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Molecular phylogeny of *Chlorella*-related chlorophytes (Chlorophyta) from Anatolian freshwaters of Turkey

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Abstract: This study aimed to investigate molecular taxonomic diversity of unicellular chlorophyte strains in the northern Anatolian freshwater habitats of Turkey. Five strains were isolated from various habitats i.e. salty lagoons, shallow lakes, or slow-flowing creeks. The habitats chosen were Cernek lagoon (Kızılırmak Delta, Samsun), Kara lake (Çubuk, Ankara), Sarıkum lagoon (Sinop), and Sırakaraağaçlar creek (Sinop). Although the observations based on light microscopy showed no significant differences in cell morphologies, phylogenetic analysis based on nucleotide sequences of nuclear 18S rDNA and chloroplast 16S rDNA regions suggested that our isolates are related to 3 chlorophyte species: *Chlorella vulgaris* Beijerinck (S705, S706, and S708), *C. sorokiniana* Shihira & Kraus (A102), and *Heterochlorella luteoviridis* (Chodat) J.Neustupa, Y.Nemcova, M.Eliás & PSkaloud (S705). This study represents the first records of *C. sorokiniana* and *H. luteoviridis* species for the algal flora of Turkey.

Key words: Trebouxiophyceae, *Chlorella*, *Heterochlorella*, molecular diversity, Turkey

1. Introduction

Many coccoid algae are very difficult to identify because of their extremely small size and simple morphology, and they have been referred to as "little green balls" (Callieri and Stockner, 2002). These little green balls have often been reported as *Chlorella* Beijerinck or *Chlorella vulgaris* Beijerinck (Fawley et al., 2004). Since *C. vulgaris* "Beijerinck strain" (SAG 211-11b) was first described and isolated in axenic culture about 120 years ago, *Chlorella* strains have been used as model organisms in plant physiology and biochemical research (Burja et al., 2001). Furthermore, mass cultures of *Chlorella* have been used in agriculture as a single cell protein both for humans and animals, in biotechnology as recovery agents for waste treatment, and in biofuel technology as microbial energy producers (Golueke and Oswald, 1964; Fogg, 1971; Soeder, 1976; Abbott and Cheney, 1982).

Among the over 100 traditionally defined *Chlorella* species, a lack of morphological characters led to the adoption of various approaches, including several combinations of physiological, biochemical, and serological studies, for identifying *Chlorella* species (John et al., 2003). The morphologically-defined species are considered very artificial and house many cryptic taxa. Using molecular data, Huss et al. (1999) demonstrated that only 5 "true" species could be regarded as part of the genus *Chlorella*. Darienko et al. (2010) revealed that 3 *Chlorella*-like strains (previously known as *Chlorella saccharophila* (Krüger) Migula, *C. ellipsoidea* Gerneck, and *C. angusto-ellipsoidea* N.Hanagata et M.Chihara) formed a monophyletic lineage within the Trebouxiophyceae; they were placed in a new genus, *Chloroidium* Nadson. Recently, Bock et al. (2011) described 7 new species and 2 new combinations.

Although previous traditional investigations have revealed the chlorophyte diversity of Anatolian freshwaters, the genus *Chlorella* is used almost as a synonym for *Chlorella vulgaris* in morphological observations. Thus, the goal of this study was to distinguish the cryptic taxonomic diversity of *Chlorella*-like strains in the following Anatolian freshwater habitats of Turkey: Cernek lagoon (Kızılırmak Delta, Samsun), Kara lake (Çubuk, Ankara), Sarıkum lagoon (Sinop), and Sırakaraağaçlar creek (Sinop), using molecular phylogenetic approaches.

2. Materials and methods

2.1. Chlorella isolations

Environmental water samples were taken from the following localities: Kara lake (Ankara, Central Anatolia), Cernek lagoon (Samsun, Kızılırmak Delta, North Anatolia), Sarıkum lagoon (Sinop, North Anatolia), and Sırakaraağaçlar creek (Sinop, North Anatolia). Serial dilutions of water samples were prepared via isotonic

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solution and were plated aseptically on Proteose medium. After 1 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 observed under a light microscope (Nikon E 600).

