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Valeriella persica sp. nov. (Chlorococcaceae, Chlorophyceae): A potential biodiesel feedstock from the hyperarid desert soil in Yazd (Iran) revealing new diagnostic criteria for green coccoids

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***Valeriella persica* sp. nov. (Chlorococcaceae, Chlorophyceae): A potential biodiesel feedstock from the hyperarid desert soil in Yazd (Iran) revealing new diagnostic criteria for green coccoids**

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## ABSTRACT

Clear-cut delineation of the species identity of the drylands-inhabiting coccoid green algae is a challenging taxonomic work that requires an in-depth polyphasic investigation. Here, we employed an integrative polyphasic approach to characterize an interesting terrestrial green coccoid belonging to the recently proposed genus *Valeriella* from Iran. Despite morphological resemblances to some established taxa, this strain is distinguished by a combination of taxonomic features. Similarities and differences with morphologically allied taxa were discussed in detail, and some new diagnostic criteria for green coccoids were revealed. Our new findings threw light on the age-old problem in the taxonomy of green coccoids. The 18S rDNA phylogenetic assignment placed our strain in a well-supported lineage within the *Valeriella* clade (Chlorococcaceae, Chlorophyceae) with close affinity to *V. minor*. Therefore, here we propose *Valeriella persica* sp. nov. as a new species based on its phylogenetic position and key taxonomic features including cell-wall ultrastructure, life-cycle stages, reproduction strategies and ecology. We also assessed lipid content and fatty-acid profiles to evaluate suitability as potential feedstock for biofuel production. The lipid content and productivity were about 27% and 130.5 mg L<sup>-1</sup> day<sup>-1</sup>, respectively, at the late exponential phase. Gas chromatography (GC) coupled with flame-ionization detection (GC-FID) unraveled twenty-six species of FAs with highly significant levels of palmitic (C16:0; 24.74%), oleic (C18:1c,  $\omega$ -9; 19.02%), linoleic (C18:2c,  $\omega$ -6; 18.15%), and  $\alpha$ -linolenic (C18:3,  $\omega$ -3; 16.28%) acids. The biodiesel properties of *V. persica* are consistent with the international standards, making it a good candidate for biodiesel production on the large-scale. Overall, these ecophysiological important compounds are well known to have major dynamic roles in regulating the fluidity and functioning of the cell and thylakoid

membranes, and they, therefore, provide ecophysiological–adaptive traits to the extremely–  
dry desert habitat of this novel green coccoid.

**Key words:** biodiesel feedstock, desert–dwelling chlorophytes, ecophysiological–adaptive  
traits, fatty acids, Iran, polyphasic study.

## 1. Introduction

Darienko and Pröschold [1] recently revised the status of a number of *Spongiochloris*  
spp. and transferred them to the new genus *Valeriella* Darienko & Pröschold derived from  
SSU rDNA sequences. *Spongiochloris* was originally described by R.C. Starr in 1955 [2],  
and contained species that had a net-like chloroplast with a single pyrenoid and the  
production of zoospores with two equal flagella and with bodies that become spherical when  
quiescent. The previously described species were transferred to the new genus *Valeriella*,  
based on “cells solitary, spherical, with smooth cell walls; mature cells multinucleate, with  
reticulate to spongiform chloroplast, and several pyrenoids; pyrenoid matrix homogeneous;  
size of mature cells less than 80 µm; reproduction by zoospores of “*Protosiphon*” type  
(without cell walls) or autospores.” Currently, this genus has only three taxonomically  
accepted species including *V. excentrica*, *V. incrassata*, and *V. minor* [3].

The biodiversity, taxonomy, and ecology of “non-motile” coccoid green algae  
inhabiting arid and hyper–arid desert habitats, especially those in the Middle East and Asia,  
remain underinvestigated [4,5]. “Non-motile greens” represent a highly heterogeneous sub-  
group of microalgae, which have been documented in over six classes encompassing 10

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6 67 different orders [6-9]. Accurate species identification of members of this group has been quite  
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8 68 challenging for algal taxonomists, owing to the high morphological uniformity and frequent  
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10 69 lack or paucity of clear-cut morphotaxonomic and ecological differentiating characteristics  
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12 70 [5,9]. Nonetheless, representatives of this algal group can nowadays be distinguished at the  
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15 71 genus, species, and infraspecific level using integrative polyphasic approaches, i.e. studies  
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17 72 combining modern morphotaxonomy (including ultrastructure), autecology, phylogenetic  
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20 73 analyses, and bioorganic data [5,9-13]. Several interesting and cryptic taxa have been  
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22 74 discovered and established in the last decades from the worldwide arid and hyperarid desert  
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25 75 ecosystems by means of combined integrative studies [5,14-16]. Physiologically, the  
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27 76 membrane-lipid classes composition and the fatty acids (FAs) profiles have been shown to  
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30 77 be useful for an understanding of the adaptations of green algal representatives to the extreme  
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32 78 desert conditions [17-19].  
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35 79 It is well known that microalgae are rich sources of naturally synthesized products for  
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37 80 food, livestock feed, fertilizers, pharmaceuticals, as well as alternative fuels [20-22].  
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39 81 Particularly, some of the green taxa are promising candidates for third-generation biofuel  
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41 82 [23,24] thanks to accumulation a large amount of lipid and polyunsaturated fatty acids  
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44 83 (PUFAs) including alpha linolenic (C18:3,  $\omega$ 3), eicosapentaenoic (C20:5,  $\omega$ 3),  
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47 84 docosahexaenoic (C22:6,  $\omega$ 3), arachidonic (C20:4,  $\omega$ 6) and gamma linolenic (C18:3,  $\omega$ 6)  
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49 85 acids [25]. Further, microalgae biodiesel rich taxa possess a considerable biomass  
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51 86 productivity and lipid content [26-30]. Besides, fatty acids composition and lipid content  
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54 87 have been used as chemotaxonomic markers to characterize closely related taxa at the generic  
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56 88 and the species levels [31,32]. Moreover, in Matsumoto's perspective, the vital role of lipids  
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59 89 for storage products and structural components of cell membranes is of great significance,  
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mainly for extremophilic algae in life-threatening conditions [33] such as terrestrial algae in arid habitats.

Dry desert ecosystems are algal–biodiversity hotspots, and also considered one of the most significant biomes for the green coccoids [5,34-37]. Representatives of these microalgae are not only important for shaping the topsoil communities, but they also interact with the other microorganisms in the ecosystems resulting in soil stability, cycling of soil nutrients, and prevention of soil erosion by increasing the water–stable aggregates stability rate (WSAR) [7,38].

In Iran, there are semi-arid to extremely arid zones in the deserts covering approximately 42.5% of the territory (~1,648,195 km<sup>2</sup>), and comprising biologically diverse and important ecosystems [39]. Until now, only few studies have been conducted to uncover the diversity of the desert-soil algal flora in Iran, and most of these reports were primarily concerned with cyanobacteria whilst green algae have been only poorly documented, using traditional morphotaxonomy [40-44]. The main objective of the present study was thus to describe and establish the novel green algal species *Valeriella persica* sp. nov. isolated from the extremely arid desert soil in Yazd (Iran) using detailed morphotaxonomy, phylogenetic placement, and autecological characterization. Another goal was to improve understanding of its ecophysiological-adaptive mechanisms to the harsh desert conditions, in particular in terms of FAs profile. Moreover, biodiesel properties were evaluated to assess the potential of the novel species as feedstock for biofuel production.

## 2. Materials and methods

## 2.1. Study area, sampling and growth conditions

Tabas crossroad (32° 2' N, 54° 12' E), located in the northwest of Yazd city and about 950 km southeast of Tehran, is characterized by an extremely arid and hot climate [39]. Mean annual precipitation equals ca. 50 mm, and mean annual air temperature is ~20 °C, and the average summer temperature frequently exceeds 40 °C [39]. Yazd is one of the ancient regions of Iran, and since 2017 it is recognized as a World Heritage Site by UNESCO.

The soil sample was collected in August 2013 from the subsurface desert soils of Tabas crossroad based on the method adopted by John [45]. Three subsurface soil samples, at a depth of c. 20 cm, were chosen and mixed together to obtain a representative combined sample for this site. The composite soil sample was used for algal cultivation and also for the determination of the soil properties. BG-11 medium, solidified with 1% agar [46], was used for the soil cultivation at  $25 \pm 2$  °C temperature and 16:8 h L:D photoperiod at an irradiance of 53  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  provided by white fluorescent lamps. Our interesting strain, *Valeriella persica*, was isolated using the plating technique method as described in Flechtner et al. [47]. The purified strain was finally inoculated on an agarized BG-11 medium, as well as in a liquid medium, for six months to observe and document all its different life stages and reproduction modes.

## 2.2. Soil analysis

Physical and chemical properties of the soil were analyzed in the soil: water extract (1:5 w/v) as described by Chapman and Pratt [48], including pH value, electrical conductivity (EC),



total dissolved solids (T.D.S.), moisture content (%), total saturation extract capability, the major cations and anions, total nitrogen, available phosphorus, and heavy elements.

### 2.3. Morphological characterization

The main diagnostic algal traits were investigated by light, fluorescent, and transmission electron microscopes. The taxon was regularly observed to document all its morphotaxonomic features. Light microscopy (LM) photomicrographs were obtained using an Olympus BH-2 light microscope (Olympus, Tokyo, Japan) equipped with a Canon camera 1200D (Canon Inc, Tokyo, Japan). Melzer's reagent was also used to determine the pyrenoid number. Morphometric data were based on a minimum of 25 measurements for each character.

For the transmission electron microscopy (TEM) examination, cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 24 h and post-fixed in 2% OsO<sub>4</sub> in the same buffer. After dehydration through an ascending ethanol and acetone series, fixed cells were embedded in Araldite and Poly/Bed<sup>®</sup> 812 mixture. Ultrathin sections were stained using uranyl acetate and lead citrate. The TEM grids were examined with a JEOL 1011 TEM (JEOL Ltd., Tokyo, Japan). TEM observations were performed using a Veleta CCD camera equipped with image analysis software Olympus Soft Imaging Solution GmbH (Münster, Germany) and later modified by Inkscape 0.92. Fluorescent DAPI staining was imaged using an Olympus BX51 microscope equipped with an Olympus DP71 digital camera (Olympus, Tokyo, Japan).

#### 2.4. PCR amplification, sequencing, and phylogenetic analysis

The genomic DNA of the strain was extracted using a DNA extraction kit (QIAGEN). PCR amplification was performed according to De Wever et al. [49]. The nucleotide sequences of 18S rDNAs were amplified using 20F (GTAGTCATATGCTTGTCTC) and CH1750R (CTTCCTCTARTGGGGAGG) primers. Amplification was done in a 26 µL volume containing 12 µL Plain Combi PP Master Mix (Top-Bio, Czech Republic), 12 µL sterile bidistilled water, 0.5 µM of each primer and 1 µL DNA, with PCR conditions as follows: 5 min at 95 °C, 35 cycles of 1 min at 94 °C, 1 min at 56 °C, 3 min at 72 °C, and followed by a final extension at 72 °C for 10 minutes. The PCR products were sequenced in SeqMe, s.r.o. (Dobříš, Czech Republic). All consensus DNA sequences were generated using Geneious Prime v. 2.22 (Biomatters, Auckland, New Zealand). The selection of representative NCBI accessions for phylogenetic analyses was performed following Darienko and Pröschold [1] and the results of BLAST searches (National Center for Biotechnology Information, Bethesda, USA; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>; accessed on 10 December 2022). Alignment was performed with the MAFFT plugin in Geneious with manual adjustment. A dataset of 18S rDNA included 69 accessions (taxa, accession number and strain name (if available) are given) belonging to the Chlorophyceae and Chlamydomonadophyceae (outgroup), 1803 aligned positions. The best evolutionary model GTR+I+G was determined using jModelTest 2.1.1 [50]. Phylogenetic trees were constructed using the maximum likelihood (ML) method in RAxML v.7.2.6 (<http://embnet.vital-it.ch/raxml-bb/>; accessed on 12 December 2022; [51] and Bayesian inference (BI) in MrBayes v.3.1.2 [52]. In BI, four runs of four Markov chains were executed for 5 million

generations, sampling every 100 generations for a total of 50000 samples. The convergence of the stationary distribution was assessed by ESS values (>200) using Tracer v.1.7.1 [53]. Convergence of the chains was assessed, and stationarity was determined according to the “sump” plot, with the first 12500 samples (25%) discarded as burn-in; posterior probabilities were calculated from trees sampled during the stationary phase. The robustness of the ML trees was estimated by examining the bootstrap percentages (BPs; [54] and posterior probabilities (PPs) in BI. Those with BPs <50% and PPs <0.95 were not considered. MEGA v.7.0.26 [55] was used to estimate interspecific pairwise distances (uncorrected *p*-distances). The new 18S rDNA sequence from this study was submitted to the NCBI GenBank database and has the accession number MN708552.

