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NOTE

RESOLVING THE PHYLOGENETIC RELATIONSHIP BETWEEN *CHLAMYDOMONAS* SP. UWO 241 AND *CHLAMYDOMONAS RAUDENSIS* SAG 49.72 (CHLOROPHYCEAE) WITH NUCLEAR AND PLASTID DNA SEQUENCES[1]

Marc Possmayer

Department of Biology, University of Western Ontario, London, Ontario, Canada N6A 5B7

Rajesh K. Gupta

Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1

Beth Szyszka-Mroz, Denis P. Maxwell, Marc-André Lachance, Norman P.A. Hüner, and David Roy Smith[2]

Department of Biology, University of Western Ontario, London, Ontario, Canada N6A 5B7

The Antarctic psychrophilic green alga *Chlamydomonas* sp. UWO 241 is an emerging model for studying microbial adaptation to polar environments. However, little is known about its evolutionary history and its phylogenetic relationship with other chlamydomonadalean algae is equivocal. Here, we attempt to clarify the phylogenetic position of UWO 241, specifically with respect to *Chlamydomonas raudensis* SAG 49.72. Contrary to a previous report, we show that UWO 241 is a distinct species from SAG 49.72. Our phylogenetic analyses of nuclear and plastid DNA sequences reveal that UWO 241 represents a unique lineage within the Moewusinia clade (sensu Nakada) of the Chlamydomonadales (Chlorophyceae, Chlorophyta), closely affiliated to the marine species *Chlamydomonas parkeae* SAG 24.89.

Key index words: **Chlamydomonas; cold adaptation; green algal phylogeny; Lake Bonney; Psychrophile**

***Abbreviations*: BS, bootstrap; ITS, internally transcribed spacer; *rbcL*, RUBISCO large subunit; SAG 49.72, *Chlamydomonas raudensis* Ettl SAG 49.72; UWO 241, *Chlamydomonas* sp. UWO 241**

Chlamydomonas sp. UWO 241 (hereafter UWO 241) is a photoautotrophic and psychrophilic unicellular green alga from the chlorophycean class of the Chlorophyta (Leliaert et al. 2012). It was isolated from water collected deep (17 m) within Lake Bonney—a perennially ice-covered lake in the McMurdo Dry Valleys of Antarctica (Neale and Priscu 1995). UWO 241 is rapidly becoming a model for investigating cold adaptation in photosynthetic eukaryotes (Dolhi et al. 2013), and has already been examined intensively with respect to the structure and function of its photosynthetic apparatus (reviewed in Morgan-Kiss et al. 2006) as well as its response to nonpermissive growth temperatures (Possmayer et al. 2011).

There is no doubt that UWO 241 belongs to the Chlamydomonadales, a well-studied order of mainly freshwater flagellates within the Chlorophyceae (Pocock et al. 2004, Leliaert et al. 2012). But its position within the Chlamydomonadales is equivocal. Cell morphological analyses initially led researchers to identify UWO 241 as *Chlamydomonas subcaudata* Wille (Neale and Priscu 1995). It was then reassigned to *Chlamydomonas raudensis* based on nuclear gene data indicating that the 5.8S rDNA, ITS1, and ITS2 sequences of UWO 241 are identical to those of *C. raudensis* Ettl SAG 49.72, hereafter SAG 49.72 (Pocock et al. 2004; the isolate UWO 241 is synonymous with CCAP 11/131, and the isolate SAG 49.72 is synonymous with CCCryo 212-05). Nevertheless, further molecular sequence analyses, including comparisons of various protein-coding gene sequences, as well as Differential Display (M. Possmayer, unpublished data) and Random Amplification of Polymorphic DNA (RAPD; Gupta 2013) indicated that SAG 49.72 and UWO 241 are not the same species. Given the importance of UWO 241 in studies of cold adaptation, it is essential that researchers have a clear understanding of its evolutionary history and position within the green algal tree of life.

