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GENOTYPE VERSUS PHENOTYPE TO DETERMINE THE DEFINITIVE IDENTIFICATION OF THE GENERA *CHLORELLA* BEIJERINCK, 1890 (CHLORELLACEAE) AND *COELASTRELLA* CHODAT, 1922 (SCENDESMACEAE)

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ABSTRACT

Conventional identification of three coccoid green algae isolates was attempted to characterize the studied algae morphologically under compound microscope, which demonstrated confusional phenomenal convergence; all were classified microscopically as the green alga *Chlorella vulgaris* Beijerinck, 1890.

Phylogenetic studies were conducted to settle the argument about the phenotype by studying the genotype. Genotype the promising field in advance classification by using 18S rRNA and compared to GenBank database using to search the related sequences. The determined sequences showed high a similarity to the strains registered in GenBank.

Phylogenetic tree of ITS within 18S RNA were analyzed: the phylogram separated into two clusters, the first cluster included C1-ITS-Iraq and C2-ITS-Iraq and put with *Coelastrella* Chodat, 1922 which was well supported in bootstrap tests (86-100%), while the second cluster included C3-ITS-Iraq and put with *C. sorokiniana* Shihira and Krauss, 1965, which have bootstrap value 100% with the mentioned species. however, in this study, the green alga *Colestrella* was identified as new record genus in Iraqi freshwater belonged to the order Sphaeropleales.

Keywords: Algae, *Chlorella*, *Coelastrella*, Genotype, Phylogenetic tree.

INTRODUCTION

"Little green balls" refer to the green coccoid shape planktonic with their small size and morphological simplicity, which often were recorded as *Chlorella Beijerinck* or *Chlorella vulgaris* Beijerinck, 1890. These simple and common green algae of this genus are classified below the order Chlorococcales and family Chlorellaceae (Hoek *et al*., 1995).

Genotype Versus Phenotype to Determine the Definitive Identification

Multi industrial and agricultural utilities of coccoid green algae had been progressed exponentially, in addition to the vast economic benefits from exploiting these algae in biofuel technology and single cell protein manufacturing for humans and animals (Soeder, 1976; Abbott and Cheney, 1982).

Confusional similarities in morphology have created a sort of dilemma to researchers by depending on the phenotypes or the morphological characters of these algae, in addition to variations in physiological characteristics which frequently varied according to changes in environmental conditions; furthermore, the morphological traits of *Chlorella* do not differentiate from the morphological traits of other similar algae (Shihira and Krauss, 1965). So, the identification of this group of algae became one of the most difficult tasks in the systematics in the 20th century because its classification remains enigmatic due to conflicts between molecular phylogenetic and morphological approaches (Krientiz *et al*., 2004).

Scenedesmaceae is a large family of Chlorophyta which have 54 genera, every one exhibiting a large morphological variability, maintained by genetic relationships resulting from autospores that support all other mutations. *Coelastrella*, named by Chodat (1922), shows a distribution in Korean rivers, Australian soil, rock surfaces, Bulgarian soil, and an alpine zone in New Zealand. Its habitats range from aerial to terrestrial with or without a relative humidity (40 %) and at high altitudes (Ancona-Canche *et al*., 2017).