## 2.5. Lipid assessment and fatty acids profiling

As described above, the unialgal strain was cultivated under standard growth conditions until reaching the late exponential phase after 30 days. The cell density was monitored regularly at a wavelength of 680 nm using a Unico 2100 Vis Spectrophotometer (Shanghai, China). The cell dry mass was determined by centrifuging 40 mL of the sample at 5000 rpm for 10 min and then drying at 60 °C until obtaining a constant weight. Biomass productivity ( $\text{g L}^{-1} \text{ day}^{-1}$ ) was assessed as the ratio of the biomass produced to the cultivation period. The total lipid content was gravimetrically extracted with a chloroform/methanol (2:1 v/v) mixture following the Bligh and Dyer’s method as modified by Kates and Volcani [56]. The lipid productivity ( $\text{mg L}^{-1} \text{ day}^{-1}$ ) was calculated as the product of the biomass productivity and lipid content of cells.

The fatty acid profiles analysis was performed by gas chromatography (GC) with flame-ionization detection (GC-FID) as described by Prada et al. [57]. FAs methyl esters (FAMES) were prepared following the procedure described by Mason and Waller [58]. Briefly, 250  $\mu$ L methanolic HCl (3M) was added into 1 mL of each lipidomic extract. The tube was then tightly capped with a Teflon-lined cap and subsequently incubated at 60 °C for 20 min. Characterization of the FAME-derivatives was conducted by gas chromatography (GC) coupled with flame-ionization detection (GC-FID) (Agilent 6890 gas chromatograph, Agilent Technologies, USA) according to the protocol described by Prada et al. [57].

## 2.6. Biofuel properties

The biodiesel properties of the investigated taxon were estimated based on the length of the carbon chain, the position of double bonds, molecular weight and degree of unsaturation of FAME. Average Degree of Unsaturation (ADU%), kinematic viscosity ( $\nu_i$ ,  $\text{mm}^2 \text{s}^{-1}$ ), density ( $\rho$ ), cloud point (CP), cetane number (CN), iodine value (IV,  $\text{g I}_2.100 \text{ g}^{-1}$  oil), Higher heating value (HHV), saponification value (SV,  $\text{mg KOH g}^{-1}$ ), long chain saturation factor (LCSF, wt%), cold filter plugging point (CFPP, °C) and oxidative stability (OS) were evaluated using the methodology proposed by Park et al. [59], Hoekman et al. [60], Ma et al. [61], Nascimento et al. [62], Song et al. [63] and Francisco et al. [64].

$$ADU = \sum N \times Mf \quad (1)$$

$$\nu_i = -0.6313 ADU + 5.2065 \quad (2)$$

$$\rho = 0.0055 ADU + 0.8726 \quad (3)$$

$$CP = -3.356 ADU + 19.994 \quad (4)$$

$$CN = -6.6684 ADU + 62.876 \quad (5)$$

$$IV = 74.373 ADU + 12.71 \quad (6)$$

$$HHV = 1.7601 ADU + 38.534 \quad (7)$$

$$SV = \Sigma(560 \times Ni)/Mi \quad (8)$$

$$LCSF = (0.1 \times C16:0) + (0.5 \times C18:0) + (1 \times C20:0) + (2 \times C24:0) \quad (9)$$

$$CFPP = (3.1417 \times LCSF) - 16.477 \quad (10)$$

$$OS(h) = (117.9295/C18p) + 2.5905 \quad (11)$$

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236 where  $N$  is the number of carbon-carbon double bonds in FA,  $M_f$  is the mass fraction of each  
 237 fatty acid,  $N_i$  is the percentage of each FAME,  $M_i$  is the molecular weight of each FAME,  
 238 C16:0, C18:0, C20:0, C22:0 and C24:0 are the weight percentage of the corresponding fatty  
 239 acids, and C18p represents the content (weight %) of linoleic (C18:2) and linolenic acids  
 240 (C18:3).

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## 242 2.7. Statistical analysis

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244 Statistical data analysis was conducted using SPSS software version 26 (Package for the  
 245 Social Sciences, SPSS Inc., Chicago ILA).

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### 3. Results and discussion

#### 3.1. Morphological observations

*Valeriella persica* sp. nov. Salehipour-Bavarsad, Riahi, Shariatmadari, Heidari, Cantonati, Nikulin et A.A. Saber (Figs 1–7; Figs S1–S9 in the Supporting Information)

**Description** Spherical cells, 10–15 (45)  $\mu\text{m}$  in diameter, rarely up to 70  $\mu\text{m}$  (Fig. 1 A-C; Fig. S1). Cell wall thin and smooth in young cells (Fig. 1 A, C), becoming distinctly thickened and layered in older cells (Fig. 1 b, E-H). The plastid is at first single, parietal in young cells (Fig. 1 C), becoming reticulate at maturity (Fig. 1 B) and rarely spongiform (Fig. S2). Usually one off-center pyrenoid or more pyrenoids present and surrounded by a prominent starch-plates layer (Fig. 1 D, E, H; Fig. S2). Mature cells accumulate orange pigments starting from their center, eventually filling most of the cell with dull-orange lipids and pigments (Fig. 1 F). Young cells are uninucleate while the older cells are distinctly multinucleated (Fig. 1 I-L). Bubble-caps, protosiphon bud-like structures and rhizopodial-like forms are present on vegetative cell walls to enter the reproductive stage (Fig. 2). Cell solitary to rarely aggregate and the colonial aggregated cells are usually embedded in gelatinous matrices and surrounded by a delicate mucilaginous envelope (Fig. 3; videos 1, 2). Active zoospores with flagella and zoosporangia were not observed. “Multimorphic-cells’ cloud” (MC) formation by progressive cleavage in giant parent cells, producing several cells differing in shape, size and color (Fig. 4; Fig. S4; Videos 3-7). Asexual reproduction by the formation of 2-many autospores, 2–4  $\mu\text{m}$  in diameter (Fig. 5 A-C; Fig. S3). Autosporangia

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5 271 15 µm or larger. Tetrad formation (Fig. 5 D-F), binary fission (Fig. 5 G, H), and budding  
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7 272 (Fig. 5 I-M) were also observed as vegetative reproduction modes. Sexual reproduction  
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9 273 occurs by the hologamy (Fig. 6 A-C), depletion (Fig. 6 D-J), and oomycete-type (Fig. 6 K-  
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11 274 M) modes. Zygotes present (Fig. 6 N-P; Fig. S5).  
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15 275 Transmission electron micrographs of *Valeriella persica* are shown in Figure 7 and  
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17 276 Figure S6. Vegetative cells have thickened bi-layered, an inner electron-dense and an outer  
18  
19 277 thinner, cell walls with distinctive hairy exopolysaccharides. At maturity, the chloroplast  
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21 278 fragmented throughout the cell to be finally reticulate with dense thylakoids and a prominent,  
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23 279 single (to numerous), almost spheroidal pyrenoid surrounded by a ring of thicker starch plates  
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25 280 ( $n = 3-5$ ). The cell is consistently multinucleated and the nuclei are located next to the  
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27 281 pyrenoid. Several cytoplasmic vacuoles filled with electron-dense and transparent contents,  
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29 282 and lipid bodies are distinctly scattered throughout the cell.  
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34 283 **Holotype** Permanent slide with the accession number HSBU-2020101 at the Phycology  
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36 284 Unit of Shahid Beheshti University, Tehran, Iran.  
37  
38

