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Assessing 18S rDNA Diversity of the Chlorophytes among Various Freshwaters of the Central Black Sea Region

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Abstract

In this study, diversity of unicellular chlorophytes isolated from various freshwater habitats in the Central Black Sea Region were assessed by the molecular phylogenetic methods. In order to determine diversity, water samples were collected from freshwater habitats including Cernek Lagoon (Kızılırmak Delta-Samsun), Kürtün Estuary (Samsun), Sarıkum Lagoon (Sinop) and a freshwater pond in Boyabat (Sinop). Axenic cultures were obtained with re-streaking the isolates from enrichment cultures on solid growth media. For characterisation of isolates, phylogenetic analyses depending on nucleotide sequences of small subunit of nuclear ribosomal DNA (18S rDNA) was used besides morphological observations. In morphological observations upon closely related unicellular chlorophytes, there were no significant morphological differences among the isolates. As a result of phylogenetic analyses, our isolates were appeared in three distinct lineages which were related with Chlamydomonadaceae (isolates 103I2 and C301), Chlorellaceae (isolate B4Riv) and Scenedesmaceae (isolates SIN-CON and N603) families.

Our results in this study clearly indicates the deficiency of the morphological observations and also the necessity of the molecular methods for true identification of chlorophytes. Additionally, it provides the first 18S rDNA haplotypes of chlorophyte families stated above from Turkish freshwaters to international databases.

Keywords: Phylogeny, SSU, Chlorophyta, rDNA.

Orta Karadeniz Bölgesindeki Çeşitli Tatlı Sularda 18S rDNA Klorofit Çeşitliliğinin Belirlenmesi

Özet

Bu çalışmada Orta Karadeniz Bölgesindeki çeşitli tatlı su habitatlarından izole edilen tek hücreli klorofitlerin çeşitliliği moleküler filogenetik yöntemler ile belirlenmiştir. Çeşitliliği belirlemek için, su örnekleri Cernek Lagünü (Kızılırmak Deltası-Samsun), Kürtün nehir ağzı (Samsun), Sarıkum Gölü (Sinop) ve Boyabat Göletini (Sinop) kapsayan tatlı su habitatlarından alınmıştır. Aksenik kültürler, zenginleştirilmiş kültürlerden katı besi ortamlarına pasajlama yapılarak elde edilmiştir. İzolatların karakterizasyonu için morfolojik gözlemlerin yanı sıra, nükleer ribozomal DNA küçük alt biriminin (18S rDNA) nükleotid dizilerine dayalı filogenetik analizler kullanılmıştır. Yakın ilişkili tek hücreli klorofitler üzerinde yapılan mikroskobik gözlemlerde izolatlar arasında önemli farklılıklar görülmemiştir. Filogenetik analizlerin sonucu olarak izolatlar *Chlamydomonadaceae* (izolatlar 103I2 ve C301), *Chlorellaceae* (izolat B4Riv) ve *Scenedesmaceae* (izolatlar SIN-CON ve N603) familyaları ile ilişkili üç farklı soy hattında yer almıştır.

Bu çalışmadaki sonuçlarımız, klorofitlerin doğru teşhisleri için morfolojik gözlemlerin yetersizliğini ve aynı zamanda moleküler metotların gerekliliğini açık şekilde göstermiştir. Buna ek olarak uluslar arası veritabanlarına Türkiye tatlı sularından yukarıda belirtilen klorofit familyalarına ait ilk 18S rDNA haplotiplerini sağlamıştır.

Anahtar Kelimeler: Filogeni, SSU, Chlorophyta, rDNA.

Introduction

The principal feature used for distinguishing the major groups (orders, classes) within the Chlorophyta has traditionally been the level of organization exhibited by the thallus (Bold and Wynne, 1985). Molecular data collected from the phylogenetic analysis depending on nucleotide sequences of several genes (18S rDNA, ITS-1/5.8S rDNA/ITS-2, 28S rDNA, actin, *rbc*L, *atp*B, and *nad*5) have, however, revealed several major chlorophytan clades (Pröschold and Leliaert, 2007). Several early

diverging lineages of unicellular algae form a paraphyletic assemblage at the base of the chlorophytan tree (Leliaert *et al*., 2012). The main lineages in the Chlorophyta are also not characterized by progressively more complex levels of organization (flagellate, coccoid, sarcinoid, filamentous, siphonocladous, siphonous) and it is been recently concluded that the traditional view of chlorophyte evolution should be abandoned (Van Den Hoek *et al*., 1995).