To accurately assess the relationship among UWO 241, SAG 49.72, and other chlamydomonadalean algae, we sequenced large amounts of DNA and

[1]Received 28 April 2015. Accepted 22 November 2015.
[2]Author for correspondence: e-mail dsmit242@uwo.ca.
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RNA from the nuclear and plastid genomes of UWO 241 and used these data for phylogenetic analyses. We computed phylogenies using nucleotide sequences encoding nuclear rDNA (18S and 28S) and the plastid-encoded *rbcL* gene. Together, these data strongly support the view that UWO 241 is a distinct species from SAG 49.72 and that it is positioned within the Moewusinia clade (sensu Nakada; Nakada et al. 2008) of the Chlamydomonadales, in a group that includes the marine species *Chlamydomonas parkeae* SAG 24.89.

Strains UWO 241 and SAG 49.72 were cultured in thermo-regulated aquaria in Suoeka's HS Medium with 10 mM sodium chloride (HS10), as described previously (Possmayer 2009, Possmayer et al. 2011). For light microscopy, cultures were grown in Bold's Basal Medium (for SAG 49.72) or Bold's Basal Medium amended with 0.07 M sodium chloride (for UWO 241; Nichols and Bold 1965). Light microscopy was performed using a Zeiss Axioimager Z1 microscope (Carl Zeiss AG, Oberkochen, Germany) at the Integrated Microscopy Facility, The Biotron, Western University.

Nucleic acids were extracted using a modified CTAB-chloroform method (Appendix S1 in the Supporting Information). DNA and RNA were selectively purified using isopropanol, lithium chloride, and ethanol precipitation techniques (Appendix S1). The ITS (5.8S rDNA, ITS1, and ITS2), partial 18S rDNA (of SAG 49.72) and D_1–D_2 regions of the 28S ribosome were amplified from whole genomic DNA by PCR (White et al. 1990, Pocock et al. 2004). Gel-purified DNA (Bio-Basics Gel Extraction Kit: Bio Basic, Markham, ON, Canada) was sequenced using an Applied Biosystems 3730 Analyzer at the Robarts Research Institute, University of Western Ontario.

The DNA sample from UWO 241 used for Illumina sequencing was gel purified and ethanol precipitated before library preparation (Appendix S1). The Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA) was used, following manufacturer's instructions, to fragment DNA and ligate sequencing adapters. The RNA samples were treated with DNase I and column purified before library preparation (Appendix S1). The Epicentre ScriptSeq v2 kit (Illumina) was used, following manufacturer's instructions, to prepare the RNA library. Libraries were paired-end sequenced (250 nt reads) on an Illumina Mi-Seq platform at the London Regional Genomics Centre (London, ON, Canada).

The Mi-Seq data were assembled using the De Novo Assembly tool of CLC Genomics workbench (version 6.5.1: CLC Bio, Aarhus, Denmark) using the default settings. The sequence of the plastid-encoded gene *rbcL* (KP981642) was identified in the UWO 241 RNA-Seq contig data with tBLASTn, using the homologous *Chlamydomonas reinhardtii* protein sequence as the query (Altschul et al. 1997; Table S1 in the Supporting Information details GenBank submissions related to this note). The UWO 241 nuclear rDNA sequence (KP313859) was identified in the DNA-Seq contigs with BLASTn (Altschul et al. 1997) using its previously published 18S rDNA sequence as the query.

Phylogenetic analyses were conducted using MEGA6 (Tamura et al. 2013). Sequences were aligned using Muscle (Edgar 2004); all gaps and missing data were eliminated. Nucleotide phylogenies were inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). Initial trees for the heuristic search were obtained by applying the Maximum Composite Likelihood approach. Trees were populated by BLASTing the sequences of SAG 49.72, UWO 241, and their close relatives against GenBank and then adding similar, but unrepresented, sequences to the analyses. The analyses were bootstrapped 1,000 times. Alignments were deposited at TreeBASE (http://treebase.org/treebase-web/home.html) with the study accession number S18437 available at http://purl.org/phylo/treebase/phylows/study/TB2:S18437.

