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Molecular identification of four *Dunaliella* strains from Ukraine via ITS2 marker

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Species of the genus *Dunaliella* are model organisms in algal physiology and widely used in phycotechnologies for commercial production of bioactive compounds, particularly carotenoids (mainly β-carotene). Reliable species identification is challenging due to low morphological variability and the absence of distinct species-specific traits in *Dunaliella*. Common traits such as cell size, shape, and pigmentation are strongly influenced by environmental factors, often leading to misidentification in culture collections and complicating comparative and applied research. Molecular approaches have thus become essential for accurate species identification. However, molecular data on *Dunaliella* from Ukraine remain scarce. The internal transcribed spacer 2 (ITS2) region is particularly informative, combining conserved secondary structures with rapidly evolving primary sequences. This study performed molecular identification of four *Dunaliella* strains from the Cherniaev Herbarium MicroAlgae Culture Collection (CWU-MACC), V.N. Karazin Kharkiv National University, using the ITS2 marker. Species identification was performed based on sequence similarity and phylogenetic analysis. Among the four Ukrainian strains, only CWU-MACC-15 corresponded to *D. salina*, while the others, including CWU-MACC-16, previously identified as *D. salina*, were assigned to *D. viridis*. The ITS2 sequences of CWU-MACC-16 and CWU-MACC-20 were identical, whereas the Henichesk Lake isolate showed a minor ITS2 variation within *D. viridis*. These results provide a molecular basis for the taxonomy of *Dunaliella* strains from Ukraine and highlight the need for expanded sampling and additional molecular markers to further resolve species diversity and phylogenetic relationships.

Key words: *Chlorophyta, Dunaliella, ITS2, DNA barcoding, phylogenetic analysis*

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Introduction

Representatives of the algal genus *Dunaliella* Teodoresco (Chlorophyta, Chlorophyceae, Volvocales, Dunaliellaceae) are widely used as model organisms to study algal physiology, including cellular tolerance to abiotic stresses, adaptation to hypersaline environments, low pH tolerance, and photosynthesis (Ben-Amotz, Avron, 1992; Gordillo et al., 2021; Ramachandran et al., 2023; Arora et al., 2025). *Dunaliella* cultures are also extensively used in phycotechnologies for the commercial production of bioactive compounds; the principal commercially valuable products are carotenoids (primarily β-carotene), lipids, glycerol, vitamins, and protein (Barbosa et al., 2023).

The genus *Dunaliella* comprises species with a simple, nearly uniform biflagellate morphology; cells lack a thick cell wall and other prominent qualitative species-specific traits (Oren, 2005). Morphological and morphometric characteristics, such as cell size, shape, and pigmentation, vary substantially with salinity, light intensity, temperature and other environmental conditions (Leung et al., 2022). This phenotypic plasticity often produces overlapping quantitative trait ranges among species (Preetha et al., 2012). Although *Dunaliella* species differ in ecological preferences (particularly with respect to salinity and light intensity), their niches frequently overlap as well (Oren, 2014). The combination of morphological similarity

and shared ecological preferences can lead to species misidentification and culture cross-contamination, which in turn may cause misinterpretation of experimental data or reduced yields in industrial cultures.

Given the limitations of morphology-based identification, molecular methods provide a reliable alternative for validating or revising taxonomic assignments. Commonly applied molecular barcodes for *Dunaliella* include plastid genes (*rbc*L, tufA) and ribosomal markers (ITS1, 5.8S rRNA, ITS2, LSU, and SSU rRNA) (Highfield et al., 2021). The internal transcribed spacer 2 (ITS2) is particularly informative because its conserved secondary structure provides phylogenetic information complementary to primary nucleotide sequence variation (Buchheim et al., 2011).

Although *Dunaliella* has been frequently reported from hypersaline environments in Ukraine (Massjuk & Lilitska, 2011), these records are based almost exclusively on morphological identification. Modern molecular phylogenetic studies of the genus have highlighted limited geographic sampling and the need for broader strain coverage (Assunção et al., 2012), and notably do not include isolates from Ukraine, leaving the taxonomic identity of Ukrainian *Dunaliella* largely unverified at the molecular level. Furthermore, species names assigned to *Dunaliella* strains in culture collections are often uncertain (Borowitzka, Siva, 2007).