There are three principal lineages of the core Chlorophyta: Ulvophyceae, Trebouxiophyceae and Chlorophyceae in the phylogenetic chlorophytan tree (Leliaert *et al*., 2012). The Treboixiophyceae was originally defined based on some ultrastructural features according to counterclockwise orientation of the basal body (CCW), non-persistent metacentric spindle and phycoplast-mediated cytokinesis (Mattox and Stewart, 1984). Several distinct lineages within this class have been redefined by molecular studies, including Treboixiales, Microthamniales, Prasiolales and Chlorellales (Pröschold and Leliaert, 2007). The latter took its name from the most famous microalgal genera worldwide, *Chlorella*. After the description of *Chlorella vulgaris* Beijerinck about 120 years ago, more than 100 species have been morphologically defined since the description of the type species (John *et al*., 2003). On the basis of biochemical and molecular data, however, Huss *et al*. (1999) denoted that there are only five "true" *Chlorella* species. Recently, seven new species and two new combinations have been distinguished in addition to the "true" ones (Bock *et al*., 2011).

The Chlorophyceae have been drastically re-described by molecular and ultrastructural studies and five major lineages have been identified in the class; Chlamydomonadales, Sphaeropleales, Chaetophorales, Chaetopeltidales and Oedogoniales (Turmel *et al*., 2008). The former is the largest group of the class and is housing the one of the largest algal genera, *Chlamydomonas*, among algae. *Chlamydomonas* contains nearly 800 morphospecies which are characterized by different cell sizes and shapes, different chloroplast shapes, the number and position of the pyrenoids within the chloroplast and the position of eyespot and nucleus (Pröschold and Leliaert, 2007). Phylogenetic analyses, however, showed that *Chlamydomonas* is extremely polyphyletic which is distributed in five distinct lineages in the Chlamydomonadales and 8 in Chlorophyceae (Leliaert *et al*., 2012). One of these lineages, Stephanosphaera is one of the clockwise orientation of the basal body (CW) group within the class and has multiple contractile vacuoles. Nonetheless, Stephanosphaera still remains unresolved and needs further study (Pröschold *et al*., 2001). Sphaeropleales form another large order of Chlorophyceae which include some of the most common freshwater phytoplankters as well as picoplanktonic members (Pröschold and Leliaert, 2007). According to the conventional systematics of the green algae, Annotated Catalogue of *Scenedesmus* revealed more than 800 morphologically described taxa (Hegewald and Silva, 1988). It was stated by Hegewald (1997) that *Scenedesmus* was firstly described by Turpin (1828) and was placed in diatoms. It was later replaced in Desmidiaceae by Ehrenberg (1834), in the Chlorococcales (family Hydrodictyaceae) by Nägeli (1849) and finally in Scenedesmaceae by Oltmanns (1904). It was concluded that shifting of the genus *Scenedesmus* to family Scenedesmaceae and molecular phylogenetic studies added complexity to delineation of genera and species in that family (Hegewald *et al*., 2013). Recently 13 genera are present in Scenedesmaceae (Krienitz and Bock, 2012).

Although previous traditional morphologic investigations have revealed chlorophyte diversity of Anatolian freshwaters, it has been substantiated that molecular phylogeny may give us more accurate and tangible knowledge of biodiversity of organisms. The goal of this study is thus to contribute to the investigation of cryptic taxonomic diversity of chlorophytes in various freshwater habitats in the Central Black Sea Region of Turkey.

Materials and Methods

Light Microscopy

Morphological observations on isolates were made via Leica DM300 (Germany) and Prior microscope (Cambridge, United Kingdom), operating in phase contrast optics. Micrographs were made using Leica plan objective under 1000x and 400x magnifications.