Revolutionary scientific methodologies in the last decades made remarkable progressing in systematic classification of micro algae, such as using many approaches of serological, physiological and biochemical studies to identify *Chlorella* species, though the cell size and the shape are markedly changed in responding to environmental parameters of the ambient (John *et al*., 2004). Likewise, molecular phylogenetic studies and electron microscopic test for cell wall structures led to shifts of genera to or from Scenedesmaceae experiments; that created more difficulties because these studies did not always come to an agreement (Krienitz *et al*., 2003). *Acutodesmus* (Hegewald) Tsarenko, 2001, *Coelastrum* Nägeli, 1849, *Enallax* Pascher, 1943, *Scotiella* Fritsch, 1912, *Scotiellopsis* Vinatzer, 1975, and *Pectinodesmus* Hegewald, Wolf, Keller, Friedl and Krienitz, 2010 belong to family Scenedesmaceae that were featured by ridges emerging from inner layer of the cell wall (Tschaikner *et al*., 2007; Hegewald *et al*., 2010). Molecular methods such as PCR techniques are useful for evaluating the genetic variations and also for accurate identification of algae (Wongsawad and Peerapornpisal, 2014). 18S rRNA is one of the most important molecular markers used for phylogenetic analysis and biodiversity screening (Meyer *et al*, 2010). Based on 18S rRNA, *Chlorella* species reduced to four true species, including C. *vulgaris, C. kessleri* Fott & Nováková, 1969, *C. lobophora* Andreyeva, 1973 and *C. sorokiniana* Shihira and Krauss, 1965 (Luo *et al*, 2006). Hence, this study aimed to spotlight on the distinctions between classical taxonomy of morphology depending classification and the systematic

classification by using molecular phylogenetic traits for three coccoid green algae isolated from fresh water in Iraq, and to an emphasis on the importance of genotype to achieve the definition in the identification of algae with convergent phenotypes.

MATERILS AND METHODS

The Specimens collection

The specimens were taken from the Tigris River in Al-Jaddria region, Baghdad province, and were collected from the higher superficial layer 20-30cm deep from the river; these specimens were transported immediately to the laboratory.

Media and culture conditions

Uni- algal cultures of the coccoid green algae were obtained using serial dilution method with 1ml of specimen inoculated into 9 ml of Chu-10 nutrient solution; the procedures were repeated many times with microscopic examination until one species was obtained; the uni-algal culture was then transferred to Chu-10 medium and incubated in illuminated incubator. The flasks were shaken well and incubated. Culturing was carried out with proper light (50 – 75 μE m-2 S-1) and incubation with temperature (23C).

Morphological characterization

The specimens were observed under microscope; the cell shape and size were monitored, measured by micrometry and documented as microphotograph. Identification of the specimens carried out by using the taxonomic publication of Prescott (1982).

DNA extraction

Extraction of genomic DNA was performed from the microgreen algae isolates according to the protocol of G-spin dna extraction kit, intron biotechnology/Korea. Electrophoresis has been done on agarose gel (1%) to determine the quality of DNA, The concentration and purity of DNA was measured via nanodrop.

Primers selection

The set of forward primer for ITS 1(5-TCCGTAGGTGAACCTGCGG-3) and reverse primer for ITS 4(5-TCCTCCGCTTATTGATATGC-3) was used for amplification of 18S rRNA for the detection of microgreen algae at the gene level (White *et al*., 1990).

Polymerase chain reaction

The PCR mixture was set up in a total volume of 25 μl and included 5μl of Taq PCR PreMix (Intron, Korea), 1 μM of each primer and 2ng/μl of template DNA with purity 1.7, and then the remaining volume was completed with nuclease free water. PCR protocol involved an initial denaturation for 3 min at 95ºC; 35 cycles of denaturation for 45 sec at 95ºC, annealing for 1min at 52ºC, extension for 1min at 72ºC then final extension for 7 min at 72ºC. After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. 5μl of PCR product was separate in 1% agarose gel electrophoresis stained with ethidum bromide and visualized on a UV transilluminator; the sizes of amplified

products were compared with the 100 pb DNA ladder to determine the exact size of these products.

Sequencing

PCR products were sent for Sanger sequencing of 18S rRNA which was performed by the national instrumentation center for environmental management (nicem), biotechnology lab, using DNA sequencer 3730XL (Applied Biosystem). Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov). MEGA7 sequence analyzing software with 1000 bootstrap value was used for constructing the phylogenetic tree.