2.2. Molecular analysis

Genomic DNA extractions from *Chlorella* samples were made by CTAB/NaCl miniprep method as explained in Temizkan and Arda (2004). For extractions, 1 mL of fresh algal cultures (in Proteose medium) grown in a rotary incubator fixed at 120 rpm and 27 °C and growth chamber of 18 h light and 6 h dark, were used. Genomic DNA was stored at –20 °C prior to use.

Our samples were identified with nucleotide sequence phylogeny of small subunit of nuclear ribosomal DNA (18S rDNA) and small subunit of chloroplast ribosomal DNA (16S rDNA). Amplifications of 18S rDNA and 16S rDNA were made with primer sets NS1/NS3 (White et al., 1990) and fD1/rD1 (Weisburg et al., 1991), respectively, under 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 of 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, OH, USA) prepared in 1X Tris-borate-EDTA (TBE) buffer. An MGW-Biotech thermal cycler was used for the amplifications in this study, and the electrophoresis gels (stained with ethidium bromide) were visualized with the GeneGenius Bio imaging system (Syngene, Synoptics Group, Cambridge, UK).

Nucleotide sequencing of 18S rDNA and 16S rDNA was performed commercially by Macrogen Inc. (Korea) with the same primers used for PCR amplifications. The SeqMan II software module of the LASERGENE 99 system (Applied Biosystems) was used to assemble nucleotide sequencings. Multiple nucleotide sequence alignments of our new haplotypes together with those obtained from GenBank (Table 2) 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) (Akaika, 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) (Saitou and Nei, 1987), maximum-parsimony (MP), and maximum-likelihood (ML) algorithms were used. NJ and MP analyses were performed with software program PAUP* v.4.0b10 (Swofford, 1998), and ML was applied with PhyML 3.0 (Guindon and Gascuel, 2003). The heuristic search approach was applied for the MP analyses using the 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 replicates for NJ and 1000 pseudo replicates for the MP and ML trees.

All new sequences obtained in this study were deposited in the EMBL data bank under accession numbers KF981992–KF982000 (Table 2).

3. Results

3.1. Morphology

All isolates examined with light microscopy (Figure 1) were found to be spherical or subspherical. Chloroplasts were parietal, lobed, and contained a single pyrenoid in all of the isolates. In morphometric observations, cell diameters were 2–20 µm for A102, 3–20 µm for S705, 5–17 µm for S706, 5–16 µm for S707, and 3–20 µm for S708.

3.2. Phylogeny

Approximately 1100 bp of the 18S rDNA gene were sequenced for 5 chlorophyte samples obtained in the study. Phylogenetic analyses of our new 18S rDNA sequences together with those downloaded from GenBank (Table 2) were performed using 1059 aligned nucleotides with 217

Table 1. PCR primers and protocols used in this study.

	fD1/rD1 (Weisburg et al., 1991)			NS1/NS3 (White et al., 1990)		
	Cycle	Time	Temperature	Cycle	Time	Temperature
Initial denaturation		5 min	95 °C		3 min	95 °C
Denaturation		45 s	95 °C		1 min	94 °C
Annealing	´40	1 min	54 °C	´40	1 min	60 °C
Extension		2 min	72 °C		2 min	72 °C
Final extension		4 min	72 °C		10 min	72 °C

Table 2. Strain names and accession numbers for 18S rDNA and cd16S rDNA of the *Chlorella*-like isolates obtained in this study (in bold) and those downloaded from GenBank.