Strain Isolation

Environmental water samples were taken from the following localities: Cernek Lagoon (Kızılırmak Delta-Samsun), Kürtün Estuary (Samsun), Sarıkum Lagoon (Sinop) and Boyabat Lake (Sinop). Serial dilutions of water samples were prepared via isotonic solution and were plated aseptically on Proteose medium. After one week of incubation at 27°C in a growth chamber fixed to 18 h light (with a photon fluence rate of 100 µmol. $m^{-2}.s^{-1}$) and 6 h dark, colonies were isolated aseptically and were observed under light microscope (Leica DM300, Germany).

Molecular Analysis

Genomic DNA extractions were made with CTAB/NaCl miniprep method as explained in Temizkan and Arda (2004). For extractions, 1ml of fresh algal cultures (in Proteose medium) that grown in a rotary incubator fixed at 120 rpm, 27°C and growth chamber of 18 hours light and 6 hours dark, were used. Genomic DNA were stored in -20°C prior

to use.

Identifications of our samples were made with phylogenetic analyses depending on nucleotide sequences of small subunit of nuclear ribosomal DNA (18S rDNA). Primers NS3/NS8 (White *et al*, 1990) were used for amplifications of 18S rDNA with the PCR conditions stated in Table 1. For all amplifications, 50 µl PCR mixtures were prepared as follows; template DNA<0.5 µg, 1.5 mM $MgCl_2$, 1.25 U Taq polymerase (Promega, Go-Taq Flexi DNA Polymerase), 0.8 mM dNTP mix (Amresco), 1X PCR buffer (Promega, Go-Taq Green Buffer), 0.4 pmol of each primer in final concentration and ddH_2O. The PCR products were electrophoresed on 1% agarose gel (Amresco, Solon, Ohio) prepared in 1X TBE (Tris-Borate-EDTA) buffer. An MGW-Biotech thermal cycler was used for the PCR amplifications and the visualization of electrophoresis gels (stained with ethidium bromide) were made with GeneGenius Bio imaging system (Syngene, Synoptics Group, Cambridge, UK).

Nucleotide sequencings of 18S rDNA were performed commercially by Macrogen Inc. (Korea) with the same primers used for PCR amplifications. SeqMan II module of the LASERGENE 99 system (Applied Biosystem) was used to assemble the nucleotide sequencings that performed from both strands. Multiple nucleotide sequence alignments of our new haplotypes together with the ones obtained from GenBank (from the closest BLAST hits and also from available literatures) were generated using ClustalX (Thompson *et al*., 1997) and optimized by hand with BioEdit (Hall, 1999). To determine the most appropriate DNA substitution model for our data sets, the Akaike information criterion (AIC) (Akaike, 1974) and Bayesian information criterion (BIC) tests were applied with jModelTest v. 0.1 package program (Guindon and Gascuel, 2003; Posada, 2008). To evaluate the phylogenetic relationships among isolates Neighbor-Joining (NJ), Maximum-Parsimony (MP) and Bayesian inference (BI) algorithms were used. NJ and MP analyses were performed with software program PAUP* v.4.0b10 (Swofford, 1998) and BI analyses with MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Pairwise and overall mean distances were calculated using software program MEGA 5.05 (Tamura *et al.*, 2011). The heuristic search approach was applied for the MP analyses using TBR swapping algorithm with 10 random repetitions and then strict consensus trees were generated from equally parsimonious trees. Bootstrap tests (Efron, 1982; Felsenstein, 1985) were performed with 10,000 pseudo replicate for NJ and with 1000 pseudo replicate for MP trees. Two parallel Markov chain Monte Carlo (MCMC) runs (each included one cold and three heated chains) were carried out for 3 million generations. Trees and parameters were sampled at every 100 generations. Convergence of the two cold chains was checked and burn-in was determined using the 'sump' command. The bootstrap and posterior probabilities for each data set are presented in phylograms.