RESULTS AND DISCUSSION

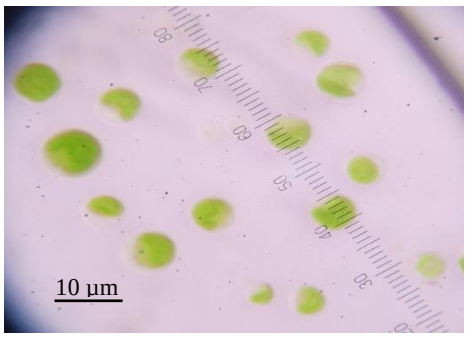
At the present time, classical morphology based on light microscope and molecular based phylogenetic analysis are used for the identification of three coccoid green algae, which are widely distributed in fresh water in Iraq. All the studied coccoidal green algae, which microscopically examined are thought to be *Chlorella* that appeared under compound microscope as unicellular, solitary or aggregated in irregular clumps; round or ellipsoid; variable in size in the same habitat. Chloroplast parietal cup or merely plate, with or without a pyrenoid; this small alga may be confused with species of *Chlorococcum*, a soil or subaerial genus and it is very simalr also to many other unicellular coccoid green algae and may be confused with motionless zoospores of some genera. The Cells of *C. vulgaris* under microscopic seem as spherical cells (Pl. 1) with parietal chloroplasts, cells 5 to 8.5 µmin diameter or sometimes reach to 10µm (Prescott, 1982).

Morphological characteristics varied noticeably depending on many parameters such as environmental and nutritional represent by cultured media; Azaman *et al.* (2017) proved that mixotrophic cultured medium and light intensity trigger morphological variations among both *C.sorokiniana* and *C. zofingiensis*; normal size of *C. sorokiniana* under standard growth conditions was 2 to 4 mm, and this size doubled when this alga grew under mixotrophic medium and under the higher light intensity with a result of increasing the size to approach even 9 mm due to an increase of cell contents. In another study, George *et al.* (2014) demonstrated several changes in shape and size of cells growing in culture medium under 150 mmol photons; Chokshi *et al.*(2015) have recorded that cells morphology varied due to the introduction of glucose to algae culture medium and the size of these cells increased 1-2 folds regardless of microalgae species.

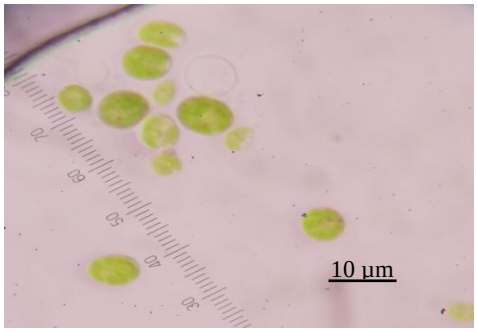
The classification of algae depending on biochemical and physiological characteristics is difficult due to certain characteristics that are not specific to species. Additionally, the shape and cell size of *Chlorella* spp. are changeable and largely depended on nutrition, age of cell and environmental factors (Wu *et al*., 2001).

Coelastrella members do not always show morphological traits identical with *Coelastrella* descriptions because of the high plasticity of phenotype, which interferes with taxonomic assignments based only in morphology (Ancona- Canche *et al*., 2017).

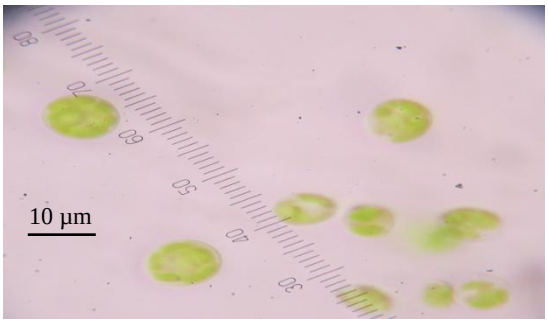
Izumo *et al.* (2007) have found that pyrenoid and stroma starch changed according to the CO_2 concentration during growth of *Chlorella* spp.



Chlorella sorokiniana



Coelastrella sp.1



Coelastrella sp.2

Plate (1): Photomicrographs of the three coccoid green algae isolates under study.

