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Journal of Applied Phycology

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J Appl Phycol (2012) 24:635–639
DOI 10.1007/s10811-011-9676-1

Phylogenetic position of *Dunaliella acidophila* (Chlorophyceae) based on ITS and *rbcL* sequences

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Received: 14 January 2011 /Revised and accepted: 1 April 2011 /Published online: 20 April 2011

Abstract *Dunaliella acidophila* is one of the most extreme acidophiles on earth and is able to survive in highly acidic habitats. This characteristic has made this organism the universal model for the study of abiotic stress. Although *D. acidophila* is currently circumscribed to the subgenus *Pascheria* within *Dunaliella* Teodoresco (Chlorophyceae), its taxonomic position has stirred controversy. The comparison of *D. acidophila* CCAP19/35 internal transcribed spacers (including ITS2 secondary structure analysis) and RuBisCo large subunit (*rbcL*) sequences with other *Dunaliella* species confirms that *D. acidophila* should maintain its phylogenetic position within the genus *Dunaliella*, suggesting its inclusion within the subgenus *Dunaliella*. Furthermore, the ITS1 and ITS2 data revealed that *D. acidophila* was highly divergent from the other freshwater species assessed, *D. lateralis*, with which it barely shares a 56.8% similarity.

Electronic supplementary material The online version of this article (doi:10.1007/s10811-011-9676-l) contains supplementary material, which is available to authorized users.

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Keywords *Chlorophyta* · *Dunaliella acidophila* · Internal transcribed spacer · Freshwater · *rbcL* · Taxonomy

Introduction

The genus *Dunaliella* Teodoresco (*Chlorophyceae*) is divided in two subgenera—*Pascheria* and *Dunaliella*—and includes both freshwater and saltwater species (Massyuk 1972; Preisig 1992; Borowitzka and Siva 2007; Polle et al. 2009; González et al. 2009). The five recognized freshwater species (*Dunaliella acidophila, Dunaliella flagellata, Dunaliella lateralis, Dunaliella obliqua* and *Dunaliella paupera*) are rare and were placed by Massyuk (1972) in the subgenus *Pascheria*; only two of these species (*D. acidophila* and *D. lateralis*) are currently available from official culture collections (Polle et al. 2009).

The taxonomic position of the freshwater species within the genus *Dunaliella* has been questioned by several authors on the grounds of cytological, ultrastructural and molecular data which suggest either their placement on the subgenus *Pascheria* or their removal from the genus *Dunaliella* (Melkonian and Preisig 1984; Preisig 1992; Borowitzka and Siva 2007; González et al. 2009) based on the following differences: (1) Unlike the saltwater species, all the freshwater species have contractile vacuoles; (2) three of the freshwater species (*D. paupera*, *D. obliqua*, *D. flagellata*) lack a pyrenoid, unlike the other *Dunaliella* species; (3) cell division in both *D. obliqua* and *D. paupera* is unusual, often producing unequal daughter cells; (4) *D. lateralis* has a lateral pyrenoid not found in other *Dunaliella* species; and (5) recent studies based on the analysis of internal transcribed spacers (ITS) sequences revealed that *D. lateralis* diverges to the same extent as *Chlamydomonas reinhardtii* from the other *Dunaliella*

species. On the grounds of these evidence, Borowitzka and Siva (2007) highlighted that the taxonomic position of *D. acidophila* needs to be reexamined.

Dunaliella acidophila (Kalina) Massyuk, originally AAGATTTCAACTAAAGCTGGCA-3′) described by Nozaki et al. (1995, 1997). DNA amplification was accomplished in a total volume of 25 μL with 1X iQ SYBR Green Supermix (Biorad, USA) and 10 pM of each

(Gimmler and Weis 1992). Life in such low pH conditions has promoted the evolution of basic transport mechanisms to maintain an intracellular neutral pH and to accumulate essential organic and inorganic elements (Pick 1999). Moreover, since the chemical bounds in biopolymers become unstable at pH 0 or 1, the extracellular components at the plasma membrane feature special structural adaptations (Pick 1999). These traits have turned this organism into the universal model system for studies of acclimation to abiotic stress (pH homeostasis). Its biology has become a challenge not only for ecologists and physiologists but also for biochemists and structural biologists (Pick 1999).