Species	Strain designation	Accession number	
		18S rDNA	**16S rDNA (cpDNA)**
Auxenochlorella protothecoides	Sag 211-7A	-	X65688[16]
Chlorella lobophora	Andreyeva 750-I	X63504[1]	-
Chlorella minutissima	C-1.1.9.	X56102[2]	-
Chlorella mirabilis	Andreyeva 748-I	X74000[3]	X65100[16]
Chlorella sorokiniana	**A102**	**KF981996**	**KF981992**
	BE1	GQ122327[4]	-
	PRAG A-14	X74001[5]	-
	SAG 211-8k	X62441[1]	X65689[16]
	UTEX 2805	AM423162[6]	-
Chlorella variabilis	NC64A	-	HQ914635[17]
Chlorella vulgaris	SAG 211-11b	X13688[7]	X16579[18]
	C-27	-	AB001684[19]
	S706	**KF981998**	**KF981993**
	S707	**KF981999**	**KF981994**
	S708	**KF982000**	**KF981995**
	CCAP 211-1e	-	D11347[20]
	CCAP211-82	AM231736[8]	-
	KMMCC FC-16	HQ702294[9]	-
	NIES-1269	AB488579[10]	-
Chloroidium saccharophila	MBIC 10037	AB183575[11]	-
	Strain 3.80	-	D11348[20]
Closteriopsis acicularis	SAG 11.86	Y17470[12]	-
Heterochlorella luteoviridis	**S705**	**KF981997**	-
	MES A5-4	AB006045[13]	-
	SAG 211-2a	X73997[5]	-
Oocystis marssonii	Krienitz 96/10	AF228688[14]	-
Oocystis solitaria	SAG 83.80	-	FJ968739[21]
Parachlorella beijerinckii	SAG 2046	AY323841[15]	-
Parachlorella kessleri	SAG 211-11g	X56105[2]	X65099[12]
	SAG 211-11h	-	D11346[20]
Pseudochlorella pringsheimii	SAG 211-1a	X63520[3]	-
	C87	-	X12742[22]
Pseudochlorella subsphaerica	CCAP 264-3	AB006050[13]	-

[1]Huss et al., 1993; [2]Huss and Sogin, 1990; [3]Krienitz et al., 1996; [4]Wan et al., 2012; [5]Huss et al., 1999; [6]de-Bashan et al., 2008; [7]Huss and Sogin, 1989; [8]Luo et al., 2006; [9]Lee and Hur, unpublished; [10]Yumoto et al., unpublished; [11]Sekiguchi et al., unpublished; [12]Ustinova et al., 2001; [13]Hanagata, unpublished; [14]Hepperle et al., 2000; [15]Krienitz et al., 2004; [16]Huss et al., unpublished; [17]Smith et al., unpublished; [18]Huss and Giovannoni, 1989; [19]Wakasugi et al., 1997; [20]Oyauzi et al., 1993; [21]Turmel et al., 2009; [22]Yamada, 1988.