39 285 **Isotypes** Permanent slide and living material with the accession number CAIA SAA-2101  
40  
41 286 was deposited at the Phycology Unit (No. 341), the Botany Department, Faculty of Science,  
42  
43 287 Ain Shams University, Cairo, Egypt; and permanent slide MUSE-LIM-FITB 1056 at MUSE  
44  
45 288 – Museo delle Scienze, Research and Collections Department (Limnology and Phycology).  
46  
47 289 An exsiccate material of the strain (HSBU-2020102) conserved at the Phycology Unit of  
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49 290 Shahid Beheshti University, Tehran, Iran.  
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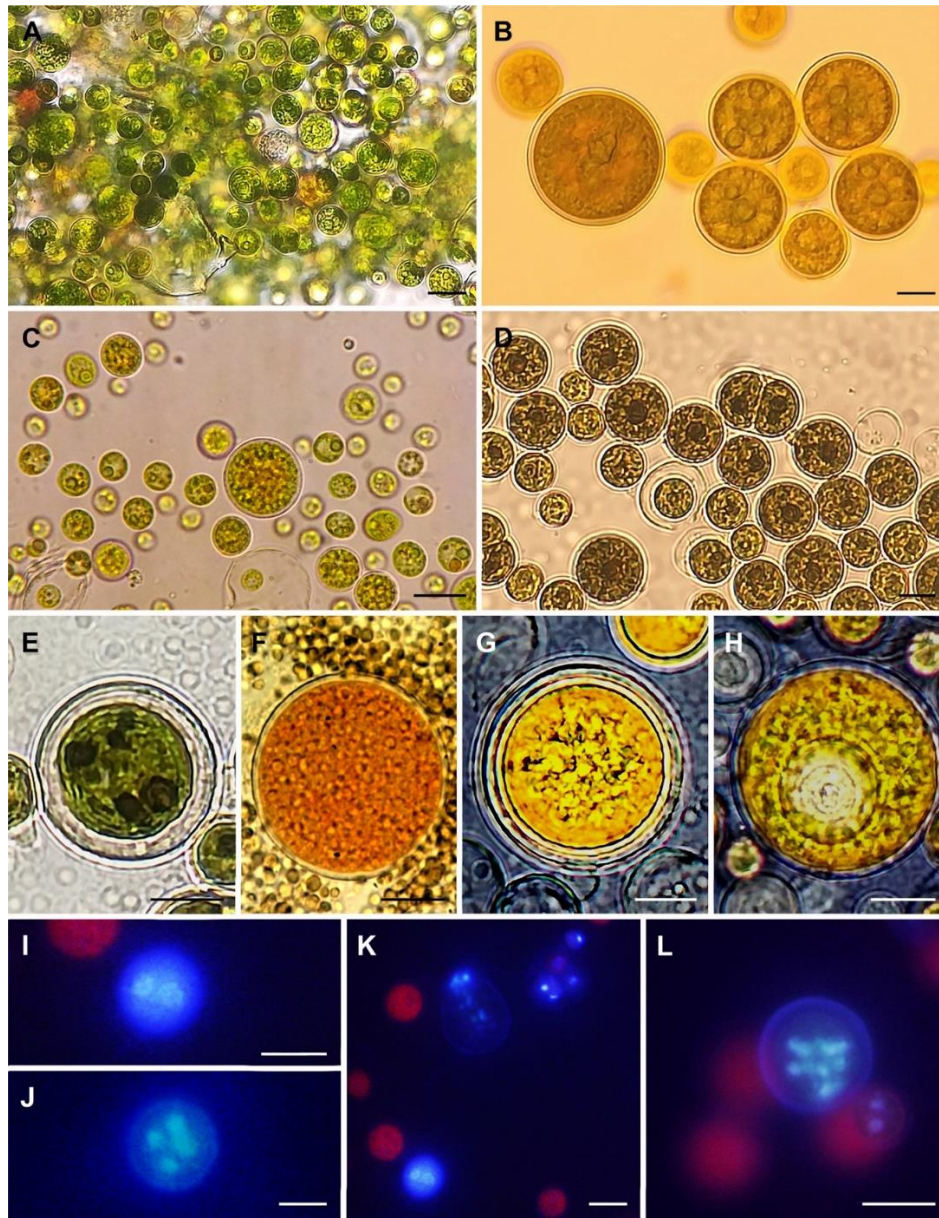
53 291 **Type locality (habitat)** Hyperarid soil, Tabas crossroad, Yazd, Iran (32° 2' N, 54° 12' E).  
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56 292 **Legit** Maryam Ahlesaadat, August 2013.  
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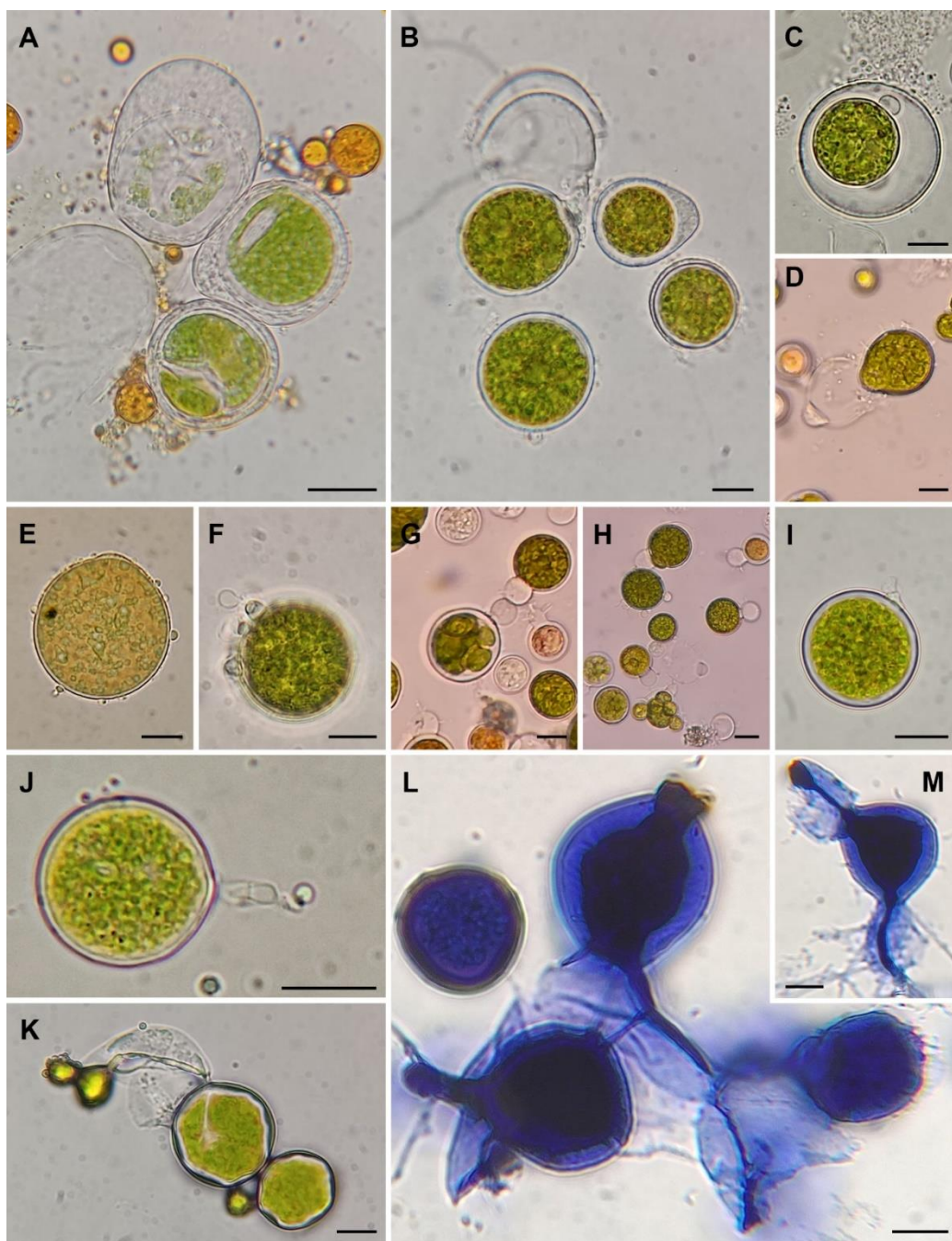
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293     **Etymology**   The specific epithet “*persica*” refers to the Latin name of ancient Iran, *Persia*,  
294     to highlight the discovery of this new algal species in this country.  
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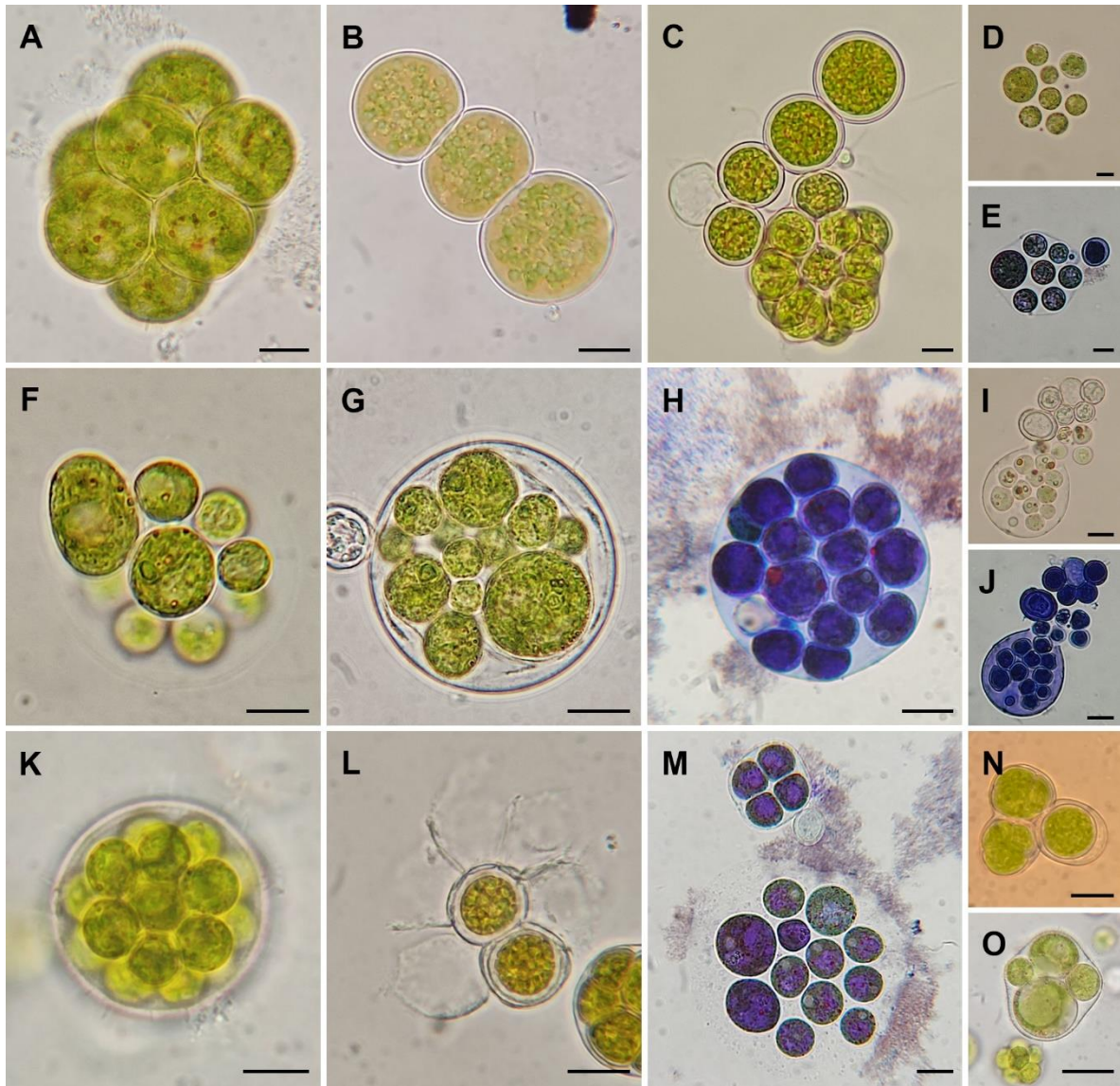


**Fig. 1.** Light micrographs of *Valeriella persica* sp. nov.: (A) young vegetative cells with thin and smooth walls and parietal plastids; (B) mature cells with thick walls, reticulate plastids and one to several pyrenoids; (C) newly formed vegetative cells with cup-shaped and parietal plastids; (D) cells stained with Melzer's reagent showing the pyrenoids; (E) mature cell showing pyrenoids and cell wall thickening; (F) 2-month-old mature cell with a thickened wall, and accumulating carotenoid pigments; (G, H) aging cells stained with the Indian ink depicting the thickened and layered cell walls, and a large pyrenoid; (I–L) cells stained with DAPI showing the nuclei. Scale bars 10  $\mu$ m.

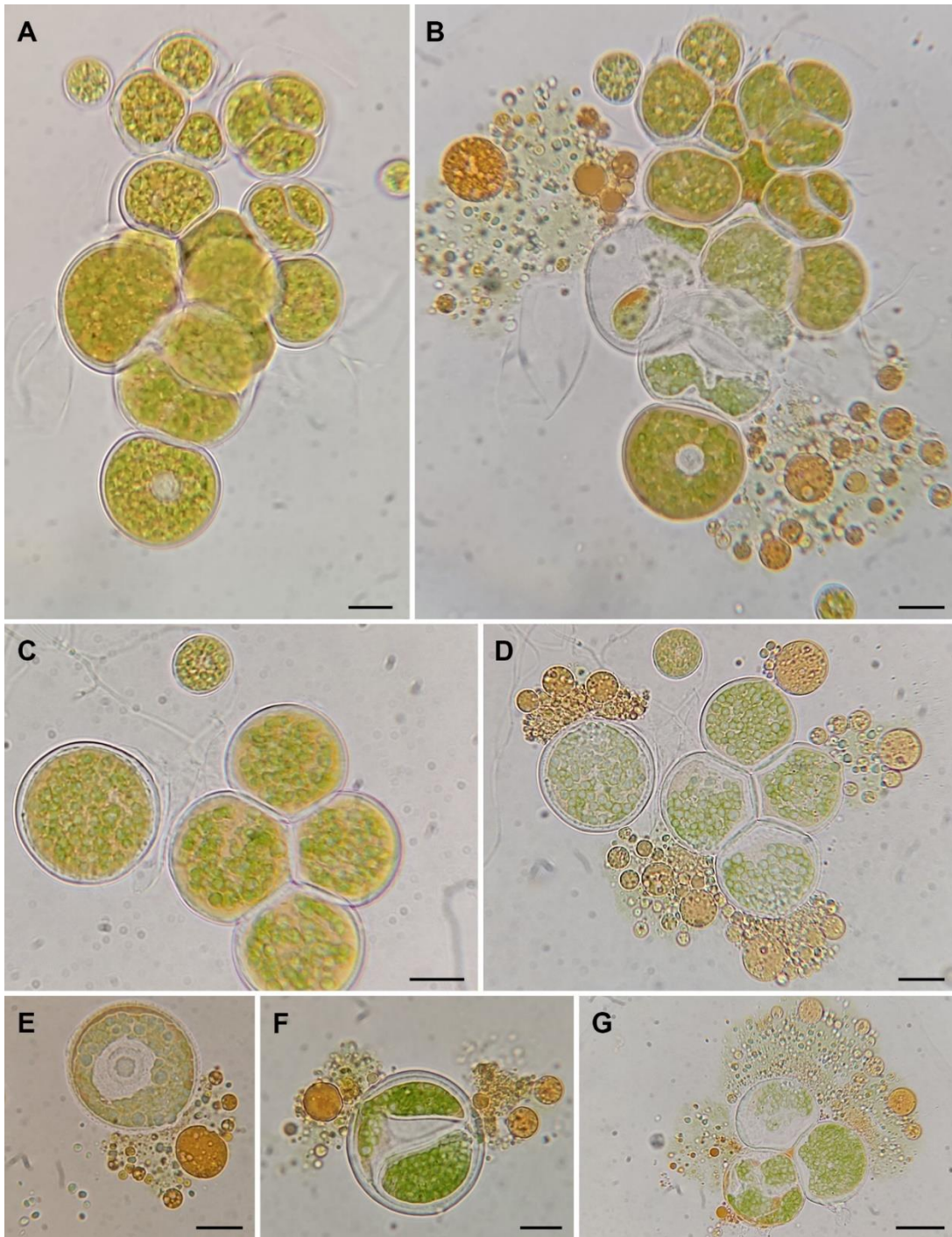


**Fig. 2.** Light micrographs of *Valeriella persica* sp. nov.: (A, B) giant cells covered by even and uneven thickened walls; (C) a cell with uneven cell wall thickening and a bubble; (D) a saccate cell out of the cell wall. Note the bubble cap; (E) a bubbly parent cell; (F) a cell with several bubbles; (G, H) cells with several spines on the caps; (I, J) cells with protosiphon-like structures on the cell walls; (K–M) cells stained with methylene blue showing rhizopodial-like structures. Scale bars 10 μm.