The aim of this study was to clarify the phylogenetic position of *D. acidophila* species based on nuclear ITS and on choroplast RuBisCo Large subunit (*rbcL*) sequences.

Material and methods

Strains, DNA extraction, PCR and sequencing

We sequenced the ITS and the *rbcL* regions of the *D. acidophila* CCAP 19/35 strain and compared it with other sequences of *Dunaliella* species available at GenBank (Electronic supplementary materials (ESM) Table 1). Although the ITS conventionally includes the entire ITS1, 5.8S and ITS2 genes of the nuclear rDNA cistron, we excluded the 5.8S gene from all data analysis because it is known to be highly conserved, being only useful for verifying the identity of the sequences and for primer design (Coleman 2003). Since it was not possible to obtain the *rbcL* sequences of all the strains used for the ITS1+ITS2 phylogenetic analysis, the *rbcL* and the *rbcL*+ITS1+ITS2 phylogenetic analysis was performed with the *Dunaliella* strains for which sequences of both regions were available.

DNA extraction of the *D. acidophila* strain was performed with a chelex-100 resin-based protocol (Richlen and Barber 2005). For the DNA amplification of the complete ITS region, we used the primers AB28 (5′-GGGATCCG TTTCCGTAGGTGAACCTGC-3′) and TW81 (5′-GGGATCCATATGCTTAAGTTCAGCGGGT-3′) described by Goff et al. (1994); for the partial amplification of the *rbcL* region, we used the primers 475-497 (5′-CGTGA CAAACTAAACAAATATGG-3′) and 1181-1160 (5′-electrophoresis in a 1.5% agarose gel to assure that a single band was present; afterwards, the PCR product was purified using the Real Clean Spin kit (REAL, Durviz S.L.U., Spain) and sequenced in both directions on an ABI PRISM 3730xl automatic sequencer (Applied Biosystems, USA) at the DNA sequencing service of Macrogen (Korea).

Phylogenetic analysis

The phylogenetic analysis was performed using the primary sequence information of the ITS1 and ITS2 spacers and the sequence and secondary structure information of the ITS2 spacer. For the primary sequence analysis, the sequences were edited and assembled using BIOEDIT v.7.0.9.0 (Hall 1999). Alignment was performed using the accessory CLUSTAL W (Thompson et al. 1994) implemented in BIOEDIT software (http://www.mbio.ncsu.edu/bioedit/bioedit.html), with manual adjustments as needed, following the indications in Kelchner (2000). Both maximum parsimony and Bayesian inference (BI) analysis were conducted for *rbcL* alone and the combined ITS1+ITS2 and ITS1+ITS2+*rbcL* sequence datasets using the sequences of *C. reinhardtii* as the outgroup in all cases. Parsimony analyses were carried out in PAUP*v4.10b (Swofford 2002) under the heuristic search option using the Tree Bisection–Reconnection (TBR) branch-swapping algorithm with 100 random stepwise addition replicates and retaining all best trees in the MulTrees option. All characters were treated as equally weighted and unordered and the gaps as missing data. The branch support was evaluated using 1,000 bootstrap (BS) replicates generated with the same options as the heuristic search (i.e. random sequence addition, equal weighting and TBR branch swapping), but holding one tree at each replicate. BI analysis was conducted with MrBayes v3.0b4 (Ronquist and Huelsenbeck 2003), where the strain *C. reinhardtii* (CCAP11/32D) was included as the outgroup. The nucleotide substitution model parameters were determined using MrModeltest 2.2 (Nylander 2004) included in MrMTgui software (http://genedrift.org/mtgui.php). Based on the Akaike information criterion (Akaike 1979), the best fitting model for our ITS1+ITS2 and *rbcL*+ITS1+ITS2 datasets was the GTR+G and for the *rbcL* dataset

was the GTR+I+G. The BI analysis was run for 10^6 generations. Four simultaneous runs with one cold and three heated chains (Markov chain Monte Carlo; Larget and Simon 1999) were sampled at intervals of 100 generations using the default priors. The first 10,001 trees were discarded as burn-in, and the remaining trees were used to estimate the topology, tree parameters and to produce a 50% majority rule consensus tree. The consensus tree was built and visualized using TreeView (Page 1996). Bayesian posterior probabilities (PP) were calculated as an estimate of clade support (Alfaro et al. 2003).