All new sequences obtained in this study were deposite in EMBL data bank under accession numbers

Results

Morphology

Morphological observations upon the isolates

Table 1. PCR protocol used in this study

	NS1/NS3 (White et al. 1990)		
	Cycle	Time	Temperature
Initial denaturation		3 min	95°C
Denaturation		1 min	94°C
Annealing	40	1 min	60°C
Extention		2 min	72°C
Final extention		10 min	72°C

Table2. Morphological characteristics of the strains isolated from various habitats of central Black Sea Region

Character	103I2	B4Riv	C301	N603	SIN-CON
Cell Shape					
-In zoospores	-nearly spherical	-	-subspherical to ovoid	-	-
-In vegetative cells	-broadly oblong	-ovoid to subspherical	-ovoid to obovoid	broadly oblong	oval to oblong ovoid
Cell Size					
-In zoospores	10-15 µ	-	20-35 µ	-	-
-In vegetative cells	4-8 x 8-11 µ	1-3 x 4-10 µ	4-9 x 9-17 µ	5-8 x 1-3,5µ	3-14 x 5-20 µ
Flagellar Length	as long as the cell	-	-	-	-
Chloroplast Shape	cup shaped	band shaped	parietal and striated	parietal	parietal
Pyrenoids	4-8	1	4-6	1	1
Contractile Vacuols	2-4	-	2-5	na	na
Nucleus position	central	central	central	central	central

were given in Table 2. Accordingly, two isolates showed palmelloid-like stages. Two measurements were taken from each isolate according to the cell formations including zoospore or vegetative forms. Zoospores of the strain 103I2 were almost spherical in shape whereas it was broadly oblong in the vegetative form (Figure 1a-b). C301 zoospores, however, varied from subspherical to ovoid while the vegetative cells were ovoid or obovoid in shape (Figure 1d-e). Both strains have a central nucleus but different chloroplast shapes. The strain 103I2 has cup shaped chloroplasts whereas C301 has parietal and striated chloroplasts. Pyrenoids of the strains 103I2 and N603 varied between 4 and 8 in number. The strains N603 and SIN-CON formed 2-celled colonies resembling coenobia of Scenedesmaceae species (Figure 1 f-g). Cells were nearly oblong in both strains. Both these have parietal chloroplasts, 1 pyrenoid and central nuclei. The strain B4Riv cells were differed from ovoid to subspherical in shape and chloroplasts were usually band shaped with 1 pyrenoid (Figure 1b).

Phylogenetics

In the current study, approximately 1100 bp of the 18S rDNA (SSU) locus was sequenced for five chlorophyte isolates obtained from different freshwaters in Northern Anatolia. Blast (Basic Local Alignment Search Tool) results pointed three chlorophyte genera, *Chlamydomonas*, *Chlorella* and *Scenedesmus*, for our isolates. Because of the high genetic diversity between these genera, we analysed them as seperate data sets. Phylogenetic analysis of our new *Chlamydomonas* related 18S rDNA haplotypes together with the haplotypes of different *Chlamydomonas* species downloaded from GenBank (stated with the accession numbers on the tree) were performed using 1012 aligned nucleotides with 253 segregating sites. For our data set, overall mean distance among haplotypes were determined as 0.072. AIC and BIC tests suggested TIM2+I+G (I: 0.586; G: 0.636) and K80+I+G (I: 0.585; G: 0.625) substitution models respectively. In this study, we considered the tree drown with TIM2+I+G model which showed the highest bootstrap values (Figure 2). MP analyses yielded 4 most parsimonious trees with 638 steps (CI:0.574; RI: 0.756 and HI: 0.426). Two of our samples 103I2 and C301 showed similar 18S rDNA haplotypes with 98.7% nucleotide sequence similarity and with 0.013 pairwise distance. The nodes combining these haplotypes were supported with 1.00 posterior probability value in BI tree and also 92% and 99% bootstrap values in the MP and NJ trees, respectively (Figure 2). *Ettlia minuta* haplotype appeared as sister to this lineage. Nucleotide sequence similarities (and pairwise distances) between isolates 103I2, C301 and *E. minuta* were 95.6% (0.044) and 95.9% (0.041), respectively. In all four equally parsimonious trees our isolates appeared as sister with *E. minuta*.