The objective of this study was to assess the species attribution of *Dunaliella* strains originating from Ukraine and maintained in a culture collection, using ITS2 sequence analysis.

Objects and methods of research

Strains

Four *Dunaliella* strains of Ukrainian origin were selected for this study, that are maintained in the Cherniaev Herbarium MicroAlgae Culture Collection (CWU-MACC), V.N. Karazin Kharkiv National University, registered in the World Data Centre for Microorganisms as WDCM 886 collection. The studied material included three subcultures originally obtained from other culture collections several decades ago, as well as one strain of *Dunaliella* sp. isolated from Henichesk Salt Lake (Table 1). The cultures were grown in the Artari nutrient medium modified by Massjuk (Massjuk, 1973) in a light cabinet equipped with neutral-light LED lamps, at 2 klx, with a 16-h light and 8-h dark photoperiod. The temperature was maintained within the range of 20-28 °C.

DNA extraction, ITS2 amplification, and sequencing

DNA was extracted from the pelletized by centrifugation algal biomass using the NeoPrep DNA Plant kit (Neogene, Ukraine) in accordance with the manufacturer's instructions. For amplification of the ITS2 region, a pair of universal primers was used: ITS2-2F (5′-ATGCGATACTTGGTGTGAAT-3′) and ITS2-3R (5′-GACGCTTCTCCAGACTACAAT-3′) (Sun, Chen, 2013; Yu et al., 2017). The expected amplicon size (~488-492 bp) was estimated based on primer binding positions identified by BLAST primer analysis of published *Dunaliella* rDNA sequences. PCR of the ITS2 region was performed with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 10 min. Bidirectional Sanger sequencing of the resulting amplicons (457-459 bp) was performed by Macrogen Inc. (South Korea) using an Applied Biosystems 3730XL DNA Analyzer.

Bioinformatic pipelines

Raw sequence reads were processed in BioEdit (Hall, 1999): low-quality caps trimmed, and forward and reverse strands were assembled using the CAP contig assembly module. Sequences were submitted to GenBank (O'Leary et al., 2016) under accession numbers PX977794-PX977797.

All 420 ITS2-containing rDNA sequences of *Dunaliella* retrieved from GenBank were combined with the newly generated sequences into a single dataset. The phylogenetically informative ITS2 region was delimited from the highly conserved flanking 5.8S and 28S motifs using the ITS2 Database annotation tool (Keller et al., 2009). This tool employs hidden Markov models (HMMs) to identify the 25 bp motif of the ITS2 proximal stem. For annotation, the "Viridiplantae" model was selected, with an E-value threshold < 0.01 and a minimum ITS2 length of 150 nucleotides. Secondary structure models of ITS2 and its proximal stem were predicted using the ITS2 Database prediction tool based on minimum free energy folding constrained by ITS2 profile HMMs (Schultz et al., 2005). Cropping the flanking motifs and the proximal stem yielded the dataset of strict ITS2 region sequences; only 225 of the initial 420 GenBank sequences contained complete ITS2 regions and were retained for further analysis.

Non-redundant sequences were extracted from the strict ITS2 dataset by clustering with CD-HIT (Li, Godzik, 2006) (Galaxy Version 4.8.1+galaxy0) on the public European Galaxy server (Jalili et al., 2020). Clustering parameters were set as follows: sequence identity threshold of 100% (1.0), local sequence

identity mode, and a minimum alignment coverage fraction for the shorter sequence (-aS) of 1.0, while all other settings were kept at their default values. Extracted non-redundant ITS2 sequences were aligned using LocARNA (Will et al., 2007), which incorporates RNA secondary structure information into multiple sequence alignment. Phylogenetic relationships were inferred using the Maximum Likelihood (ML) method implemented in IQ-TREE (Nguyen et al., 2015). The best-fitting nucleotide substitution model (SYM+I+R3) was selected automatically based on the Bayesian Information Criterion (BIC). Branch support was assessed using 1000 ultrafast bootstrap replicates (Hoang et al., 2018).