ITS region of the nuclear rRNA is one of the most extremely utilized regions for phylogenetic analysis at the species and generic levels (Coleman, 2003); primers specific for ITS1 and ITS4 within18S rRNA gene were amplified from three coccoid green algae and resulted in 650 bp fragments visualized in electrophoretic gel (Pl.2).

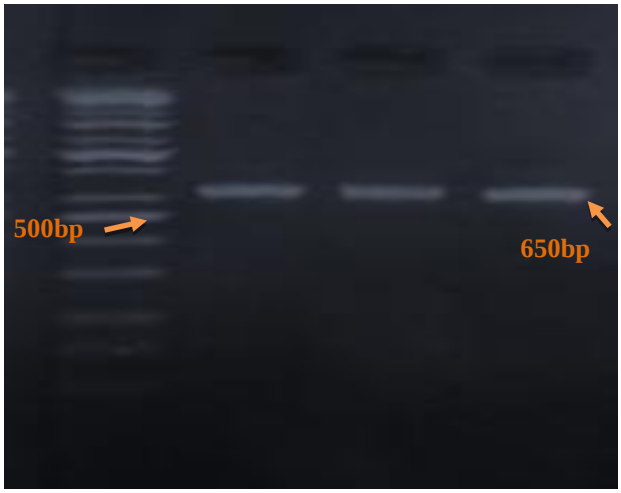


Plate (2): PCR product of the 18S rRNA gene (650 bp) of three coccoid green algae isolated from freshwater samples. The product was electrophoresis on 2% agarose at 5 volt/cm2. 1x TBE buffer for 90 min. Lanes 1-3: freshwater samples. M:100 bp DNA ladder.

Phylogenetic studies were conducted using 18S rRNA which explained a significant diversity in the green algae genes *Chlamydomonas* (Buchhein *et al*., 1997).

The sender nucleic acid sequences of the 18S rRNA gene were compared withGenBank database using BLAST in NCBI website to search for the related sequences. The determined sequences showed high similarity to the strains registered in GenBank (Tab. 1).C1-ITS-Iraq (1) and C2-ITS-Iraq (2) observed 97% and 99% homology respectively to *Coelastrella* sp. whereas C3-ITS-Iraq observed 97% homology to *C. sorokiniana*.

Table (1): Coccoid green algae isolates identified according to the results of a BLAST on the GenBank database in NCBI.

Isolates	Accession number in GenBank	Closest species in GenBank database	Similarity Index			
			Score(bit)	E-value	Identity	Gap
C1-ITS-Iraq (1)	KM061471.1	*Coelastrella sp.*	800	0.0	97%	0%
C2-ITS-Iraq (2)	KX940913.1	*Coelastrella sp.*	1153	0.0	99%	0%
C3-ITS-Iraq	KM514851.1	*C. sorokiniana*	937	0.0	97%	0%

Phylogenetic tree of ITS within 18S rRNA were analyzed. The phylogram was separated into two clusters (Diag. 1). The first cluster included C1-ITS-Iraq and C2-ITS-Iraq and was put with *Coelastrella* species which was well supported in bootstrap tests (86-100%) while the second cluster included C3-ITS-Iraq and was put with *C. sorokiniana* which have bootstrap value 100% with the mentioned species.

The employ of molecular techniques to estimate the species diversity of environmental samples has been used in many fields such as taxonomy, ecology, and oceanography; many of 18S rDNA sequences are available in the GenBank and provide a major source for the selection of a target DNA region; indeed, 18S rDNA was used to classify algae such as those belonging to Trebouxiophyceae and Chlorophyceae that are allowed to locate the phylogenetic positions of closely related taxa (Haddad *et al.*, 2014).

According to the molecular classification, the genus *Coelastrella* is not recorded in Iraqi freshwater in the checklist of algae of Maulood *et al*.(2013); however, this coccoid green alga was identified as new record genus in Iraqi freshwater belonged to the order Sphaeropleales.