Phylogenetic analysis of 18S rDNA haplotypes of our new *Chlorella* related isolate, B4Riv and different *Chlorella* species downloaded from GenBank (stated with the accession numbers on the tree) were performed using 1109 aligned nucleotides with 209 segregating sites. For our data set, overall

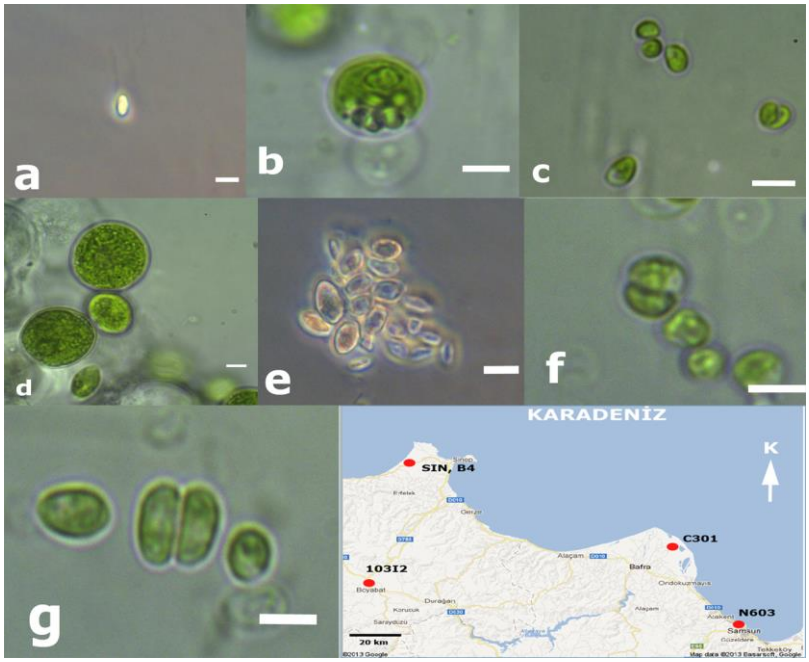

Figure 1. a-g) micrographs of the strains isolated from the freshwater habitats of the central Black Sea Region. a) the vegetative form of the strain 103I2, b) the palmelloid stage of the strain 103I2, c) uncellular strain B4Riv, d) zoospores of the strain C301, e) the vegetative forms of the strain C301, f) single cells and the colony with coenobia of the strain N603, g) single cells and the colony with coenobia of the strain SIN-CON. White bar scales indicates 10 µm, h) A scaled map of the samppling area.