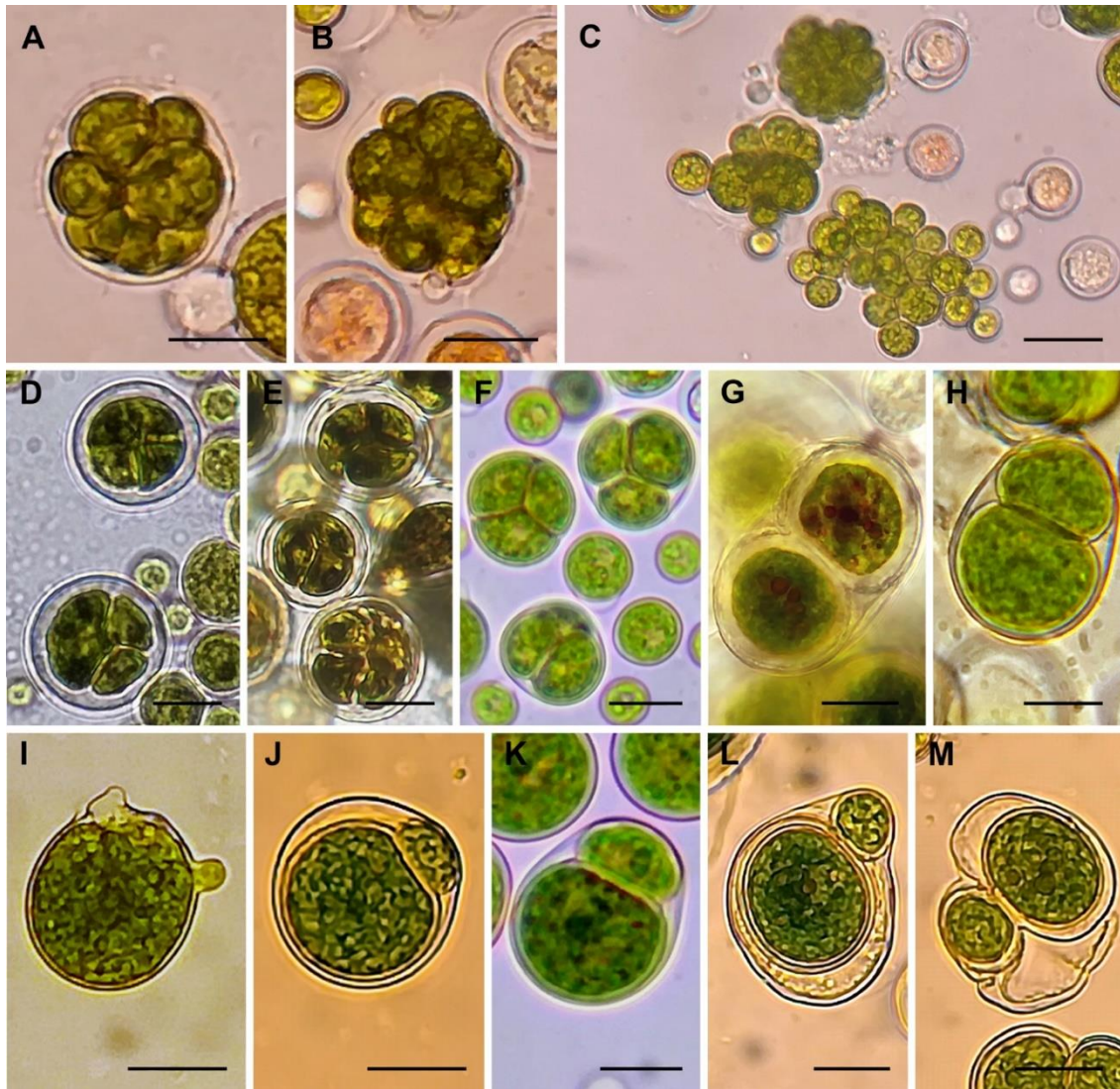


**Fig. 3.** Light micrographs of *Valeriella persica* sp. nov.: (A) aggregated giant cells; (B, C) cell aggregation in pseudo-filamentous form; (D–F) colonial cells in jelly matrices surrounded by weakly visible envelopes; (G–J) colonial cells surrounded by thick walls of parent cells; (K) a coenobial-like colony; (L) two cells of seven cells in a coenobial-like colony. Note the connections; (M) colonial cells stained with methylene blue surrounded by envelope; (N, O) colonial cells with independent division. Scale bars 10  $\mu$ m.



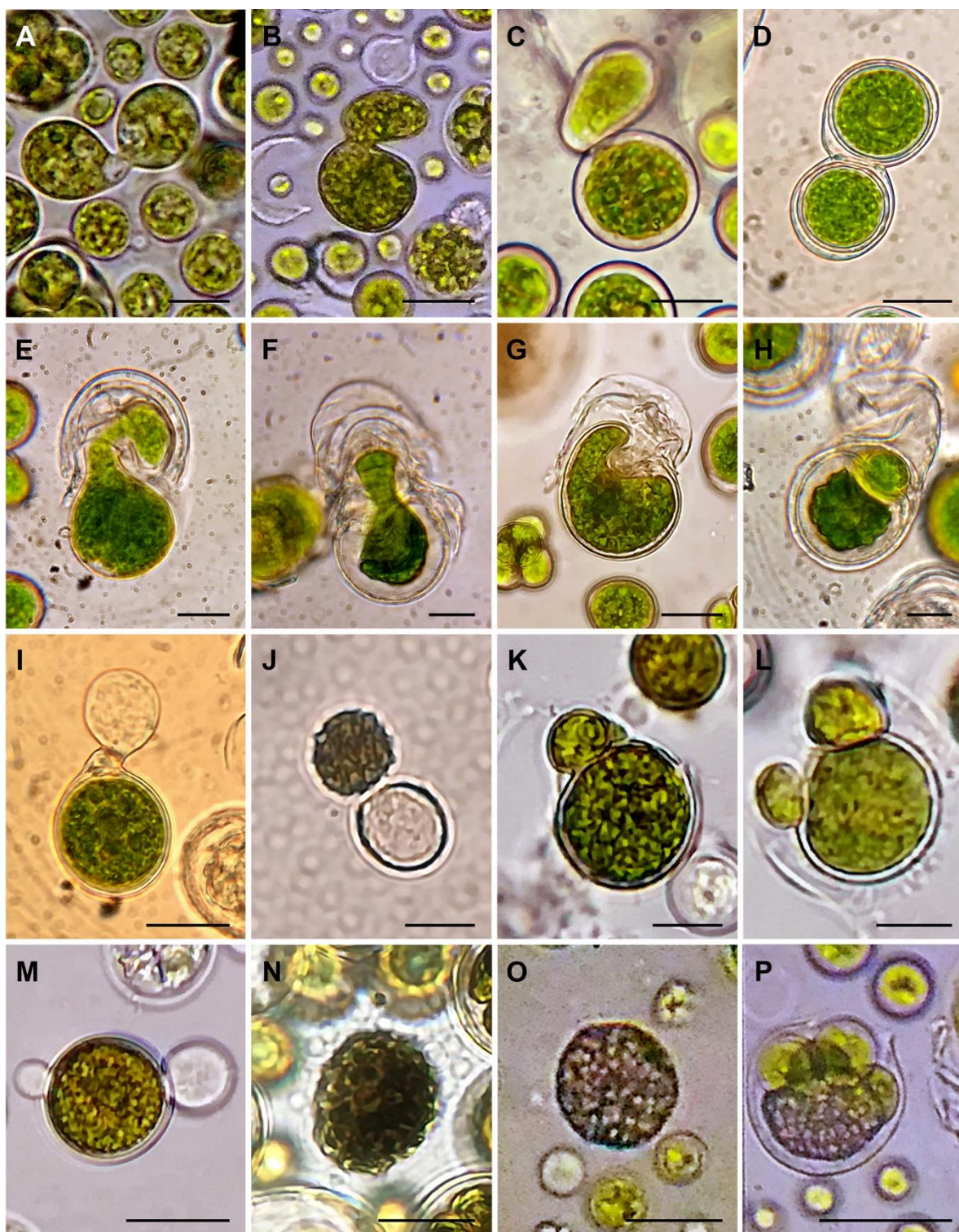
**Fig. 4.** Light micrographs of *Valeriella persica* sp. nov.: (A, C) aggregated giant cells before releasing multimorphic-cells' cloud; (B, D-G) giant parent cells after releasing multimorphic-cells' cloud. Note the different size, shape and color of the released cells. Scale bars 10 μm.





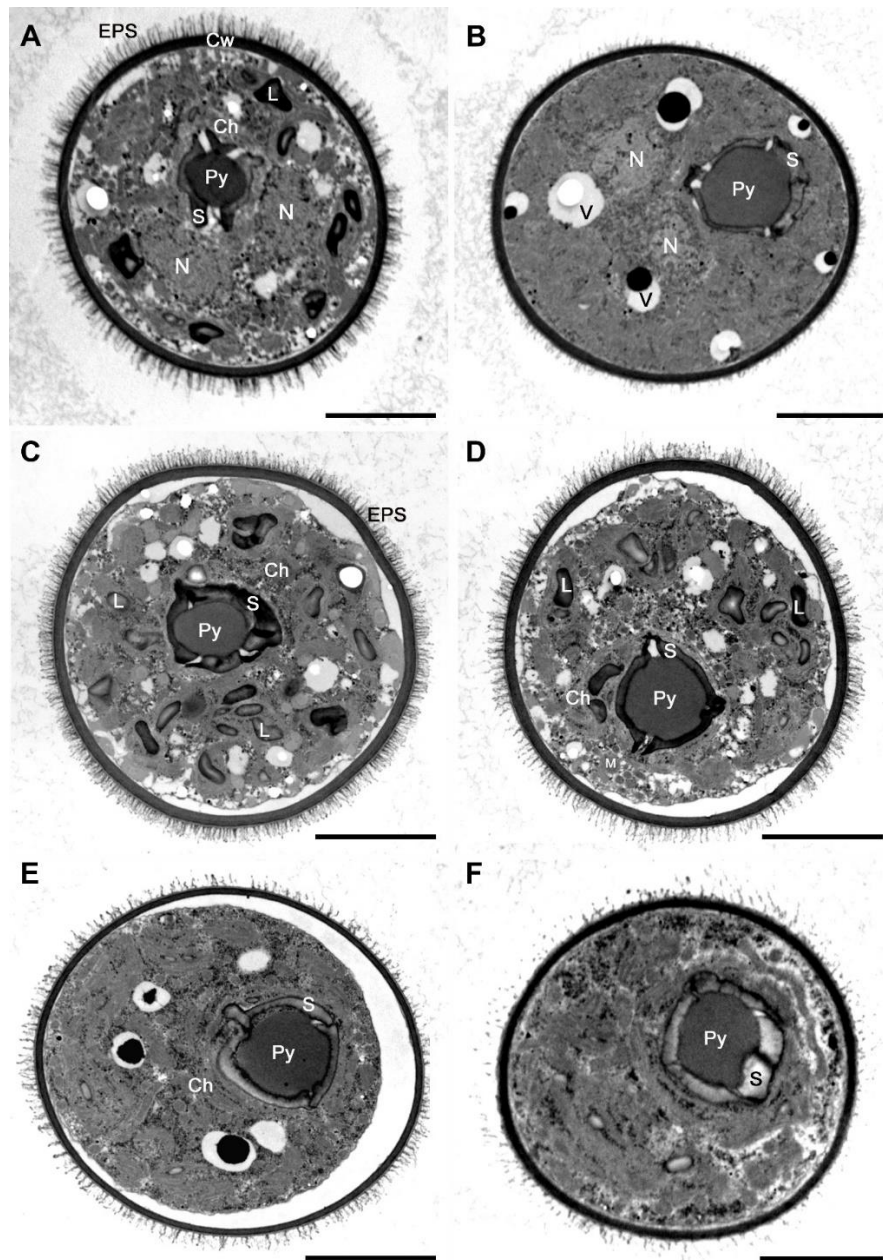
**Fig. 5.** Asexual and vegetative reproduction of *Valeriella persica* sp. nov.: (A–C) autosporangia with numerous autospores; (D–F) tetrad formation; (G, H) binary fission; (I–M) budding. Scale bars 10 μm.