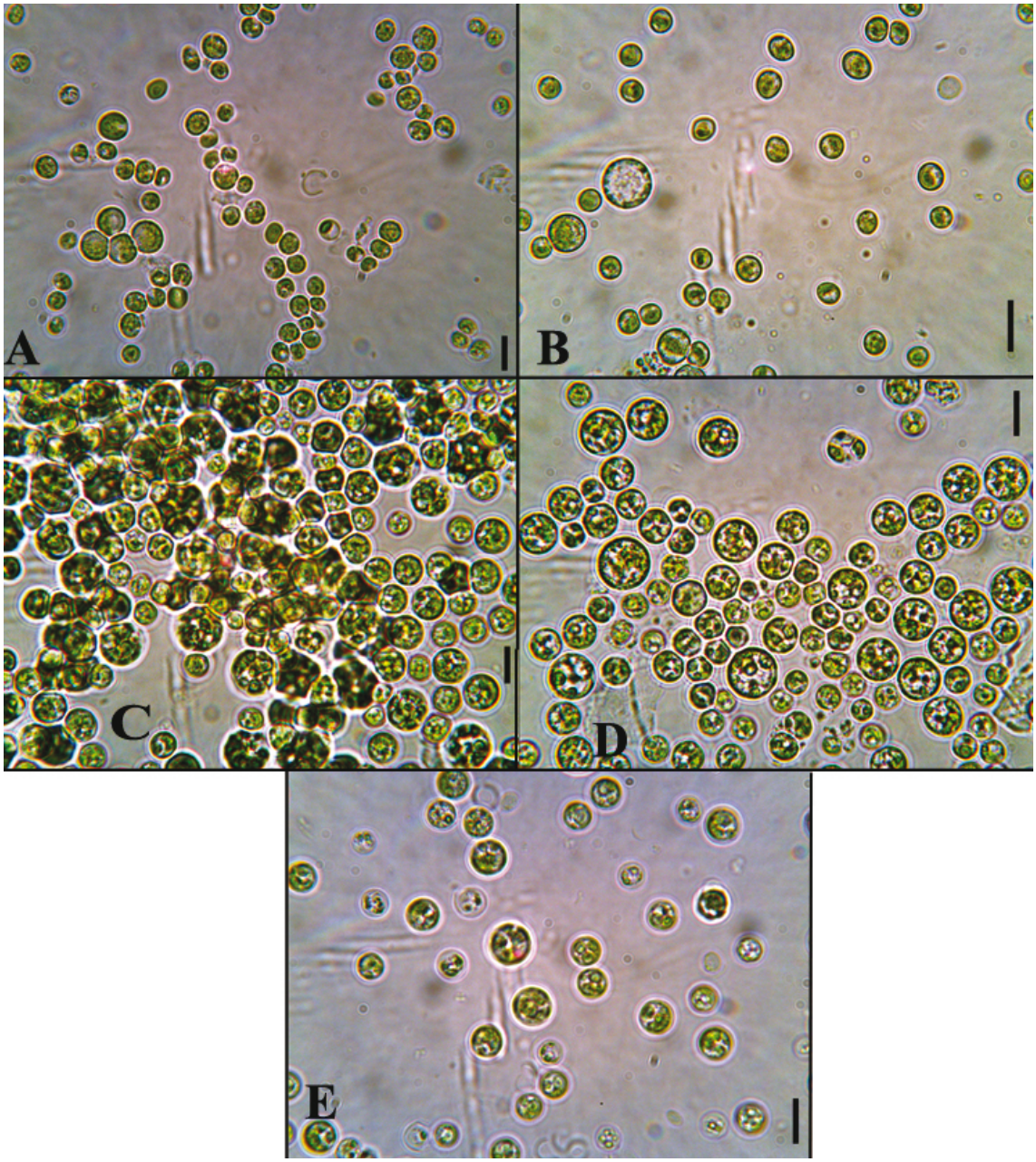

Figure 1. Light micrographs of the young and mature vegetative Chlorophyta cells. A- strain A102, B- strain S705, C- strain S706, D- strain S707, E- strain S708. Scale bar = 10 μm.

polymorphic sites. For our data set, the AIC and BIC tests suggested TrN+I+G (I: 0.561; G: 0.704) and TrNef+I+G (I: 0.561; G: 0.708) substitution models, respectively. In this study we considered the tree drawn with the TrNef+I+G model which showed the highest bootstrap values (Figure 2). In MP analyses the 8 most parsimonious trees had 374 steps (CI: 0.706; RI: 0.747; and HI: 0.294). All phylogenetic methods (NJ, MP, and ML) produced similar tree topologies with minor variations. Two of our samples, S707 and S708, showed the same haplotype as the type strain of *Chlorella vulgaris* (SAG 211–11b) and other *C. vulgaris* samples (KMMCC FC-16, CCAP 211/82, NIES-1269). The isolate S706 appeared as sister to this haplotype, with 98.7% nucleotide similarity. This monophyletic group was supported with 90%, 56%, and 81% bootstrap values in NJ, MP, and ML trees, respectively (Figure 2). The isolate A102 showed the same 18S rDNA haplotype as *Chlorella sorokiniana* Shihira & Kraus isolates (BE1, SAG 211-8k, Prag A14) and also grouped with another *C. sorokiniana* isolate UTEX 2805 with 99.9% nucleotide similarity. Our

