of this genus await characterization. The habitat of *D. sundellii*, the biotic crust of a sodic–saline slick in Warren Prairie, is very unusual, with high levels of sodium ions that cause the breakdown of clay aggregates into fine dust. In addition, the hydrology of the slick is such that a hardpan is formed below the surface. This hardpan prevents the movement of water either upward to the surface during dry periods or downward

during wet periods (U.S. Fish and Wildlife Service 1993). These conditions result in a hydroxeric condition, where the soil surface is extremely hot and dry during summer and fall, but submerged in water during periods of heavy rainfall in winter and spring (Foti & Witsell 2013). *Diplosphaera sundellii* appears very well adapted for these conditions. The life cycle is such that the very small, thin– walled cells can reproduce very rapidly by binary fission during wet periods, but then likely form a quite different morphology during hot, dry periods, represented by the morphology that we observed for old cultures grown on agar. These larger cells have thick walls, accumulate a large number of lipid droplets in the cytoplasm, and develop short filaments or clumps of cells. All these features are adaptations to reduce desiccation (Holzinger & Karsten 2013). However, when rain returns and the slick is flooded, the large cells can undergo sporulation to return to the small, thin–walled morphology and reproduce rapidly.

The habitat of *D. sundellii* in Warren Prairie strongly resembles desert biotic crusts, and *Diplosphaera* has been described from desert biotic crusts in North America (Lewis & Flechtner 2002; Lewis & Lewis 2005; Flechtner et al. 2013). However, the 18S rDNA sequences for the two strains from Lewis & Lewis (2005) differ from the sequence of *D. sundellii* and they are not likely to be the same species. Both ITS and rbcL data are not available for these strains. However, some lichen photobionts are linked to *D. sundellii* by phylogenetic analysis. The 4 ITS strains grouped with the WPNA strains were derived from the lichen photobiont *Dermatocarpon luridum*. Three of these strains (FontaineNi15–3 JX645011; FontaineNe29–2 JX645013; FontaineP28–1 JX645011) were from lichens growing on granite boulders along the margins of lakes in the Mistik Creek watershed in Manitoba, and 1 strain (FontaineM18–1 JX645045) was from the same habitat in the Austrian Waldaist, a tributary of the River Danube (Fontaine et al. 2012). The ITS region sequences of these strains differ by only 4–6 substitutions compared to the sequence from *D. sundellii*, which suggests that they could be included in *D. sundellii*. However, the habitats of sodic–saline slick and lichen photobiont appear to be so different that the photobionts may be a distinct species or variety. For now, we recommend referring to these four photobionts as *Diplosphaera* cf. *sundellii*, as we have suggested for identifications without microscopy (Fawley & Fawley 2020). A list of some GenBank sequences that we feel should receive this designation is given in Supplemental Table S2. The breadth of this list suggests that *D. sundellii* may be a common lichen photobiont. Moreover, the results of rbcL sequence analysis also suggest that a lichen photobiont is closely related to *D. sundellii* (Fig.2). Strain L624 (JN573860) is a photobiont of *Agonimia repleta* and was collected from sand pebbles in Uličské Krivé, Poloniny, Slovakia (Thüs et al. 2011). In this case, there is enough difference between the *D. sundellii* sequence and JN573860 to suggest that these strains may be different species. Strain L624 should be listed as *Diplosphaera* sp. instead of the current *D. chodatii*. At first glance, the environments of biotic crust and lichen photobiont seem quite different. It seems unlikely that these habitats would be home to very similar (or the same) species of *Diplosphaera*. However, both the sodic–saline slicks and the lichens harboring these photobionts undergo dramatic fluctuations in moisture levels. These dynamic ecological factors may govern the success of *Diplosphaera sundellii* and related species.

Much work remains to properly define additional species of *Diplosphaera*. Over 150 ITS sequences from *Diplosphaera* are available in GenBank; however, many of them are not represented by an actual culture. There are also some rbcL sequences, but the ITS data and rbcL data are mostly from different sources. In order to produce a robust species–level phylogeny and define species boundaries, both the complete ITS region and rbcL sequence data should be used, along with habitat and biogeography data.

This paper is the first in a series of studies of the soil algae of Warren Prairie Natural Area. We have evidence for a number of additional new species, as well as new distribution records for other unusual algae which will be presented in future papers.

After this paper was completed, we learned of the additional new species of *Diplosphaera*, *D. elongata* Chiva & Barreno (Chiva et al. 2023). *Diplosphaera elongata* is in a lineage that includes the strain S1MWC–04 in our analysis of ITS data (Fig. 1) and is well separated from *D. sundellii*. Thus, there are now 3 species of *Diplosphaera*.

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Supplementary material

The following supplementary material is available for this article:

Fig. S1. The ITS2 secondary structures for *Diplosphaera chodatii* (Type strain SAG 49.86, MT078182) and *D. sundellii* sp. nov. (strain WPWO2–8).

Table S1. Soil chemical analysis from sodic-saline slick in Warren Prairies Natural Area.

Table S2. Partial list of ITS sequences that are likely *D. sundellii*.

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

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