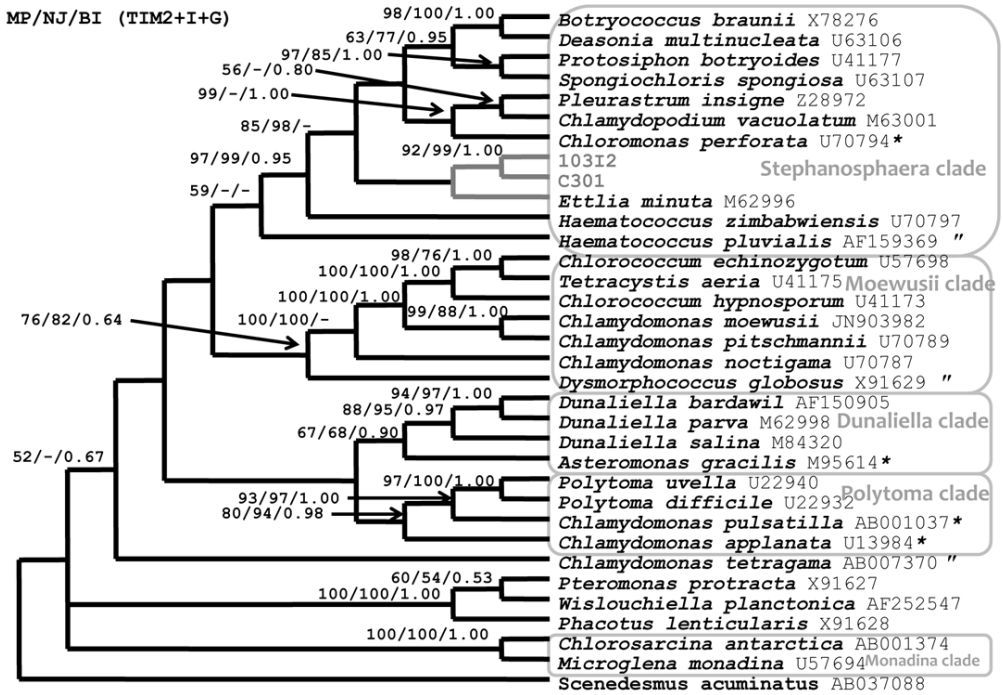

Figure 2. MP tree showing the phylogenetic relations among *Chlamydomonas* related 18S rDNA haplotypes obtained in this study (103I2 and C301) and the ones obtained from NCBI (stated with the GenBank accession numbers on the tree) database. On the tree bootstrap values (greater then 50%) from MP and NJ analysis and also probability values from Bayesian tree are given in the same order. Sequences with asterisks indicates taxa needing emendation nomenclaturally. Sequences with double prime demonstrates phylogenetically indistinct taxa.

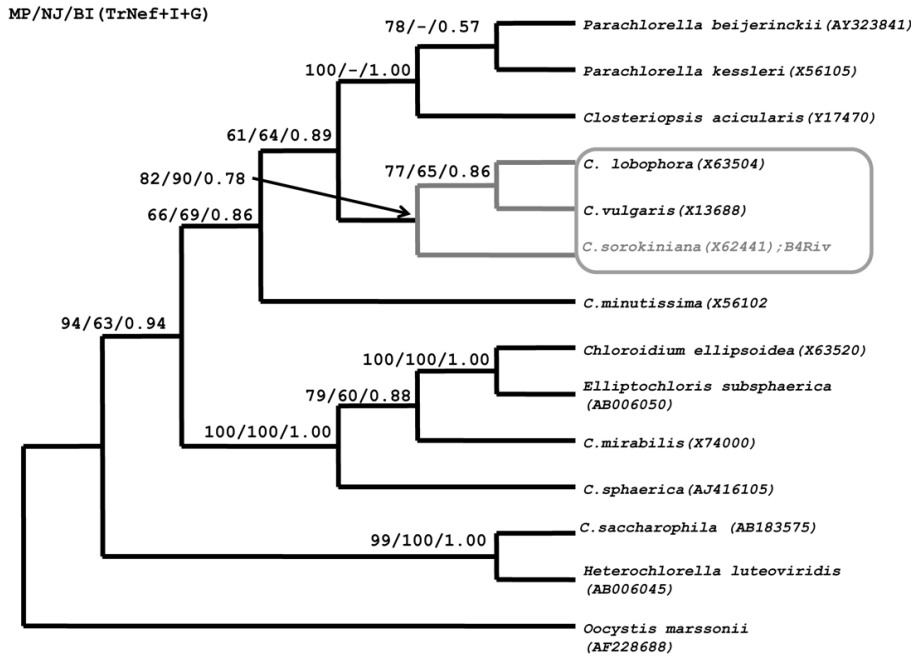

Figure 3. MP tree showing the phylogenetic relations among *Chlorella* related 18S rDNA haplotype obtained in this study (B4Riv) and the ones obtained from NCBI (stated with the GenBank accession numbers on the tree) database. On the tree bootstrap values (greater then 50%) from MP and NJ analysis and also probability values from Bayesian tree are given in the same order. Sequences with asterisks indicates taxa needing emendation nomenclaturally. Sequences with double prime demonstrates phylogenetically indistinct taxa.

mean distance among haplotypes were determined as 0.066. AIC and BIC tests suggested TrN+I+G (I: 0.563; G: 0.538) and TrNef+I+G (I: 0.563; G: 0.542) substitution models respectively. Because the highest