Species identification was performed based on a combination of sequence similarity searches against GenBank and phylogenetic analysis. ITS2 sequences of the studied strains were compared with reference sequences in GenBank, and assignment to a species was made when 100% sequence identity with a named reference was observed and supported by phylogenetic clustering with the corresponding taxa.

Results and discussion

The dataset of 227 complete ITS2 sequences collapsed into 97 unique sequence types. Of these, 28 represented clusters of identical sequences, each containing between 2 and 50 entries, while the remaining 69 sequences were unique and ungrouped. The four newly sequenced Ukrainian strains exhibited 100% ITS2 sequence similarity with GenBank entries annotated at the species level (Table 1), and their taxonomic assignment was further supported by phylogenetic analysis (Fig. 1).

Table 1. Representative ITS2 sequence clusters identified by CD-HIT at 100% sequence identity, containing the studied *Dunaliella* strains

GenBank Accession Number	GenBank species ID	Strain	Country of isolation
	Cluster of *Dunaliella salina*		
PX977795	***D. salina***	**CWU-MACC-15**	**Ukraine (this study)**
MW471055	*Dunaliella* sp.	CCAP 19/15	Israel
DQ116740	*D. salina*	OUC 38	China
DQ116741	*D. salina*	OUC 66	China
DQ116742	*D. salina*	“Inner-mongolia” (hd 5)	China
DQ116743	*D. salina*	“Israel” (hd 6)	Israel
		SK-2019 isolate	
MN628171	*Dunaliella* sp.	Bazangan_Lake3	Iran
JQ080301	*Dunaliella* sp.	ABRIINW-UB	Iran
KF573420	*D. salina*	RR102	India
GQ337903	*D. salina*	MSI-1	Iran
JX014241	*D. salina*	MSI-2	Iran
KX870020	*Dunaliella* sp.	ABRIINW-B1	Iran
KC477401	*D. salina*	MSI-3	Iran
KJ756826	*D. salina*	CCAP 19/12	Israel-
MH880103	*Dunaliella* sp.	ABRIINW-I1	Iran
FJ164063	*Dunaliella* sp.	ABRIINW U1/1	Iran
JX134754	*Dunaliella* sp.	ABRIINW-G4	Iran
KJ094621	*Dunaliella* sp.	CCAP 19/15	Israel
AY512973	*D. salina*	DCCBC2	South Korea
KF229733	*D. salina*	ATCC 30861	Israel
OQ418484	*D. minutissima*	TAU-MAC 1220	Greece
	Cluster 1 of *Dunaliella viridis*		
PX977796	***D. viridis***	**CWU-MACC-16**	**Ukraine (this study)**
PX977794	***D. viridis***	**CWU-MACC-20**	**Ukraine (this study)**
HQ864830	*D. viridis*	MSV-1	Iran
JN587238	*Dunaliella sp.*	ABRIINW-S1.5	Iran
JN383918	*Dunaliella sp.*	ABRIINW-Sh1.2	Iran
	Cluster 2 of *Dunaliella viridis*		
PX977797	***D. viridis***	**CWU-MACC-17**	**Ukraine (this study)**
MH399207	*D. viridis*	DU3	Iran
AY828228	*D. viridis*	SAG 44.89	Ukraine