A Maximum Likelihood phylogeny was constructed using plastid-encoded *rbcL* nucleotide sequences from various chlamydomonadalean algae and other chlorophyte taxa (Fig. 1). The resulting tree placed UWO 241 (KP981642) as a distinct lineage within the Chlamydomonadales, and indicated that SAG 49.72 is more closely related to *Chlamydomonas moewusii* than to UWO 241 (Fig. 1).

To position UWO 241 and SAG 49.72 more precisely within the Moewusinia clade, 18S and 28S rDNA phylogenies were constructed using sequences from a broad range of chlamydomonadalean and chlorophyte species (Figs. 2 and 3). Both of the rDNA-based trees replicated the division of the Moewusinia clade observed in the *rbcL* phylogeny, and this division was again supported by high bootstrap values. As with the *rbcL* phylogeny, the two rDNA-based phylogenies placed UWO 241 in a clade with *C. parkeae*, to the exclusion of SAG 49.72 and *C. moewusii*, which were grouped in their own clade. The topologies of the rDNA trees support the idea that UWO 241 is a distinct lineage from SAG 49.72 and that the latter is more closely related to *C. moewusii* than to UWO 241 (Figs. 2 and 3). Indeed, for the 18S rDNA tree, there was strong bootstrap support for the clade containing *Chlorococcum elkhartiens*e, SAG 49.72 and *C. moewusii* (BS = 100) as well as for the SAG 49.72-*C. moewusii* sub-clade (BS = 100), and the sub-clades containing UWO 241 (KP313859; BS = 96, 98; Fig. 2 and Fig. S1 in the Supporting Information). The 28S rDNA-based phylogeny (built using the D1-D2 region) strongly supported the clade containing SAG 49.72 (KP981641) and *C. moewusii* (BS = 98) as well as that containing UWO 241 (KP313859) and *C. parkeae* (BS = 76; Figs. 3 and S2 in the Supporting Information). These rDNA-based phylogenies clearly indicate that UWO