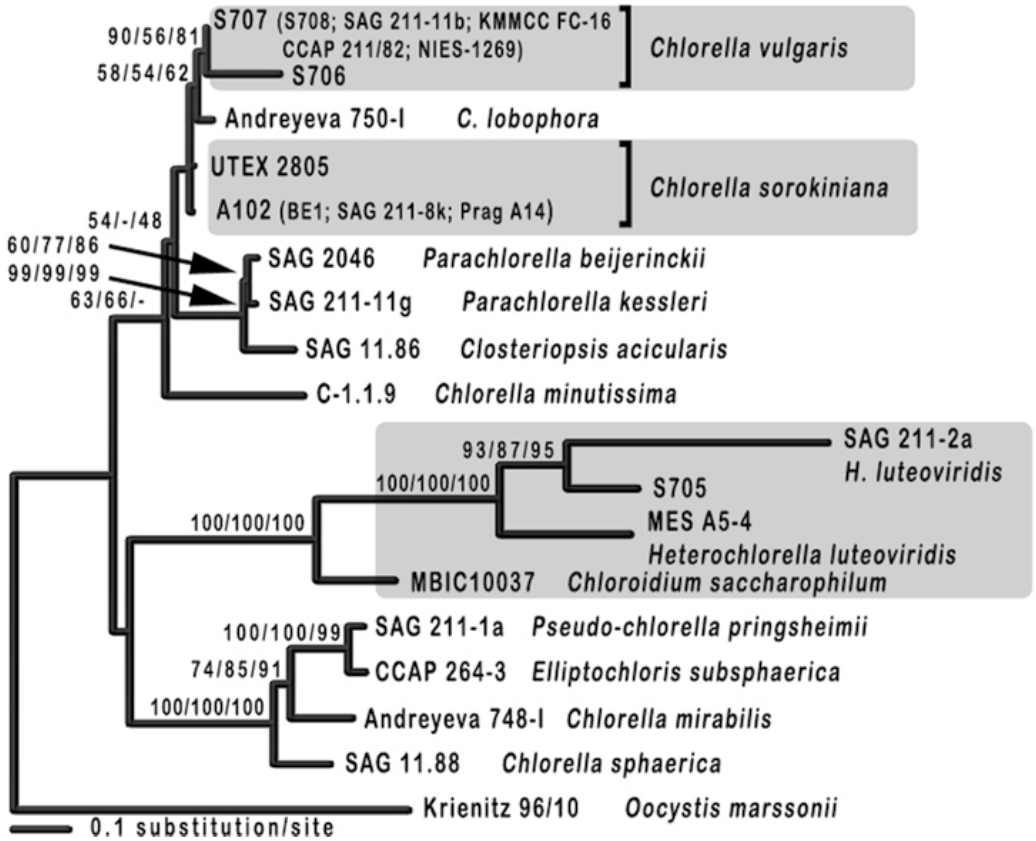

Figure 2. NJ tree showing the phylogenetic relations among 18S rDNA haplotypes obtained in this study and those obtained from GenBank (Table 2). Bootstrap values (>50%) for NJ, MP, and ML trees stated on the tree with the same order.

other isolate S705 grouped with *Heterochlorella luteoviridis* with 94.9% nucleotide similarity, and this group was supported with 93%, 87%, and 95% bootstrap values in NJ, MP, and ML trees, respectively (Figure 2).

Approximately 1200 bp of the chloroplast 16S rDNA gene for our Chlorophyta samples was sequenced. Phylogenetic analyses of our new 16S rDNA haplotypes together with those downloaded from GenBank (see Table 2) were performed using 946 aligned nucleotides with 158 polymorphic sites. For our data set, AIC and BIC tests suggested TIM3+I+G (I: 0.552; G: 0.783) and TPM3+G (G: 0.136) substitution models, respectively. The tree drawn with the TIM3+I+G substitution model was chosen for the study because it showed the highest bootstrap values (Figure 3). MP analyses yielded 4 most parsimonious trees with 223 steps (CI: 0.857; RI: 0.893; and HI: 0.143). All 3 phylogenetic trees drawn with NJ, MP, and ML approaches showed the same topologies. Our samples S706, S707, and S708 formed a monophyletic group with *Chlorella vulgaris* isolates 211-11b (Beijerinck strain), C-27, and CCAP 211-1e, which were supported with relatively high bootstrap values in the NJ tree. The nucleotide sequence similarity within this lineage was between 99.8% and 99.5%. On the other hand, isolate A102 showed the same cp16S rDNA haplotype as *Chlorella sorokiniana* type strain 211-8k. *C. sorokiniana* lineage appeared as sister to *C. vulgaris* lineage with 98%, 99%, and 98% bootstrap values in the NJ, MP, and ML trees, respectively (Figure 3).