**Fig. 6.** Sexual reproduction of *Valeriella persica* sp. nov.: (A–C) hologamy; (D–J) depletion; (K–M) oomycete-type; (N–P) zygotes. Scale bars 10  $\mu$ m, except (A, B, J, O and P) 20  $\mu$ m.





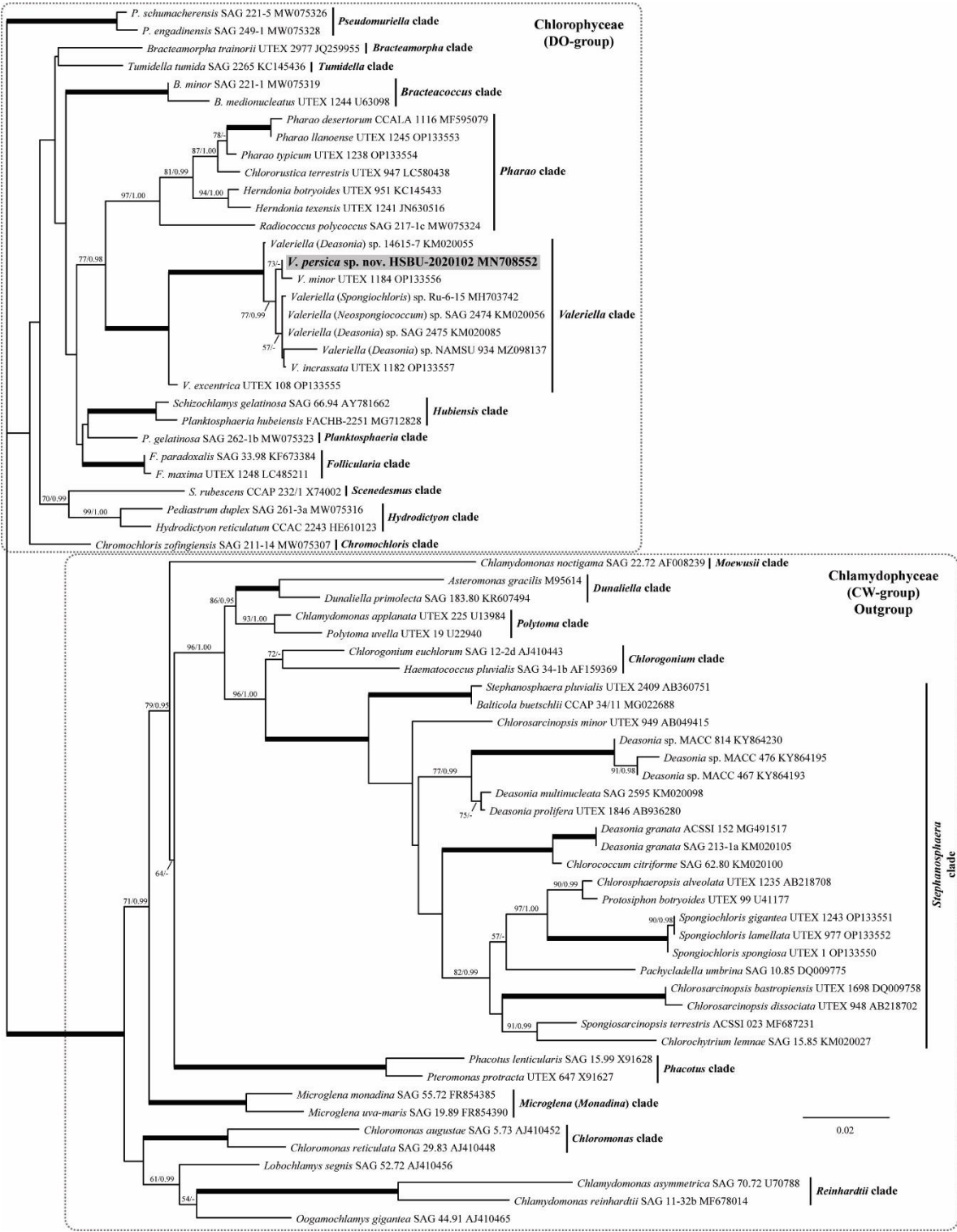
**Fig. 7.** Transmission electron micrographs of *Valeriella persica* sp. nov.: (A, B) details of vegetative cells showing a dense cytoplasm, large pyrenoid, two nuclei per cell, and the thickened cell wall with hairy exopolysaccharides; (C–F) details of cells showing structure of the starch plates, numerous lipid bodies in the cell, and vacuoles filled with electron–dense and transparent contents. Ch, chloroplast; Cw, cell wall; EPS, exopolysaccharides; N, nucleus; Py, pyrenoid; S, starch plates; V, cytoplasmic electron–dense and transparent vacuoles. Scale bars 3 μm.

### 3.2. Molecular phylogeny

In order to determine the correct taxon assignment, a phylogenetic inference was performed using the 18S rDNA gene sequencing of our strain and some other related sequences retrieved from the NCBI GenBank. As shown in Fig. 8, the accessions belong to two groups of the Chlorophyceae: the Chlamydoephyceae (Clockwise-group; CW) and the Direct-Opposite (DO) group of the Chlorophyceae sensu Mattox and Stewart [65]. Based on the phylogenetic analysis of the 18S rDNA gene region, our isolate (with the accession number MN708552) was placed in a well-supported lineage (100/1.00 by PP/BI) inside the Chlorophyceae comprised *Valeriella* species, and a few taxa with questionable identification (*Deasonia* sp. 14615-7 KM020055, *Spongiochloris* sp. Ru-6-15 MH703742, *Neospongiococcum* sp. SAG 2474 KM020056, *Deasonia* sp. SAG 2475 KM020085, *Deasonia* sp. NAMSU 934 MZ098137). The latter taxa we consider to belong to the genus *Valeriella* on the basis of phylogenetic data. The strong placement of *V. excentrica* UTEX 108 OP133555 (100/1.00) followed by diversification to more basal *Deasonia* sp. 14615-7 KM020055 lineage and the two diverged subclades (77/0.99). The first subclade harbored *V. persica* sp. nov. and *V. minor* UTEX 1184 OP133556, while the second subclade comprised *V. incrassata* UTEX 1182 OP133557 and the rest probably undescribed *Valeriella* taxa.



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**Fig. 8.** ML phylogenetic tree (GTR+I+G model) of the Chlamydomonadales and Chlorophyceae (Chlorophyta) showing position of the new species *Valeriella persica* (showed boldface) based on 18S rDNA sequence data (1803 aligned positions of 69 sequences). The strain designations and GenBank accession numbers of all sequences used in the analyses are given. Support [(BP)  $\geq$  50% and (PP)  $\geq$  0.95: ML/BI] are provided above/below the branches. Branches with 100% BP and 1.00 PP are shown in boldface. Clade designations follow Darienko & Pröschold [1]. *V. persica* was nested in phylogenetic proximity with *V. minor* (73/-, where «-» means no significant support), but differed by three base substitutions in the conservative 18S rDNA.

Intragenetic *p*-distances between *Valeriella* accessions ranged from 0 to  $2.31 \pm 0.6\%$  (Table 1). *Spongiocloris* sp. Ru-6-15, *Neospongiococcum* sp. SAG 2474, *Deasonia* sp. SAG 2475 shared extra small evolutionary dissimilarly distances (from 0 to  $0.11 \pm 0.07\%$ ) with *V. incrassata* UTEX 1182. The authentic strains of the aforementioned taxa should be revised taxonomically using the state-of-the-art taxonomy standards to precisely define their taxonomic positions. 18S rDNA sequence of the new strain differed from those in other sequences in  $0.18 \pm 0.11\%$  (with *V. minor*) to  $1.21 \pm 0.25\%$  (with *V. excentrica*). On average, from the rest of the sequences, the *V. persica* differed by 0.33%, which not exceeded the difference between known *Valeriella* species. Besides its distinctive 18S rRNA phylogenetic assignment, *V. persica* still differs from the genus *Deasonia* (placed in the outgroup; see Figure 8), and other phylogenetically close *Valeriella* isolates, by a combination of taxonomic characters including the chloroplast shape, pyrenoid number, multiple nuclei, the vegetative, asexual and sexual reproductive manner. Considering all the combined phylogenetic assignment and morphotaxonomic features, our isolate can be resolved taxonomically as a new species of genus *Valeriella* under the green algal family Chlorococcaceae, class Chlorophyceae.

**Table 1.** Percent dissimilarity (100 x uncorrected *p*-distances) between aligned 18S rDNA sequences of *Valeriella persica* sp. nov. and closely related taxa. Standard error estimates are shown above the diagonal.

	1	2	3	4	5	6	7	8	9
1 <i>V. excentrica</i> UTEX 108		0,26 %	0,27 %	0,25 %	0,25 %	0,28 %	0,26 %	0,26 %	0,60 %
2 <i>V. minor</i> UTEX 1184	1,32 %		0,17 %	0,11 %	0,17 %	0,17 %	0,16 %	0,16 %	0,27 %
3 <i>V. incrassata</i> UTEX 1182	1,43 %	0,46 %		0,15 %	0,16 %	0,07 %	0,06 %	0,06 %	0,25 %
4 <i>V. persica</i> sp. nov.	1,21 %	0,18 %	0,30 %		0,14 %	0,16 %	0,13 %	0,13 %	0,25 %
5 <i>Valeriella (Deasonia)</i> sp. 14615-7	1,09 %	0,51 %	0,40 %	0,36 %		0,14 %	0,14 %	0,14 %	0,37 %
6 <i>Valeriella (Spongiochloris)</i> sp. Ru-6-15	1,43 %	0,46 %	0,11 %	0,30 %	0,40 %		0,06 %	0,06 %	0,29 %
<i>Valeriella (Neospongiococcum)</i> sp.									
7 SAG 2474	1,37 %	0,40 %	0,06 %	0,24 %	0,34 %	0,06 %		0,00 %	0,25 %
8 <i>Valeriella (Deasonia)</i> sp. SAG 2475	1,37 %	0,40 %	0,06 %	0,24 %	0,34 %	0,06 %	0,00 %		0,25 %
9 <i>Valeriella (Deasonia)</i> sp. NAMSU 934	2,31 %	0,68 %	0,54 %	0,54 %	0,95 %	0,68 %	0,54 %	0,54 %	

### 3.3. Taxonomic perspectives

In the present study, a novel coccoid green algal species, named *Valeriella persica* sp. nov., was discovered from the extremely hyperarid Tabas crossroad soil in Yazd city (Iran) based on an integrative polyphasic investigation. According to modern taxonomic concepts, the strain belongs to the recently established algal genus *Valeriella*. Analysis of the molecular data indicated its close affiliation with this genus, being most closely related to *V. minor*. At the morphological level, the shared characteristics were: chloroplast type, pyrenoid features, and nuclei numbers. From the ecological standpoint, *Valeriella* spp. are usually found in terrestrial environments, particularly desert soils (Table 2). The differences between the Iranian strain and its closest relatives are: (1) relatively smaller size of vegetative cells; (2)

diversity in cell shape; (3) the presence of EPS bristles; (4) cell organization; and (5) remarkable thickness of the cell wall. Another specific feature is the MC formation. Furthermore, asexual, sexual and unusual modes of reproduction are quite unprecedented (Table 2). Our results are key findings in the taxonomic identification of green microalgae, which are all pointing phycologists toward features that may as well be relevant for other coccoids.