For the ITS2 sequence and secondary structure analysis, we followed the instructions described by Schultz and Wolf (2009). The software used for the ITS2 sequence–structure analysis can be obtained from http://its2.bioapps.biozentrum.uni-wuerzburg.de/cgi-bin/index.pl?about. Briefly, secondary structures were obtained using the homology prediction implemented in the ITS2 Database v1.0 (Schultz et al. 2006; Selig et al. 2008; Koetschan et al. 2010) via SOAP interface using an ITS2-specific scoring matrix (Wolf et al. 2005). The accepted homology models had a highly significant sequence similarity ($p<10^{-16}$). During the transfer of the structure to the homologous sequence, only the sequences that resulted in a high-quality model and contained all four helices (percentages of or close to 100/100/100/100, never <75%) were used in this study, as specified by Schultz and Wolf (2009).

To perform the alignment based on sequences and their individual secondary structures, the 4SALE v1.5 (Seibel et al. 2006, 2008) was used. For the phylogenetic reconstruction of the ITS2 region, we used the ProfDistS software (Müller et al. 2004; Friedrich et al. 2005; Rahmann et al. 2006; Wolf et al. 2008), which calculates trees considering both sequence and structure information. In ProfDistS, phylogenetic relationships, based on primary and secondary structure information, were reconstructed by profile neighbour joining through the use of an ITS2 sequence/structure-specific and general time reverse (GTR) substitution model.

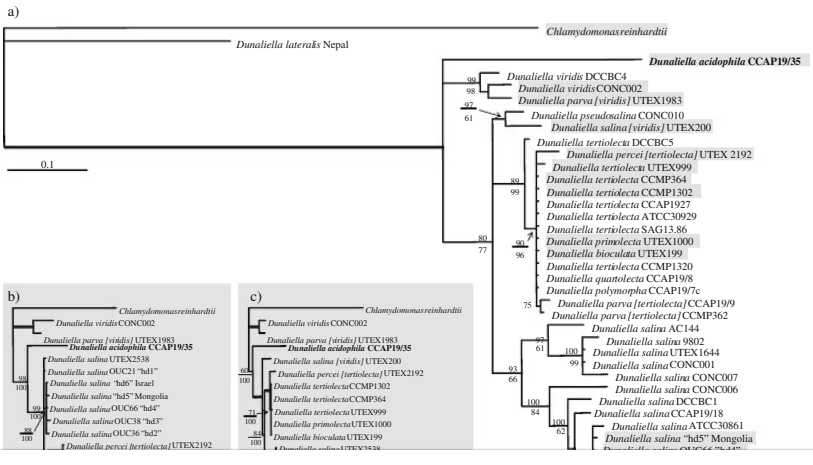

Fig. 1 Bayesian 50% majority rule phylogenetic trees based on ITS1 +ITS2 (**a**), *rbcL* (**b**) and *rbcL*+ITS1+ITS2 (**c**) sequences of different *Dunaliella* taxa. *C. reinhardtii* was included as the outgroup. *D. acidophila* CCAP 19/35 was positioned within the genus *Dunaliella* in all cases. The values *above and below the branches* indicate PP and BS values. The *thicker branches* are those with maximum PP and BS support. *Hyphenated lines* indicate branches that collapse in the strict consensus tree. The *taxa underlined in grey* are common to the three phylogenetic trees. The re-identification of some strains is written *within brackets* (see ESM Table 1)

The resulting tree was visualized with TreeView (Page 1996), and the identity/similarity of each two sequences was calculated with the BIOEDIT software.

Results

The aligned ITS1+ITS2 sequences contained 487 characters (223 constant, 127 parsimony uninformative and 135 parsimony informative), with ITS1 and ITS2 yielding 64 and 71 parsimony informative characters, respectively. The non-aligned ITS sequence lengths ranged from 411 bp in *D. lateralis* from Nepal to 448 bp in *Dunaliella salina* UTEX1644. The combined *rbcL*+ITS1+ITS2 alignment yielded 1,151 characters (804 constant, 221 variable but parsimony uninformative and 126 parsimony informative). The aligned *rbcl* sequences contained 667 characters (551 constant, 74 variable but parsimony uninformative and 42 parsimony informative), and no insertions/deletions were observed. Genetic distance values between taxa were higher for ITS1+ITS2 than for *rbcL* sequences (data not shown).