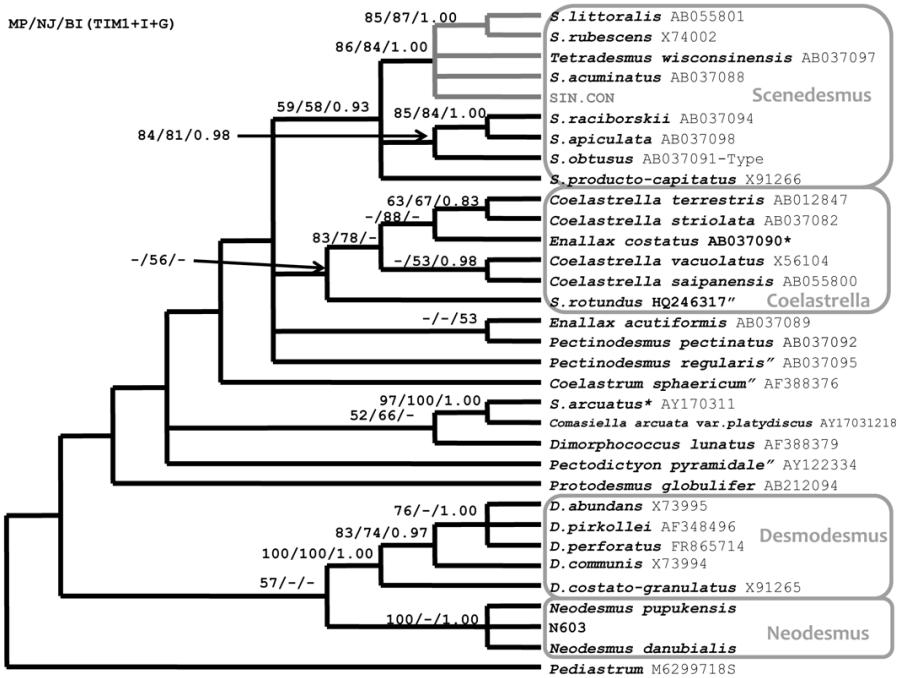

Figure 4. MP tree showing the phylogenetic relations among *Scenedesmus* related 18S rDNA haplotypes obtained in this study (SIN-CON and N603) and the ones obtained from NCBI (stated with the GenBank accession numbers on the tree) database. On the tree bootstrap values (greater then 50%) from MP and NJ analysis and also probability values from Bayesian tree are given in the same order. Sequences with asterisks indicates taxa needing emendation nomenclaturally. Sequences with double prime demonstrates phylogenetically indistinct taxa.

bootstrap values were obtained from the tree drawn with TrNef+I+G model this tree was considered in the study. MP analyses yielded a single most parsimonious tree with 347 steps (CI:0.735; RI: 0.719 and HI: 0.265). Our isolate B4Riv showed the same 18S rDNA haplotype with *Chlorella sorokiniana* isolate. The lineage comprised of *C. lobophora* and *C. vulgaris* was appeared as sister to B4Riv and *C. sorokiniana* with 99.4% and 99.6% nucleotide similarities, respectively. Additionally, pairwise distances between *C. lobophora* and *C. vulgaris* haplotypes and B4Riv (*C. sorokiniana*) were 0.005 and 0.004, respectively. The node combining these two lineages were supported with 0.78 posterior probability value in BI tree and also 82% and 90% bootstrap values in the MP and NJ trees, respectively (Figure 3).