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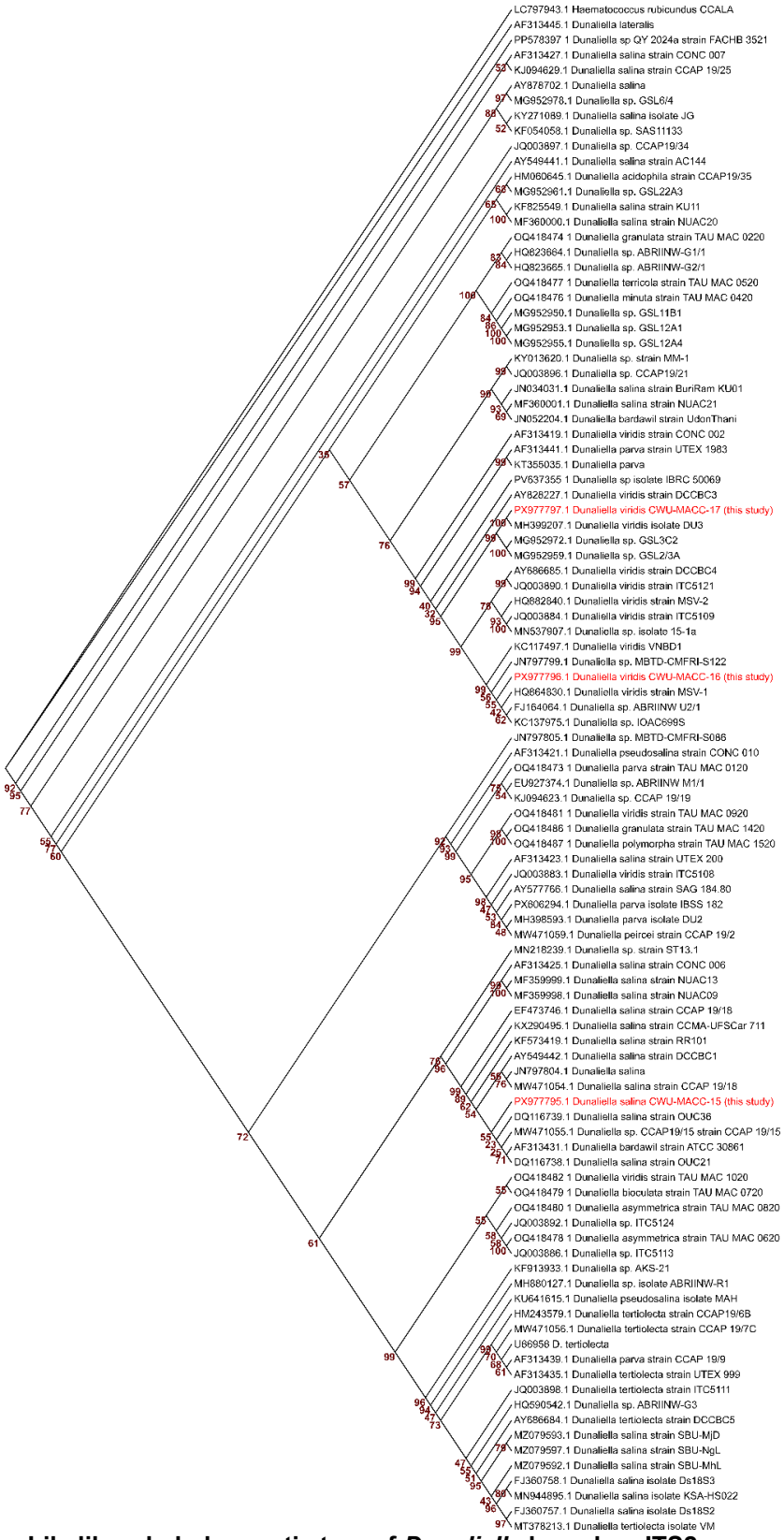

Fig. 1. Maximum Likelihood phylogenetic tree of *Dunaliella* based on ITS2 sequences (LocARNA alignment; SYM+I+R3 model). Numbers at the nodes indicate ultrafast bootstrap support values (1000 replicates). Identical ITS2 sequences belonging to the same CD-HIT cluster (Table 1) were represented in the phylogenetic tree by the first sequence of each cluster