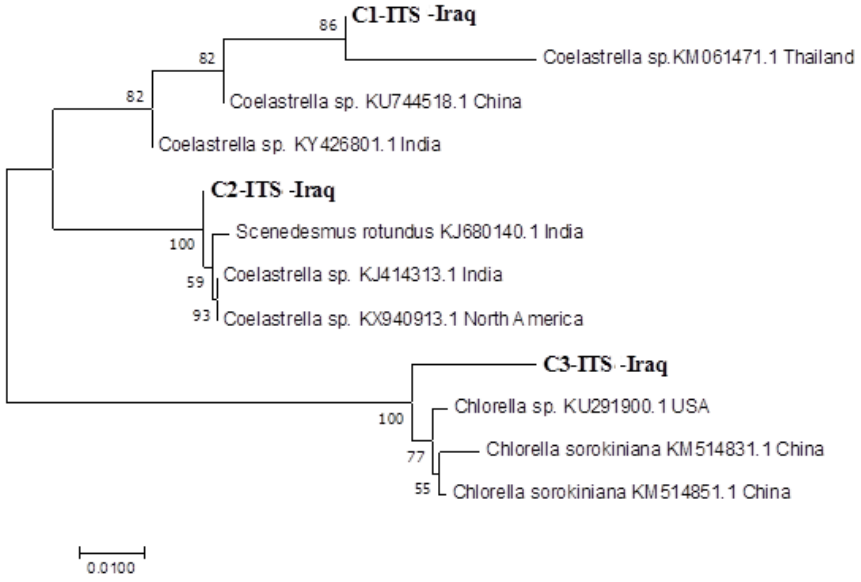


Diagram (1): Neighbor-joining phylogenetic analysis 18S rRNA for three coccoid green algae; the analysis performed using MEGA7.
(Value above the nodes marked to percentage of bootstrap test)

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النمط الوراثي مقابل النمط المظهري لتحديد التشخيص النهائي للجنسين *Chlorella* Beijerinck, 1890 (Chlorellaceae) و *Coelastrella* Chodat, 1922(Scendesmaceae)

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الخلاصة

اجريت محاوله للتشخيص التقليدي لثلاث عزلات من الطحالب الخضراء الكروية الدقيقة والتي شملت وصف الطحالب المدروسة شكليا تحت المجهر الضوئي المركب والذي أظهر التقارب الهائل فيما بينها، وقد شُخصت جميعها على أساس انها الطحلب الأخضر *Chlorella vulgaris* Beijerinck, 1890.

أجريت دراسات للتطور الوراثي لتفسير الحجة حول النمط المظهري من خلال دراسة النمط الوراثي والذي يمثل الحقل الواعد في التصنيف باستخدام 18S rRNA ومقارنتها مع قاعدة بيانات بنك الجينات باستخدام البحث في التسلسلات ذات الصلة، اذ أظهرت التسلسلات المحددة تشابهاً عالياً مع السلالات المسجلة في بنك الجينات.

جرى تحليل الشجرة التطورية لمنطقة ITS ضمن 18S rRNA؛ وقسم المخطط التطوري إلى مجموعتين، ضمّت المجموعة الأولى العينات C1-ITS-Iraq و C2-ITS-Iraq وتم تشخيصها مع جنس *Coelastrella* Chodat, 1922 و التي تم دعمها بشكل جيد في اختبارات bootstrap (٨٦-١٠٠٪) في حين ضمت المجموعة الثانية عينة C3-ITS-Iraq وضعت مع النوع *Chlorella sorokiniana* Shihira and Krauss, 1965 وبقيمة bootstrap ١٠٠% مع النوع المذكور، علما ان الطحلب *Coelastrella* قد سُجّل في هذه الدراسة كجنس جديد في المياه العذبة العراقية والعائد الى رتبة Sphaeropleales.