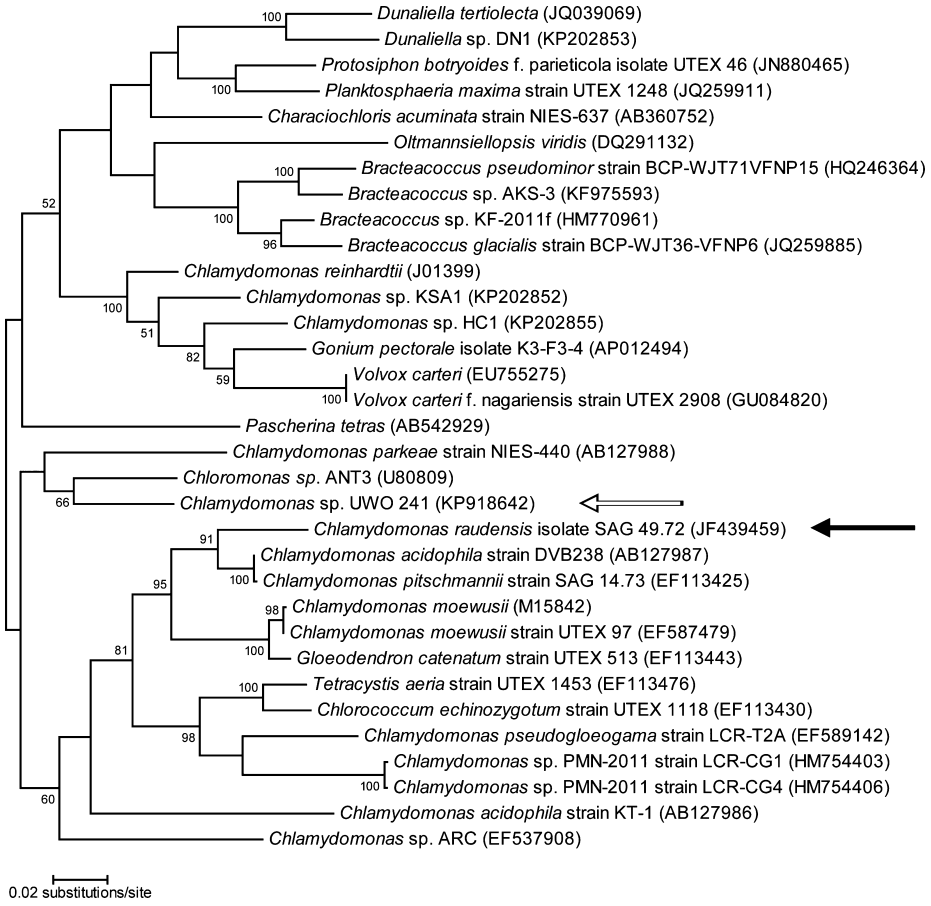

FIG. 1. Molecular phylogenetic analysis of *rbcL* nucleotide sequences of various green algal species by Maximum Likelihood method. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. There were a total of 1093 nucleotide positions in the final data set. GenBank Accession numbers appear in brackets beside organism names. Arrows indicate species considered in this study.

241 and SAG 49.72 belong to different sub-clades of the Moewusinia (sensu Nakada).

Although revealing a close relationship between UWO 241 and SAG 49.72, both the nuclear and plastid phylogenies support the hypothesis that these two algae are distinct species. These data conflict with previously published results reporting that UWO 241 and SAG 49.72 have identical nuclear 5.8S rDNA, ITS1, and ITS2 sequences (Pocock et al. 2004). To address this contradiction, we re-sequenced the 5.8S rDNA, ITS1, and ITS2 regions from both isolates. An alignment of these two sequences revealed many mismatches between UWO 241 and SAG 49.72 (KP981643; Fig. S3 in the Supporting Information), including single-nucleotide substitutions as well as insertion–deletion mutations. These observations are consistent with previous RAPD results comparing UWO 241, SAG 49.72, and several strains of *Chlamydomonas reinhardtii* (Gupta 2013). Thus, all of the data presented here indicate that SAG 49.72 is much more closely related to *C. moewusii* than to UWO 241. Finally, to ensure that the sequencing results generated by this study are otherwise consistent with those previously published for SAG 49.72, its 18S rDNA was re-sequenced. The newly sequenced 18S rDNA was identical to the previously sequenced 18S rDNA from this alga (GenBank JN903891; Fig. S4 in the Supporting Information).

We also performed light microscopy-based morphological characterizations of strains SAG 49.72 and UWO 241 (Fig. S5, A, B, and C for UWO 241 and D, E and F for SAG 49.72 in the Supporting Information). Observations of these two strains were consistent with earlier descriptions with the exception that under the growth conditions used in this study the cell size of UWO 241 was slightly less than that previously reported (Ettl 1976, Pocock et al. 2004). UWO 241 exhibited ellipsoidal to sub-fusiform cells, 7–12-μm long and 5–7-μm wide as well as two flagella, up to 1.5× the cell body length (Fig. S5, A and B). We observed a single cup-shaped chloroplast which was only present on the cell periphery in the apical half of the cell (Fig. S5C). The prominent basal pyrenoid was flattened on the apical side in many individuals. The eyespot of UWO 241 was small, round to sub-ellipsoidal and considerably more prominent in cells from stationary phase than mid-log phase cultures. In contrast to SAG 49.72, UWO 241 formed prominent palmelloids of up to 25 μm in diameter consisting of two to approximately 16 individual cells in mid-log phase as well as stationary cultures. SAG 49.72 exhibited ovate to ellipsoidal cells, which were