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The strain CWU-MACC-15 showed 100% ITS2 sequence identity with multiple GenBank entries annotated as *Dunaliella salina* (Table 1), all originated from southern Europe and Asia (Greece, Israel, Iran, China, South Korea), and fell within the corresponding clade together with well-characterized culture collection strains such as CCAP 19/18 (*Dunaliella Salina*. (n.d.)., *Phytozome*. (n.d.).) (Fig. 1), supporting its identification. For *D. salina*, no universally accepted authentic molecular reference strain appears to exist, and current species identification therefore relies on comparison with well-characterized culture collection strains. The CD-HIT clusters included strains from geographically diverse regions, suggesting a broad distribution of the identified ITS2 sequence type. At the same time, the presence of sequences annotated as *Dunaliella* sp. or other taxa within the same cluster reflects inconsistencies in public database annotations or the known taxonomic complexity of *D. salina* (Borowitzka & Siva, 2007; Assunção et al., 2012).
The other three strains, including CWU-MACC-16 (previously classified as *D. salina*, Table 2), were assigned to *D. viridis*. The ITS2 sequences of CWU-MACC-16 and CWU-MACC-20 were identical, indicating that they represent the same ITS2 haplotype. The isolate from Henichesk Salt Lake, CWU-MACC-17, displayed minor ITS2 sequence variation relative to CWU-MACC-16 and CWU-MACC-20 strains, comprising eight SNPs at positions 16, 29, 38, 39, 78, 87, 223, and 224. The ITS2 region folds into a conserved RNA secondary structure consisting of a proximal stem followed by four helices (Zhang et al., 2015). The structure is stabilized by compensatory base changes (CBCs) in paired regions and variable loops in the distal parts (Coleman, 2009) (Fig. 2). All substitutions in CWU-MACC-17 were located in loop or distal stem regions and did not affect the conserved species-specific secondary structure (Fig. 2). All three strains fell into CD-HIT *D. viridis* clusters together with isolates from Iran. Henichesk Salt Lake isolate CWU-MACC-17 also appeared to have 100% ITS2 sequence identity with one of a few sequenced *Dunaliella* strains of Ukrainian origin, an authentic strain (*SAG 44.89 Dunaliella viridis, n.d.*) (Table 1). Furthermore, all three

Table 2. Molecular identification of Ukrainian *Dunaliella* strains based on ITS2 sequences

CWU-MACC ID	Original strain ID	Original Identification	Source / Origin	ITS2 Sequence Length (bp)	Accession number	Closest GenBank Match	% Identity	Final Identification
CWU-MACC-15	IBSS-1	*D. salina*	Syvash bay, Crimea, 2001, obtained from A.O. Kovalevsky Institute of Biology of the Southern Seas, National Academy of Sciences of Ukraine, Sevastopol, 2005	237	PX977795	*D. salina* ATCC 30861 KF229733	100%	*D. salina*
CWU-MACC-16	IBASU-AD-11	*D. salina*	Heroiske salt works (Prohnoi), Kherson oblast, 1962, obtained from M. G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, Kyiv, 1995	232	PX977796	*D. viridis* MSV-1 HQ864830	100%	*D. viridis*
CWU-MACC-20	IBASU-AD-29	*D. viridis*	Sasyk-Syvash salt works, Crimea,1965, obtained from M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, Kyiv, 1989	232	PX977794	*D. viridis* MSV-1 HQ864830	100%	*D. viridis*
CWU-MACC-17	Henichesk Salt Lake	*Dunaliella* sp.	Henichesk Salt Lake, Kherson Oblast, 2020, original isolate	232	PX977797	*D. viridis* SAG 44.89 AY828228	100%	*D. viridis*

Note: CWU-MACC accession numbers are given according to (Komarysta, 2021)