4. Discussion

To date, *Chlorella* species *C. citriformis* Snow, *C. ellipsoidea* Gerneck, *C. longiseta* Lemmerman, *C. saccharophila*, and *C. vulgaris* have been reported from freshwaters of Turkey using traditional methods such as microscopic observations of cellular morphology (Gönülol et al., 1996; Aysel, 2005). The main problem with traditional methods is that they are not sufficient to distinguish the cryptic species of genus *Chlorella*. For instance, *C. vulgaris* and *C. sorokiniana* are morphologically very similar (both in shape and size) and could be only distinguished from the glucosamine content of the cell wall (Huss et al., 1999) or the phylogenetic analysis of several genes (Burja et al., 2001). Our morphological observations also supported this information. Unfortunately, this fact renders previously

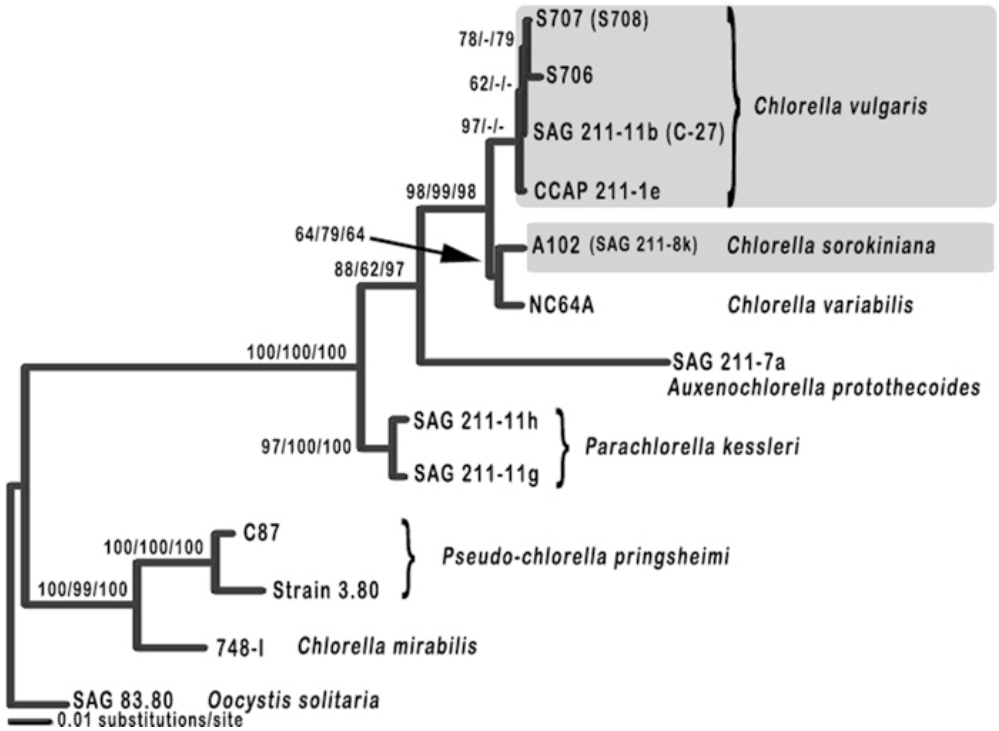

Figure 3. NJ tree showing the phylogenetic relations among 16S rDNA haplotypes obtained in this study and the ones obtained from GenBank (Table 2). Bootstrap values (>50%) for NJ, MP, and ML trees stated on the tree in the same order.

identified *C. vulgaris* isolates from Turkey doubtful. In this study, phylogenetic analysis of both cp16S rDNA and nuc 18S rDNA revealed that our isolates S706, S707, and S708 are clearly related to *Chlorella vulgaris* type strain SAG 211-11b (Beijerinck strain) and other *C. vulgaris*-related isolates (211-1e and C-27). From this perspective our isolates (S706, S707, and S708) can be considered the first true identified *C. vulgaris* isolates from Turkey. On the other hand, isolates A102 and S705 showed a close relationship with *Chlorella sorokiniana* and *Heterochlorella luteoviridis* species, respectively.

In conclusion, although previous studies concerning molecular identification of some algae from other Middle East countries (Attaran-Fariman and Javid, 2013; Attaran-Fariman and Bolch, 2014) are available, to the best of our knowledge, this preliminary study is the first phylogenetic investigation to determine the algal diversity of Anatolian freshwaters and contains the first reports for *Chlorella sorokiniana* and *Heterochlorella luteoviridis* species. In addition to these 2 new records, it also contains the first phylogenetically identified true *Chlorella vulgaris* isolates. However, many cryptic infra/intra-species remain unresolved, and more investigations are urgently needed in order to unravel the cryptic diversity present in Anatolian freshwaters.

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