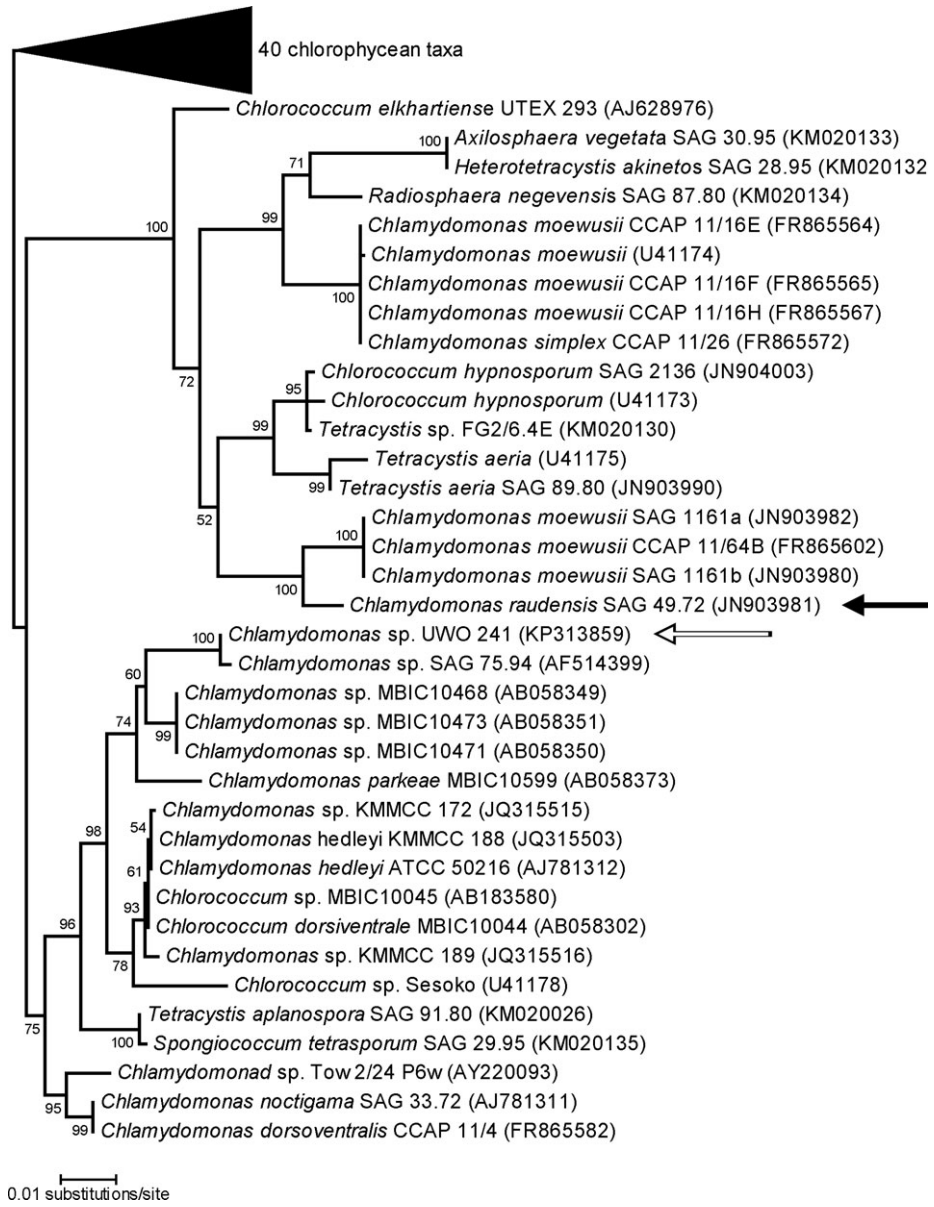

FIG. 2. Molecular phylogenetic analysis of 18S rDNA sequences of various green algal species by Maximum Likelihood method. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Collapsed clades are represented by triangles at branch ends. There were a total of 1676 nucleotide positions in the final data set. GenBank accession numbers appear in brackets beside organism names. Arrows indicate species considered in this study. The full tree without a collapsed clade appears as Figure S1.

basally rounded with dimensions of 8–11-μm long and 5–8-μm wide with two flagella up to 1.5× cell body length (Fig. S5, D and E). SAG 49.72 also exhibited a single lobed chloroplast (Fig. S5F) with a central pyrenoid, which was rounded in small cells but somewhat irregular in larger cells, with an ellipsoidal eyespot near the pyrenoid. Two apical vacuoles were observed near the flagellar bases of SAG 49.72 which formed palmelloids (of up to eight cells) only in stationary phase cultures.