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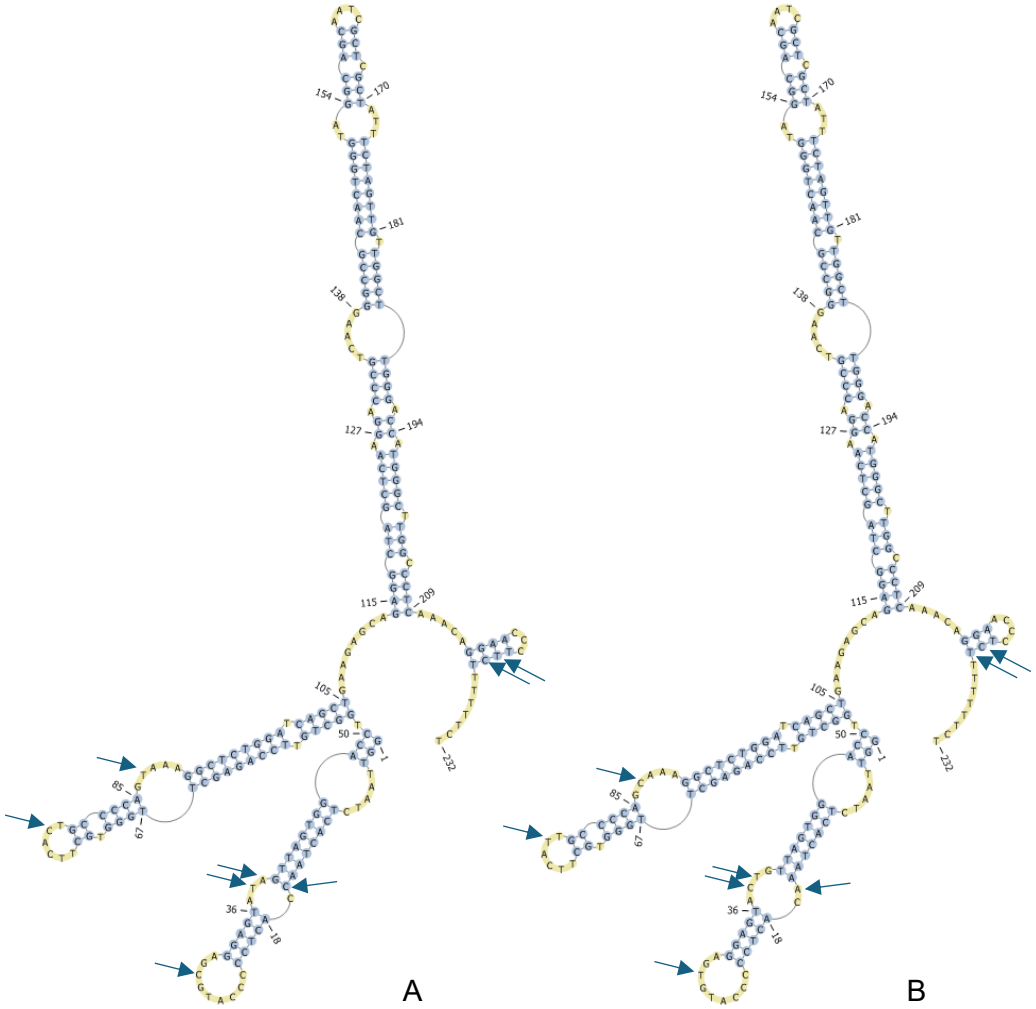

Fig. 2. Secondary structure models for ITS2: *Dunaliella viridis* CWU-MACC-16 and CWU-MACC-20 (A), *D. viridis* Henichesk Salt Lake isolate CWU-MACC-17 (B). The structures are shown using DNA notation (T instead of U), as provided by the ITS2 Database. Arrows indicate single nucleotide polymorphisms

putative *D. viridis* strains belonged to a common phylogenetic clade with another *D. viridis* reference strain ITC5109 (Assunção et al., 2013) (Fig. 1). As in the case of *D. salina*, the presence of sequences annotated as *Dunaliella* sp. within these clusters reflects inconsistencies in GenBank annotations and warrants cautious interpretation.

The previous identification of *D. salina* CWU-MACC-15, derived from strain IBSS-1, by morphological (cell size and shape) and biochemical (ability to turn brick-orange due to accumulation of β-carotene) traits was confirmed (Table 2). On the other hand, the results of phylogenetic analysis suggest that strain CWU-MACC-16, originated from IBASU-A D-11, appears to have been previously misidentified as *D. salina* or affected by cross-contamination prior to the present study. This conclusion is supported by the earlier observation that this strain lacks the ability to change cell color, which is a typical trait of *D. salina* due to β-carotene accumulation (Pasiuga et al., 2013).

Despite the presence of many habitats suitable for *Dunaliella* in Ukraine, especially in the near-Black Sea region, and the extensive work of N. P. Massjuk (1973), the internationally recognized monographer of the genus *Dunaliella* (Oren, 2005), molecular data on Ukrainian *Dunaliella* strains remain scarce and sometimes ambiguous. Overall, this study provides a molecular insight into the diversity of *Dunaliella* in Ukraine, supporting future research on species distribution and the genetic basis of important physiological traits and highlighting the importance of molecular confirmation of morphologically identified strains in culture collections used for experimental studies.

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Conclusions

Among four Ukrainian *Dunaliella* strains from the CWU-MACC collection, two species were identified based on ITS2 sequence analysis: *D. salina* (one strain, CWU-MACC-15) and *D. viridis* (three strains, CWU-MACC-16, CWU-MACC-20, and the isolate from Henichesk Salt Lake, CWU-MACC-17). The strain CWU-MACC-16, previously identified as *D. salina*, was re-identified as *D. viridis*.