**Table 2** Comparison of morphometric data of *Valeriella persica* sp. nov. with the most closely related species.

Morphological characteristics	<i>Valeriella persica</i>	<i>Valeriella minor</i>	<i>Valeriella incrassata</i>	<i>Valeriella excentrica</i>
cell shape	spherical, sometimes irregular, saccate, pyriform, ovoid or subspherical	spherical at all ages	spherical	spherical
cell size	10–15 µm,	36–40 µm	38–54 µm	30–60 µm
cell organization	up to 70 µm solitary to aggregated	solitary	solitary	solitary
nucleus	uni- to multinucleate	uni– to multinucleate	uni– to multinucleate	uni– to multinucleate
chloroplast	parietal to spongiform and reticulate	parietal to spongy and reticulate	parietal to spongy and reticulate	parietal to spongy and reticulate
pyrenoids	one or more	one or more	one or more	one or more
starch sheath	smooth and discontinuous	–	–	–
pyrenoid matrix	homogenous	homogenous	homogenous	homogenous

extracellular	EPS bristles	—	—	secreting a gelatinous substance
matrix				
asexual reproduction	autospores, multimorphic–cells’ cloud, tetrad formation, binary fission and budding	autospores and zoospores	autospores and zoospores	autospores and zoospores
sexual reproduction	hologamy, depletion, and oomycete–type	not observed	not observed	not observed
zygotes	always present at the late stationary phase	not observed	not observed	not observed
zoospores type	not observed	protosiphon–type (without cell walls)	protosiphon–type (without cell walls)	protosiphon–type (without cell walls)
habitats	soil	soil	soil	soil
reference	this study	[66]	[66]	[67]

A key point needs to be discussed here: on the basis of morphotaxonomic criteria, *Valeriella persica* revealed a high similarity to some established genera of the Chlorophycean coccoids. Morphologically, the most resembling genus is *Deasonia* H. Ettl & J. Komárek, that was established to include the multinucleate species of the genus *Neospongiococcum* Deason. In this genus cells are coenocytic with parietal to reticulate chloroplasts, storage vacuoles present, and cell wall thickening at maturity. These characteristics are in common with the new species *V. persica*, in addition to their shared ecological preferences for soil habitats. Other relevant similarities are: MC formation, starch sheath and pyrenoid features,

as well as cell size and the diversity in cell shape from spherical to saccate cells. In other words, at the morphological level, our strain presents greater similarity to *Deasonia* rather than to *Valeriella*, due to missing data for the latter. Thus, in spite of the fact that the Iranian coccoid and *Deasonia* represent independent clades on the phylogenetic tree, they apparently share similar morphological features and MC formation. In the authors' view, the present identification key of *Valeriella* is in need of fundamental revision, in spite of having been published only some months ago. To avoid contributing to the proliferation of genera with a premature new genus description, we have introduced *V. persica* as a new species. Taxonomic interpretation of morphotaxonomic and ultrastructural characters of *V. persica* is described in more detail in the following.

### 3.3.1. Cell wall structure and EPS bristles

*Valeriella persica* possesses a unique cell wall ultrastructure. To be more specific, the cell wall is composed of distinctive bi-layers including a thicker electron-dense inner layer and a thinner outer layer with lower electron density (Fig. 7; Fig. S6). This arrangement of the cell wall layers is also in contrast to the *Chlorella* cell wall [68]. On the other hand, distinctive hair-like exopolysaccharides (EPS) appendages are clearly visible on the cell wall surface using TEM. It is similar to hyaluronan hair-like fibers protruding from the outer layer of *Chlorella vulgaris* [68]. According to Graves et al. [69], these fibers have a possible functional role as defense/prevention against superinfection by the PBCV-1 virus. Furthermore, the EPS matrix in *V. persica* may provide a protective mechanism against UV radiation. It has been documented that the external polysaccharide layer acts as a protective sheath in extremophilic species [70]. Presumably, this matrix might provide a humid

environment around the cells to reduce the rate of evaporation. It is similar to adaptation strategies in xerophyte plants to survive in hyper-arid habitats.

### 3.3.2. Cell wall thickening

Similar to green coccoids, the cell wall of *Valeriella persica* can be thickened “evenly” or “unevenly” with the factor of age. “Even” cell wall thickening has been observed in *Valeriella* spp. (Fig. 1 E-H) and some coccoid-related taxa [5,9,71-73]. “Uneven” cell wall thickening as a unique character was pictured in some species of the family Chlorococcaceae including *Chlorococcum elkhartiense*, *Neosporangiococcum vacuolatum*, *Nautococcus pyriformis* and *Nautococcus soluta* [73,74]. In addition to “uneven” thickening, different terms have been used for this phenomenon such as the “uni- or bipolar” thickening in *Parachlorococcum turfosum* [73] and the “external protuberance” in *Scotinosphaera* [75]. It is worth mentioning that the vegetative cell wall of *V. persica* is unevenly thickened, particularly in liquid medium (Fig. 2 A-C). There is no report of such thickenings in *Valeriella* spp. Archibald [74] believed that the uneven thickening observed in *Nautococcus* is a cap-like structure of the cell wall material on one side of the vegetative cell. She stated that caps are common on vegetative cells of all ages, but most frequently in the stationary phase of growth. It is noted that the presence of caps is subject to type of species, populations and culture conditions [74]. According to Watanabe and Lewis [73], *Chlorococcum elkhartiense* is unevenly thickened at one or two opposite poles to assume a cap-like form in older cells. Thus, cap-like structures are special form of the uneven cell wall thickening. Here, we are of the opinion that cell wall thickening and cap-like structures are two distinct

cell phenomena of the cell function; the latter of which represents unusual cell division as we shall see later.

### 3.3.3. Bubble–cap, protosiphon bud–like structure and rhizopodial–like form

Stated as such, there are cap–like structures in young and mature cells of *Valeriella persica*. The number of bubbles is varied from one, two to many, for instance, bubbly–parent cells bulge out with numerous bubbles (Fig. 2 E). Infrequently, there are several delicate tapering spines on the caps. Although these spines are not always present, they have been also seen on the cell wall in addition to the bubble–caps (Fig. 2 G, H). Occasionally in liquid medium culture, a bubble is placed on another bubble to form a protosiphon bud–like structure (Fig. 2 I, J) that is hollow and segmented. This structure is totally different from the saccate cells of *Deasonia saccata*, which are also observed in *V. persica* (Fig. S7 A, C, D). As Deason [71] pointed out, *Deasonia saccata* is delimited by the feature of the tendency to form protosiphon–like sacs on agar at the species–level. It also differs from *Protosiphon* by having ovoid than sac–like cells and *Chlamydomonas*–type zoospores at the genus–level. Moreover, the rhizopodial–like form is another structure in aggregates of *V. persica*, which was not observed in any closely related taxa (Fig. 2 K–M). It seems like colorless cytoplasmic strands for connecting the progeny cells before releasing them from the parent cell. This feature needs to be evaluated separately that is inextricably bound up with the “social behavior” of the unique alga, *V. persica*.

### 3.3.4. Social behavior



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5 482 The nonmotile cells of *Valeriella persica*, whether they are vegetative or reproductive, are  
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8 483 solitary to rarely aggregate (Fig. 3). Although they are usually solitary spherical cells, their  
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10 484 tendency to aggregate in normal conditions is undeniable. These cell aggregations are the  
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13 485 results of different modes of cell division that will be discussed in the following subsection.  
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15 486 Among the cell aggregates, “double”, “quadruple” and “multiple” spherical packs are  
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18 487 abundantly observed in both liquid and solid culture media. These multicellular structures  
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20 488 are comparable to that found in the life cycle of *Chlamydomonas* as the social behavior. This  
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23 489 genus can form aggregates [76] when confronted with harsh stress conditions. Socialization,  
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25 490 as a protective mechanism for better resistance to severe stress, helps vegetative cells adapt  
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28 491 to some stressors like cold conditions [77,78]. Cell aggregations of *V. persica* are not always  
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30 492 in a globe form, and the cells are sometimes arranged along each other linearly or spirally in  
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32 493 a row (Fig. 3 B, C). These pseudo-filamentous forms were found in both young and mature  
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35 494 cells in liquid growth media. Similarly, the progeny cells of *Kirchneriella* form a short  
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37 495 filament while releasing at first and then dissociate into independent unicells [79]. We are  
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40 496 dubious about any cell-cell communication via cytoplasmic bridges in the filamentous form;  
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42 497 only physical contact may be established through the connection of the cell walls. More  
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45 498 interestingly, other colonial cells are held together in a jelly matrix. In this case, the colonial  
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47 499 cells are usually different in size (Fig. 3 D-J), and when they are the same, resembling  
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50 500 autospores within the parent cell (Fig. 3 K). The cells are placed together without any  
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52 501 connection, at a distance from each other, in an invisible sphere. This gelatinous sphere may  
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55 502 be surrounded by a delicate mucilagenous envelope or not. The deposition of gelatinous  
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57 503 material around cells is a valid taxonomic character for green coccoids. By way of  
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59 504 illustration, in the family Chlorococcaceae, common matrices are present in  
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*Neospongiococcum mobile*, and also the cells of *Neospongiococcum gelatinosum* are coherent by their individual matrices [80,81]. Based on the literature, there is no such report for *V. minor* and *V. incrassata*, but cells of *V. excentrica* sometimes secrete a gelatinous substance demonstrable with dilute methylene blue [63]. By contrast, extracellular matrix of *V. persica* cannot be detected with methylene blue or India ink at solitary phase. It seems that to form the gelatinous colonies, the cells of *V. persica* are embedded in gelatinous material of the parent cell without secreting the gelatinous substance. Not only the presence or absence, but the quantity of gelatinous matrix is a determining feature in the taxonomy of coccoids, which depends on ecophysiological conditions of taxa [73]. It was also observed that several cells sometimes form coenobia-like colonies with or without a mucilaginous envelope (Fig. 3 K, L). Most probably these cells retain within parental cell wall, which are interconnected by cell wall connections. These cell connections are simply elucidated by the observed lacunas in the parent cells (Fig. 3 L). It may be hypothesized that the thick walls of the long-lasting aggregates form the coenobia-like colonies. To conclude, these aggregates are colonies of the cells living as clumps with no specialization, similar to the colonial green algae. The colonial cells are often embedded in a gelatinous matrix that may be surrounded by a common envelope. The point is that each cell can survive on its own and divide independently (Fig. 3 N, O). Moreover, the number of cells is not fixed like a coenobium.

### 3.4. Reproductive strategies

*Valeriella persica* represents both singular and colonial forms of microscopic green algae. For this algal group, several levels of organization are introduced, including coccoid,

sarcinoid, coenobial, filamentous and siphonous. Therefore, all packet-like colonies of *V. persica* can be considered as the sarcinoid habit comparable to *Chlorosarcinopsis* and *Chlorosarcina* [79,82]. The sarcinoid green algae can be dissociated into single cells through enzymatic dissolution within the parent cell. It has been demonstrated that the various modes of reproduction in multicellular structures lie at the root of timing in cell division, such as timing of cytokinesis or timing of cell wall deposition. For example, in *Chlorosarcinopsis*, wall formation around the progeny cells is right after each mitotic division, while in *Kirchneriella* there is delayed time after the final deposition of wall material to produce four or more progeny cells by several divisions of the parent cell. Furthermore, there is no crucial distinction between the pattern of development in releasing the progeny cells that was observed in coccoids or multicellular algae [79].