Phylogenetic analysis of 18S rDNA haplotypes of our new *Scenedesmus* related isolates, SIN-CON and N603 together with different *Scenedesmaceae* familia members downloaded from GenBank (stated with the accession numbers on the tree) were performed using 1087 aligned nucleotides with 149 segregating sites. For our data set, overall mean distance among haplotypes were determined as 0.031. AIC and BIC tests suggested TIM1+I+G (I: 0.707; G: 0.643) and K80+I+G (I: 0.706; G: 0.64) substitution models respectively. In the current study the tree drown with TIM1+I+G model considered because it showed the highest bootstrap values (Figure 4). MP analyses yielded 63 most parsimonious trees with 276 steps (CI:0.699; RI: 0.807 and HI: 0.301). In all trees created using NJ, MP and BI algorithms, one of our isolates, SIN-CON formed a lineage with *Scenedesmus acuminatus* with 99.7% nucleotide sequence similarity and 0.003 pairwise distance. The other isolate N603 grouped with *Neodesmus pupukensis* and *Neodesmus danubialis* with 99.8% and 99.7% nucleotide sequence similarity and 0.002 and 0.003 pairwise distances. In all 63 equally parsimonious trees our isolates (SIN-CON and N603) showed exactly the same phylogenetic relations.

Discussion

The morphological observations indicated that the strains 103I2 and C301 may be posited in Chlamydomonadales according to the description in Algaebase (Guiry and Guiry, 2013) or in Volvocales described by Pentecost in the algal identification guide of John *et al.* (2003), since both categories include the non-motile palmelloid stages and vegetative motile cells with 2 equal flagella. The lack of morphological characters under light microscope, however, makes these strains very difficult to identify even at the order level. The ultrastructural classification concept of Chlorophyta (Mattox and Stewart, 1984) based on the ultrastructure of the basal body in flagellated cells may be a solution for identifying these strains. Nonetheless, difficulties of ultrastructural studies not only create identification or classification problems but it has also been argued

that ultrastructural characters in Chlorophyta are insufficient (Van den Hoek *et al.*, 1995). The Maximum Parsimony (MP) tree based on the 18S rDNA nucleotide sequences showed the same 5 lineages of Pröschold *et al*. (2001). These were Dunaliella, Moewusii, Monadina, Polytoma and Stephanosphaera lineages. The latter enclosed the strains 103I2 and C301 of this study as sisters to *Ettlia minuta*.

According to the characteristics of the cells of the strain B4Riv (1-3 µm wide and 4-10 µm long, cells differed from ovoid to subspherical in shape and had band shaped chloroplasts), we can regard this isolate as *Chlorella* sp. These characteristics are, however, not sufficient to identify the strain at species level. For example, we observed ovoid-ellipsoidal and subspherical cells (Figure 1c) in the same liquid culture. This observations posits the strain B4Riv both to *C. saccarophila* and *C. vulgaris* according to John *et al*. (2003). Furthermore, some species of this genus were classified with biochemical characters i.e. peptidoglican compositions in the cell wall (Huss *et al*., 1999), which make the strain B4Riv impossible to identify with only morphological characters. Our phylogenetic analysis depending on 18S rDNA nucleotide sequences clearly showed that isolate B4Riv is related to *C. sorokiniana* rather than *C. vulgaris* and *C. lobophora* which are morphologicaly undistinguishable with *C. sorokiniana*.

The strains N603 and SIN-CON can be assumed to be in Scenedesmaceae due to their coenobia (Figure 1f-g). The MP tree of representative Scenedesmaceae sequences agreed with four clades (*Scenedesmus*, *Coelastrella*, *Desmodesmus* and *Neodesmus*) of Krienitz and Bock (2012). The strain SIN-CON was placed in the core *Scenedesmus* group which was characterized by smooth cell wall, curved coenobia and absence of spines and these characteristics were well agreed with our observations (Table 2). *S. acuminatus* is the closest species to the isolate SIN-CON. Isolate N603 is, however, placed in the *Neodesmus* lineage and is affiliated with *N. pupukensis* and *N. danubialis*.

Our findings in this study clearly show that the morphological characterisation of *Chlamydomonas*, *Chlorella* and *Scenedesmus* genera do not adequately congruent with phylogenetic relations and therefore molecular studies are essential for true identification of algal isolates. We also presented first molecular records belonging to coccoid chlorophytes for the Turkish freshwater algal flora. There is, however, much work to be performed in order to unreveal cryptic taxonomic diversity of the chlorophytes in Turkish freshwaters.

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