While this study provides initial molecular insights into Ukrainian *Dunaliella*, its main limitations include the small number of strains analyzed and the use of a single marker (ITS2). Future studies should expand both strain sampling and the set of molecular markers to improve taxonomic resolution.

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Молекулярна ідентифікація чотирьох штамів *Dunaliella* з України за допомогою маркера ITS2

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Види роду *Dunaliella* є модельними об’єктами у фізіології водоростей і широко використовуються у фікотехнологіях для промислового виробництва біологічно активних сполук, зокрема каротиноїдів (передусім β-каротину). Надійна видова ідентифікація ускладнена через низьку морфологічну варіабельність і відсутність чітких видоспецифічних ознак у *Dunaliella*. Такі ознаки, як розмір клітин, форма та пігментація, значною мірою залежать від умов середовища, що часто призводить до помилкової ідентифікації в колекціях культур і

ускладнює порівняльні та прикладні дослідження. Тому молекулярні підходи стали необхідними для точної видової ідентифікації. Водночас молекулярні дані щодо *Dunaliella* з території України залишаються обмеженими. Ділянка внутрішнього транскрибованого спейсера 2 (ITS2) є особливо інформативною, оскільки поєднує консервативні елементи вторинної структури з швидко еволюціонуючими первинними послідовностями. У цьому дослідженні проведено молекулярну ідентифікацію чотирьох штамів *Dunaliella* з колекції мікроводоростей гербарію Черняєва (Cherniaev Herbarium MicroAlgae Culture Collection, CWU-MACC) Харківського національного університету імені В.Н. Каразіна з використанням маркера ITS2. Визначення видової належності здійснювали на основі подібності послідовностей і філогенетичного аналізу. Серед чотирьох українських штамів лише CWU-MACC-15 відповідав *D. salina*, тоді як решта, включно з CWU-MACC-16, який раніше був ідентифікований як *D. salina*, були віднесені до *D. viridis*. Послідовності ITS2 штамів CWU-MACC-16 і CWU-MACC-20 були ідентичними, тоді як ізолят з Геніческого солоного озера демонстрував незначну варіацію ITS2 у межах *D. viridis*. Отримані результати створюють молекулярну основу для таксономії штамів *Dunaliella* з України та підкреслюють необхідність розширення вибірки штамів і використання додаткових молекулярних маркерів для кращого розкриття видового різноманіття та філогенетичних зв'язків.

Ключові слова: *Chlorophyta, Dunaliella, ITS2, ДНК-баркодинг, філогенетичний аналіз*

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Authors contribution: K. Fursova performed DNA extraction and amplification, bioinformatic data analysis, visualization of results, preparation of the first draft of the manuscript, and manuscript editing. M. Horpynchenko performed DNA extraction and amplification, provided funding within the framework of the research project No. 1-16-25 "DNA barcoding as an effective method for biomonitoring of freshwater bodies under climate change and the impact of military activities" (state registration No. 0125U001468), and participated in manuscript editing. O. Utevska was responsible for the conceptualization and methodological design of the study and participated in manuscript editing. V. Komarysta performed strain cultivation, submission of sequences to GenBank, visualization of analytical results, manuscript editing, and overall scientific supervision of the study. / **Внесок авторів:** К. Фурсова здійснювала екстракцію та ампліфікацію ДНК, біоінформатичний аналіз даних, візуалізацію результатів, підготовку першої версії рукопису та редагування тексту. М. Горпинченко здійснював екстракцію та ампліфікацію ДНК, забезпечував фінансування дослідження в межах НДР № 1-16-25 «ДНК-баркодинг як ефективний метод біомоніторингу прісних водойм в умовах зміни клімату та впливу військових дій» (державний реєстраційний номер № 0125U001468) та брав участь у редагуванні рукопису. О. Утєвська відповідала за концептуалізацію та методологічне забезпечення дослідження, а також брала участь у редагуванні рукопису. В. Комариста здійснювала культивування штамів, депонування послідовностей у GenBank, візуалізацію результатів аналізу, редагування рукопису та загальне наукове керівництво дослідженням.

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