The multinucleated species *Valeriella persica* possesses two types of cell division; progressive cleavage and successive bipartition (Fig. S4). The MC of *V. persica* is produced by progressive cleavage in which cells are different in size shape and color. The final result of progressive cleavage is the formation of incompletely divided cells due to several nuclear divisions, which is followed by later divisions of the protoplast into smaller units. This interesting feature was also observed in the genus *Deasonia* ([71]: Fig 5). Besides, *V. persica* has another type of cell division, successive bipartition, in which a nuclear division followed by the protoplast division into two equal parts. Successive bipartition was also observed in many of the uninucleate taxa that produce uniform zoospores [71,83]. The successive bipartition is responsible for producing progeny cells from mature vegetative cells or encysted zygotes. The progeny cells of *V. persica* which are produced normally can be

considered autospores of the same size. However, unequal-sized autospores may be formed by repeated bipartition in each progeny cell independently (Fig. 5 A-C; Fig. S4).

It should be noted that several methods were applied for zoospore induction [72,84], but we could neither observe zoosporangia nor active zoospores. The formation of MC was based on the method of Starr [85] by transferring the mature (not old) cells of *Valeriella persica* from agar to liquid media; to accelerate the process, we added some alcohol (2% ethanol).

MC formation in *Valeriella persica* is interestingly similar to that observation in *Chlorococcum diplobionticum*. In the words of Herndon [86], protoplast division is induced within the intact parent wall when the protoplast size is increased by water absorption through the flexible wall of the mother cell. In this study, mature cells of *V. persica* become giant cells with a tendency to aggregate by absorbing water (Fig. 4). The wall of these giant cells is more flexible in the presence of alcohol (2% ethanol), and after a few minutes, numerous motile cells and coenocyst-like cells are formed through successive cleavage. There are several remarkable points here. Firstly, the released cells are very different in size, shape and color. The color varies from cyan to green and orange. Secondly, there are probably some motile cells inside the larger orange coenocyst-like cells. Thirdly, with the explosion of the parent cell, all the new cells are usually pushed out along with the sticky matrix. These interesting features were not reported for the other species of *Valeriella*. In opposite to *Chlorococcum diplobionticum*, we could not observe any active zoospore within the parent cell prior to release. The released small cells probably continue to grow to mature, which may be zoospores or spherical vegetative cells. The latter seems more logical to happen as the mature zoospores were not observed in the subsequent investigations. This kind of

zoospore maturity has also been discussed briefly in *Chlorococcum diplobionticum*; the typical green coloration of the zoospores is regained after completed gametogenesis [86]. Notably, we observed some colorless ovoid to ellipsoidal cells, with no flagella. It seems that giant cell explosion depends on temperature, light and circadian rhythm. Under the laboratory conditions, MC released more in the afternoon, while the giant cells tended to become cysts in the morning.

There are number of possible outcomes when MC formation occurs (Fig. S8). By absorbing water, the giant vegetative cells undergo numerous cleavages to form motile cells. The giant cell explosion may occur or not, and the latter forms resistant cyst (Fig. S8 E, F). If the cell wall rupture occurs, three states may happen: (1) almost all the cell contents (the resulting division) are ejected through a pore; (2) a small amount of the cell contents are released through a pore; (3) all the cell contents are driven out through several pores. Each of the released cells will have a different destiny; (1) the small motile cells grow to form vegetative cells or zoospores; (2) the coenocyst-like cells may turn green vegetative cells or gray cysts. Interestingly, all released cells can grow independently, aggregate or merge together. After the rupture, several cells sometimes retain within the parental cell wall that will have different destinies. The remaining cells may form equal/unequal autospores, cyst or colony. Understanding the reproductive process and the consequences will help the taxonomist to identify the challenging group of microalgae. For example, to form the colonial genera *Coelastrum* and *Scenedesmus*, the progeny cells are retained and remain attached to each other [9]. It seems that the ruptured cell wall can be repaired, especially when the wall is present in colonial forms and cysts. There are some evidences of cell wall decoration.

Below are different reproductive strategies of *V. persica* leading to different forms of this interesting alga from the desert land.

#### *3.4.1. Asexual reproduction*

The novel taxon *Valeriella persica* reproduces asexually via equal autospores by multiple fission. A large parent cell divides into several non-spherical compact cells (Fig. S3), and then numerous small progeny cells are produced (Fig. 5 A-C; Fig. S3). Finally, thin-walled autospores are released through cell wall rupture (Fig. 5 C; Fig. S3). Autospore formation is the most common type of reproduction in *V. persica*, and, generally, in green microalgae enclosed by cell walls [87]. Autospores are morphologically similar to vegetative cells and there is no specific difference between them. These autospores within the parent cells may form a miniature colony that can be considered an autocolony or coenobium [79]. Apart from the aforementioned reproduction “tetrad formation” was also observed in *V. persica* (Fig. 5 D-F). It is a form of vegetative cell division similar to Brown and Bold’s [88] observations in *Spongiococcum tetrasporum*. This 2–4 autospore formation has also been reported in some other genera such as *Chlorosarcinopsis* [89]. In this study, both types of tetrahedral or isobilateral tetrads were observed. The significance of reproductive traits is important to such an extent that the formation of either tetrahedral or isobilateral tetrads led to the differentiation of the two genus *Tetracystis* and *Chlorococcum* [90]. The tetrahedral tetrads are considered as an ancestral trait comparing to the isobilateral tetrads within *Moewusinia* [73].

Two other modes of vegetative propagation were observed in *Valeriella persica*: binary fission and budding. As shown in Figure 5 (G, H), *V. persica* propagates by binary fission.

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5 618 The parent cell–wall gradually expands, and covers the progeny cells during the progression  
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8 619 of cleavage. It is similar to the binary fission type in *Nannochloris* as described by Yamamoto  
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10 620 et al. [91]. In addition, *V. persica* regenerates by budding (Fig. 5 I-M). In this process, small  
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12 621 bulb–like cellular outgrowths are formed from a vegetative cell. The buds keep on increasing  
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14 622 in size, and then detach to make new cells. In addition to yeasts, the budding process has also  
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16 623 been observed in some green algae such as *Marvania* and *Protosiphon* [91,92].  
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20 624 In the authors’ opinion, there are other vegetative reproductive strategies in this genus  
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22 625 that needs more investigation. For example, the spherical cells of *Valeriella persica* form  
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24 626 many irregular shapes before cell division (Fig. S7). Here, we observed the kind of “cell  
25  
26 627 elongation” that was described in *Chlorosphaeropsis alveolata*. Herendon [89] made a valid  
27  
28 628 point that the vegetative cells of *C. alveolata* become elongated to form sacs by analogy with  
29  
30 629 young cells of *Protosiphon*. To draw a comparison, *Protosiphon* develops long tubular sacs  
31  
32 630 by keeping cell elongation, while *Chlorosphaeropsis* undergoes vegetative cell division. This  
33  
34 631 process was also observed in *Deasonia saccata*. Moreover, there is probably an unusual cell  
35  
36 632 division similar to “protocytotomy” that was reported in *Microglena*, *Paludistella* and  
37  
38 633 *Chloromonas* [93-95]. In this process, there is a new elastic cell wall that enlarges within the  
39  
40 634 old parent cell wall. Therefore, the complete cell division takes place inside the parent cell,  
41  
42 635 including the simultaneous division of the protoplast and the cell wall. This division is very  
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44 636 similar to the late binary fission when each progeny cell has its own cell wall (Fig. S7).  
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#### 54 638 3.4.2. Sexual reproduction

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56 639 Contrary to a widely held erroneous belief, sex seems fairly common among  
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59 640 sphaeroplealean lineages under specific environmental conditions [96]. So far, different types  
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of reproduction have been reported in Sphaeropleales including fusion of unusual quadriflagellate gametes in Neochloridaceae, anisogamy or oogamy in Sphaeropleaceae, isogamy in Bracteamorphaceae and Tumidellaceae, etc. [96-98]. Unlike the common types of sexual reproduction, here we have introduced three different modes of sexual reproduction, in which the involved cells are non-flagellated. More to the point, all three types are the fusion of unspecialized gametes. Interestingly, asexual zoospores of *Pediastrum* Meyen were investigated ultrastructurally as gametes to form colonies in this genus [99].

*Valeriella persica* is a member of the green hologamous algae that does not form specialized flagellated gametes. Hologamy is the simplest type of sexual reproduction in which the two involved cells are morphologically indistinguishable from other vegetative cells. The cells become physiologically differentiated as gametes and fuse directly to form zygotes (Fig. 6 A-C). This reproductive manner has already been observed in several members of the Chlorophyta, e.g. *Chlamydomonas*, *Dunaliella*, and *Polytoma*. Another type of genetic integration is the complete depletion of one cell into another through a cytoplasmic bridge (Fig. 6 D-J). The two involved cells often have multiple mucilaginous membranes where the outer layers are destroyed later during ontogenesis. First, two mature cells are placed next to each other. The protoplast of one cell completely moves into another cell through the cytoplasmic bridge. Eventually, the products will be a large syncytium and an empty cell. As illustrated by West [100], the depletion mode is roughly comparable to the fusion of gametes in the genus *Chlamydomonas*. Moreover, a rare type of sexual process was reported herein for *V. persica* (Fig. 6 K-M). It can be considered as a variant of oogamy due to the non-motile reproductive cells. Non-flagellate male gametes are also observed in the sexual reproduction of Rhodophyta as spermatia. Fusion of non-flagellate male and female



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5 664 gametes of dissimilar size via fertilization tubes, resulting in the formation of thick-walled  
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8 665 resistant zygotes. It is noteworthy that sometimes more than one male gamete is involved.  
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10 666 Therefore, this type of reproduction is more similar to that of oomycetes than to that of  
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13 667 Rhodophyta. Due consideration of reproductive modes as important biological characters  
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15 668 supports the impression that our new species *V. persica* might be a cryptic taxon. The best  
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18 669 example to support the rationale for using the term “oomycetes-type” is the sexual  
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20 670 reproduction of *Microglena* [94]. Based on the literature, there are two types of sexual  
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22 671 reproduction in the genus *Microglena*; “oogoniogamy” and “advanced anisogamy” which  
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24  
25 672 are different in (1) possessing flagellated or non-flagellated gametes and (2) the behavior of  
26  
27 673 the macrogamete. In oogoniogamy, the protoplast of non-flagellated macrogamete remains  
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30 674 in its cell wall, unlikely in advanced anisogamy, the gametes are flagellated that lose their  
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32 675 flagella before the fusion. The oomycete-type of sexual reproduction in *V. persica* is  
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35 676 somewhat different from the reproduction of *Microglena*. First, the vegetative cells are in  
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37 677 charge of gametes which are not flagellated. Second, there is one macrogamete with the  
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39 678 function to receive the protoplast of the microgamete. Third, the number of involved cells  
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42 679 that is usually three gametes including one macrogamete and two microgametes. Although  
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44 680 the first two reasons confirm oogoniogamy, the third one is reminiscent of the reproduction  
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47 681 of oomycetes. Furthermore, there are two differences between oomycetes-type and budding.  
48  
49 682 In budding, small bulb-like cellular outgrowths keep on increasing in size to form new cells  
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52 683 while in oomycetes-type, such growth has not been observed. Plus, what is visible at the end  
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54 684 of the budding process is bud scars, but the empty cells of microgametes remain for a while  
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56 685 on the fertilized macrogamete in the oomycetes-type. Moreover, microgametes attach to the  
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outer wall layer of macrogamete not the adjacent protoplasm in the oomycetes-type, whereas in budding outgrowths bulge out from the inner layer.

Zygote formation in *Valeriella persica* is stimulated by nutrient depletion in old cultures, implying that the cells must be decent-sized and sexually-competent. Similarly, this competency can be seen in *Dunaliella salina* [101,102]. Fusion of equally- or unequally-sized cells forms a fused cell (zygote) which is initially green or orange in color and surrounded by one to several layers. The zygote eventually turns gray, loses its surrounding layers, and develops a thick wall. As observed in *Dunaliella salina*, zygote size hinges on the size of the cells involved [101,102]. We also emphasize that not only the size of the zygote, but also its color and number of layers depend on the fused cells (Fig. 6 N-P; Fig. S5).