In summary, phylogenetic analysis of the plastid-encoded *rbcL* established a close relationship between UWO 241, SAG 49.72 and *C. moewusii*. Additional phylogenies using nuclear rDNA sequences further supported the affiliation of UWO 241 with the Moewusinia clade (sensu Nakada; Nakada et al. 2008). The nuclear 18S and 28S rDNA phylogenies, in particular, reproduced the broad division of the Moewusinia clade into two groups (Nakada et al. 2008), one of which contains *C. moewusii* and SAG 49.72, and the other UWO 241. One of the closest known relatives of UWO 241 as indicated by the *rbcL*, 18S, and 28S phylogenies is the marine species *C. parkeae*. This observation and the high salinity of the water in Lake Bonney at the depth at which UWO 241 was collected (0.7M) suggest that UWO 241 had a marine ancestor.

Pair-wise alignment of the newly sequenced 5.8S rDNA, ITS1, and ITS2 regions of UWO 241 and SAG 49.72 uncovered many differences and little sequence similarity outside the central, conserved 5.8S rDNA region, conflicting with the findings of Pocock et al. (2004), which reported that these regions were identical between the two algae. The study of Pocock et al. (2004) emanated from the same laboratory as the present report, and the identical UWO 241 strain was used in both studies—as

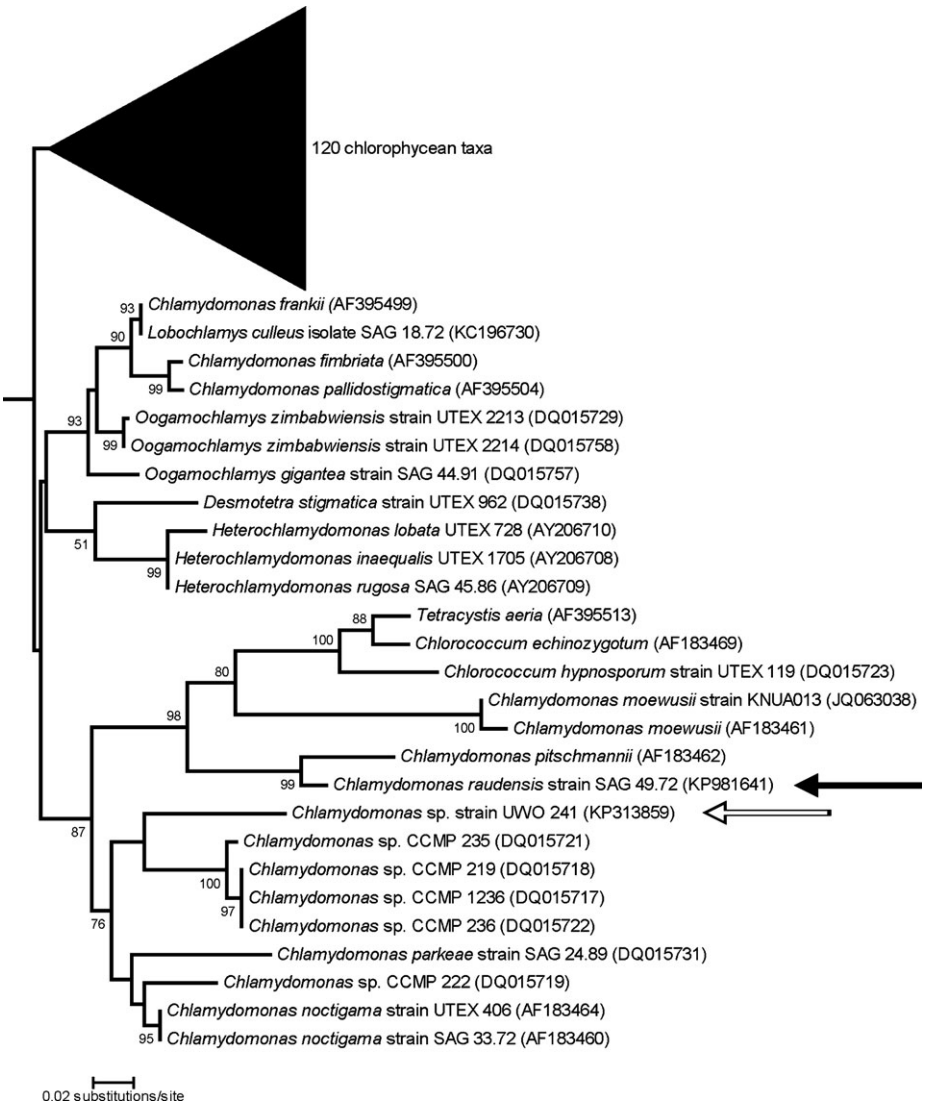

FIG. 3. Molecular phylogenetic analysis of 28S rDNA sequences (D1–D2 region) of various green algal species by Maximum Likelihood method. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Collapsed clades are represented by triangles at branch ends. There were a total of 498 nucleotide positions in the final data set. GenBank accession numbers appear in brackets beside organism names. Arrows indicate species considered in this study. The full tree without a collapsed clade appears as Figure S2.