The ITS1+ITS2 data revealed that *D. acidophila* was highly divergent from the other freshwater species (*D. lateralis*), with which it only shared a 56.8% similarity. This result could not be confirmed with the *rbcL* data because it was neither possible to obtain the *rbcL* sequence from GenBank nor a representative strain of *D. lateralis* for sequencing. Unexpectedly, *D. acidophila* was phylogenetically closer to the species belonging to the subgenus *Dunaliella* (Fig. 1). The closest strain to *D. acidophila* was *Dunaliella parva [viridis]* UTEX1983, with a similarity proportion of 80.3%, 88.9% and 92.8% for ITS1+ITS2, *rbcL*+ITS1+ITS2 and *rbcL* datasets, respectively. Although *D. acidophila* was the single taxon within the *Dunaliella* subgenus clade in the ITS1+ITS2 phylogenetic tree, it was positioned between *D. viridis* and *D. salina*/*D. tertiolecta* strains in the *rbcL* and *rbcL*+ITS1+ITS2 trees (Fig. 1). The results obtained with the ITS2 secondary structure analysis also showed that *D. acidophila* was phylogenetically closer to the *Dunaliella* species belonging to the subgenus *Dunaliella* than to the other freshwater species (ESM Fig. 1). While *D. viridis* strains were phylogenetically closer to *D. tertiolecta* strains than to *D. salina* with the ITS1+ITS2 analysis (Fig. 1), *D. viridis* strains were closer to *D. salina* than to *D. tertiolecta* with the ITS2 secondary structure analysis (ESM Fig. 1).

Discussion

Both ITS and *rbcL* genes have been extensively used for phylogenetic reconstruction despite the fact that ITS is known to evolve much faster (Calonje et al. 2009). In the case of *Dunaliella*, several phylogenetic studies based on the ITS region have been published (Gomez and Gonzalez 2004; González et al. 1999, 2001, 2009; Polle et al. 2008; Buchheim et al. 2010; Hejazi et al. 2010), as opposed to the *rbcL* chloroplastic region for which fewer studies exist (Nozaki et al. 2003; Buchheim et al. 2010).

Our results unequivocally show that *D. acidophila* should not only maintain its position within the genus *Dunaliella* but perhaps might even be considered within the subgenus *Dunaliella*. Based on the 18S rDNA nuclear gene sequence, Ciniglia et al. (2009) showed that two field strains of *D. acidophila* were also positioned within the subgenus *Dunaliella*, a finding which is consistent with our results with ITS and *rbcL* genes. This is in contrast to the results obtained with the ITS analysis of the species in the subgenus *Pascheria* (González et al. 2001, 2009; this study) which support the conclusion that *D. lateralis* should no longer be placed within genus *Dunaliella*. These results emphasize that (1) the attribute that was used in the past to put these five species in the same group (freshwater habitat) should no longer be used for taxonomic circumscription and that (2) there may not be a substantial phylogenetic distance between some freshwater and saltwater *Dunaliella* species. On the whole, this investigation bolsters the lack of correlation between molecular data and morphological attributes used so far for taxonomic circumscription within the genus *Dunaliella*, as already noted by other authors (González et al. 1999). Our future research efforts in this genus will be addressed to investigate the intraspecific *ITS* and *rbcL* polymorphism within *D. acidophila* through increasing the number of sampled strains.

Acknowledgements This research was supported by BANGEN-Banco Genético de la Macaronesia (INTERREG-IIIB, MAC/1/C070). We thank the Cabildo de Gran Canaria for allowing the collaboration of the Departamento de Biodiversidad Molecular y Banco de ADN at the Jardín Botánico Canario "Viera y Clavijo"–Unidad Asociada CSIC, and for continuous support to all its research lines. We also thank Dr. Hisayoshi Nozaki (University of Tokyo, Japan) and Dr. Mark A. Buchheim (The University of Tulsa, OK, USA) for their recommendations regarding *rbcL* primers. Finally, we would like to thank Dr. Matthias Wolf (University of Wuerzburg, Germany) for his help with the software for the ITS2 secondary structure analysis.

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