confirmed by the fact that our newly sequenced 5.8S rDNA, ITS1, and ITS2 loci (Fig. S3) were identical to those previously sequenced by our laboratory and available in GenBank. Again, re-sequencing of both UWO 241 and SAG 49.72 confirmed that the UWO 241 strain used in the present study was identical to that reported by Pocock et al. (2004), but significantly different from that of SAG 49.72 (Fig. S3). Furthermore, our new SAG 49.72 18S rDNA sequence was identical to that available in GenBank, confirming that the mesophile used in this study was indeed SAG 49.72.

The cause of the earlier misidentification of UWO 241 as *C. raudensis* is unknown. One possibility is that an error was made by Pocock et al. (2004) in the previous sequencing of the 5.8S rDNA, ITS1, and ITS2 regions of SAG 49.72, although the cause of this error remains unclear. Alternatively, there is reason to believe that SAG 49.72 was mis-transferred sometime after 2004 and mixed up with another strain (T. Proeschold, unpublished results). Whatever the cause, the data presented here clearly show that UWO 241 is a distinct species from SAG 49.72. We suggest that, hereafter, this strain be referred to as *Chlamydomonas* sp. UWO 241.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Molecular phylogenetic analysis of 18S rDNA sequences of various green algal species by Maximum Likelihood method.

Figure S2. Molecular phylogenetic analysis of 28S rDNA sequences (D1–D2 region) of various green algal species by Maximum Likelihood method.

Figure S3. Nucleotide sequence alignment of the ITS1, 5.8S rDNA and ITS2 regions from *Chlamydomonas raudensis* SAG 49.72 (KP981643) and *Chlamydomonas* sp. UWO 241.

Figure S4. Nucleotide sequence alignment of GenBank JN903981 and the resequenced partial 18S rDNA sequence from *Chlamydomonas raudensis* SAG 49.72.

Figure S5. Light micrographs of *Chlamydomonas* sp. UWO 241 (panels A, B and C), and *Chlamydomonas raudensis* SAG 49.72 (panels D, E and F). Labels indicate cells' chloroplast (C), eyespot (E), flagellum (F), pyrenoid (P) and apical vacuole (V).

Table S1. GenBank accession numbers for sequences referenced for the first time in this note.

Appendix S1. Supplemental methods.