Reproductive strategies are probably responsible for the multilayered nature of some aging cells. In budding, the pressure is from the inside. A small bulge grows and eventually the outer layer ruptures. Thus, the number of the layers decreases. During depletion, the outer layer usually remains wrinkled if the depleted cell is large. But in the oomycetes-type reproduction mode, the outer layer remains like an empty balloon. The latter has the least amount of rupture. *V. persica* most often reproduces by autosporulation while other reproductive strategies occur under stress conditions like nutrient depletion. As Borowitzka [103] states, zygote germination is an alternative way to avoid chronic stress as an ‘avoidance’ response to stressors. A thick-walled resting stage was also observed herein under unfavorable conditions, e.g. nutrient limitation (Fig. 6 N-P). Therefore, *V. persica* enters a stress-resistant life cycle stage similar to what observed in several species of green microalgae [102]. Resting cysts with thick cell walls are resistant to extreme environmental conditions such as nutrient deficiency, temperature fluctuations, UV and desiccation [103].

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### 710 3.5. Soil characterization and co-occurring taxa

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712 The subsurface Tabas crossroad soil (Yazd city) was alkaline, with an average pH value  
 713 of 7.81. Conductivity of the soil extract was relatively high, with an average value of 5650  
 714  $\mu\text{S}\cdot\text{cm}^{-1}$ , and the soil moisture was distinctly low (~1%).  $\text{Ca}^{2+}$ ,  $\text{Na}^{+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cl}^{-}$ , and  $\text{SO}_4^{2-}$   
 715 were the major ions in the Yazd desert soil, while nutrients (total N and available P) were  
 716 very low with average concentrations of 0.185% and 5.195 ppm, respectively. Fe and Mn  
 717 were the major heavy elements having prominent concentrations (20426 and 507 ppm,  
 718 respectively), reflecting the nature of the desert bedrock of Yazd province situated near both  
 719 the Dasht-e Kavir and Dasht-e Lut deserts (Table 3). The most frequent and abundant co-  
 720 occurring taxa included the green alga *Tetrademus* G.M. Smith sp., and several  
 721 cyanobacteria: *Oscillatoria tenuis* C.Agardh ex Gomont, *Stenomitos frigidus* (F.E. Fritsch)  
 722 Miscoe et J.R. Johansen, *Jaaginema pseudogeminatum* (G. Schmid) Anagnostidis et  
 723 Komárek, *Jaaginema* Anagnostidis et Komárek sp., *Phormidium* Kützing ex Gomont sp.,  
 724 *Nostoc edaphicum* Kondratieva, *Nostoc hatei* S.C. Dixit, *Nostoc* Vaucher ex Bornet et  
 725 Flahault sp., *Trichormus* (Ralfs ex Bornet and Flahault) Komárek and Anagnostidis sp.,  
 726 *Calothrix* C. Agardh ex Bornet and Flahault sp., and *Chroococcus minimus* (Keissler)  
 727 Lemmermann.

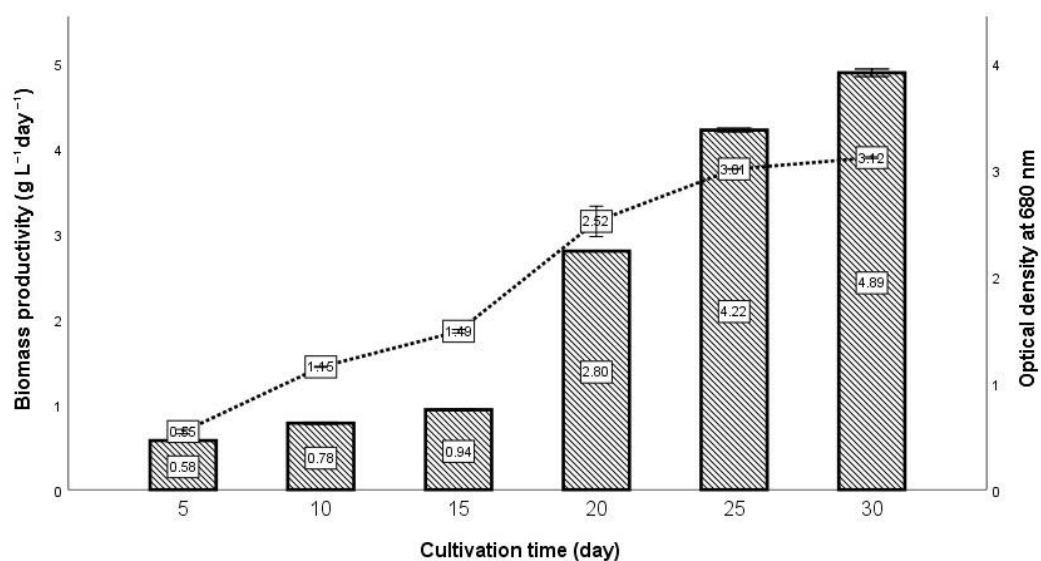
728 **Table 3** Physical and chemical characteristics of the Tabas crossroad soil (Yazd city, Iran) where *Valeriella*  
 729 *persica* was recorded in Tabas crossroad, Yazd (Iran) (values are means  $\pm$  SD).

Parameters	Tabas crossroad soil (Yazd city, Iran)
Soil texture	78.2% sand, 10.4% silt, and 11.4% clay
pH	7.81 $\pm$ 0.28

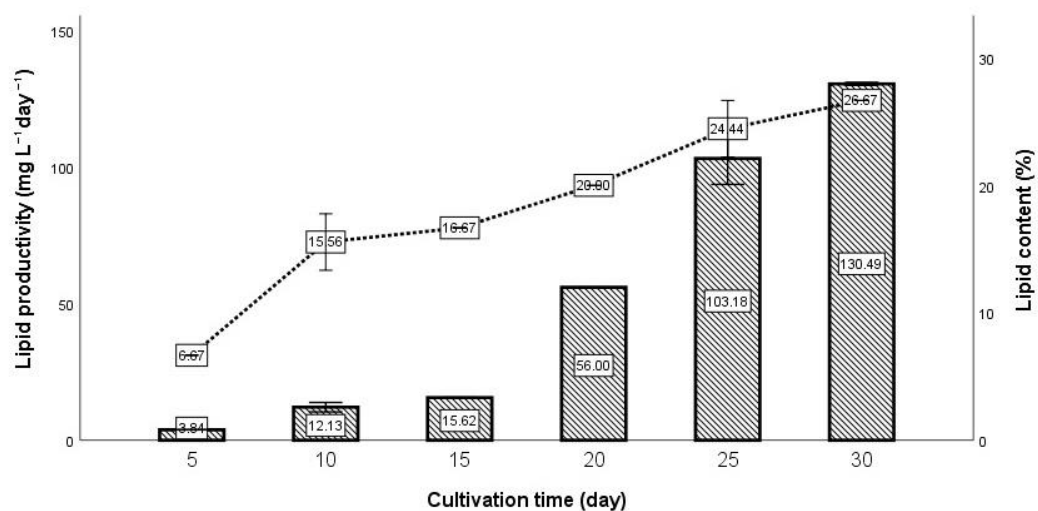
EC ( $\mu\text{S.cm}^{-1}$ )	$5650 \pm 1.73$
T.D.S. (ppm)	$3510 \pm 3.6$
Moisture content (%)	$1.039 \pm 0.01$
$\text{Ca}^{2+}$ (ppm)	$900 \pm 4.1$
$\text{Mg}^{2+}$ (ppm)	$520 \pm 2.65$
$\text{Na}^{+}$ (ppm)	$573.0 \pm 1.4$
$\text{K}^{+}$ (ppm)	$64.70 \pm 1.15$
Total saturation extract capability (ppm as $\text{CaCO}_3$ )	$311 \pm 2.65$
$\text{HCO}_3^{-}$ (ppm)	0.0
$\text{CO}_3^{2-}$ (ppm)	$311 \pm 1.22$
$\text{SO}_4^{2-}$ (ppm)	$600 \pm 1.55$
$\text{Cl}^{-}$ (ppm)	$1401 \pm 0.93$
Total N (%)	$0.185 \pm 0.001$
Available P (ppm)	$5.195 \pm 0.01$
Cu (ppm)	$19.94 \pm 1.1$
Fe (ppm)	$20426 \pm 2.15$
Mn (ppm)	$506.87 \pm 3.05$
Zn (ppm)	$52.57 \pm 0.82$

### 3.6. Characterization of algal growth, fatty acids and biodiesel properties

The algal growth pattern (over 30 days) in the BG-11 culture medium is shown in Fig. 9. The final biomass productivity at the end of the late exponential growth phase reached about  $4.90 \text{ g L}^{-1} \text{ day}^{-1}$ . The cell lipid content at late exponential phase reached  $26.7\% \text{ mg g}^{-1}$  of cell dry weight and the lipid productivity was  $130.5 \text{ mg L}^{-1}$  (Fig. 10).



**Fig. 9** Growth curve based on optical density at 680 nm (line) and biomass productivity (column) of *Valeriella persica* sp. nov. The data represent means and standard deviations of three biological replicates.



**Fig. 10** Lipid content (line) and lipid productivity (column) of *Valeriella persica* sp. nov. The data represent means and standard deviations of three biological replicates.

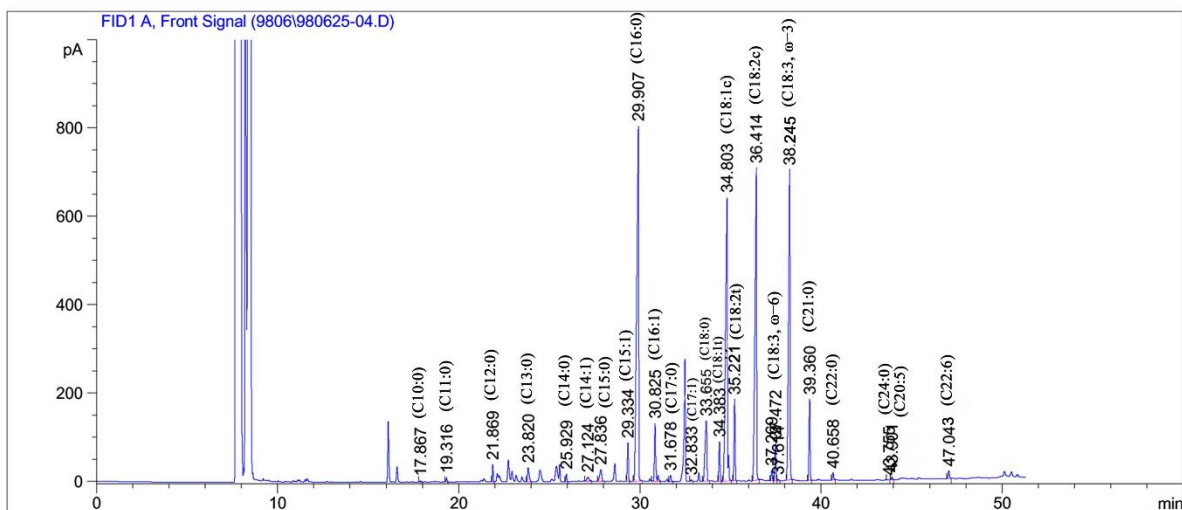
The FA composition of *Valeriella persica* biomass grown under standard growth conditions until the late exponential phase was analyzed using gas chromatography (GC) coupled with flame-ionization detection (GC–FID). The FAs identified ranged from C10:0 to C24:0 (Fig. 11) and the detailed composition is reported in Table 4. Twenty–six different species of FAs were identified. They mainly included saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated (PUFAs) FAs. The SFAs and PUFAs constituted the main bulk with percentages of 35.43% and 39.47%, respectively, of the total FAs, whilst the MUFAs had a percentage of 25.10% of the total FAs (Fig. 12). The SFAs were represented by 13 different species, and palmitic acid (C16:0), in particular, was the predominant one with a percentage of 24.74% (of the total FAs). As far as the MUFAs are concerned, 7 species were identified, with oleic acid (C18:1c,  $\omega$ –9) having the highest percentage (19.02% of the total FAs). Linoleic (C18:2c,  $\omega$ –6) and  $\alpha$ –Linolenic (C18:3,  $\omega$ –3) acids represented the two major PUFAs (from the 6 species unraveled) with relative percentages of 18.15 and 16.28%, respectively, of the total FAs (Table 4).

**Table 4** Fatty acid percent composition (% of FAMES) of *Valeriella persica* sp. nov. grown for four weeks in BG-11 medium.

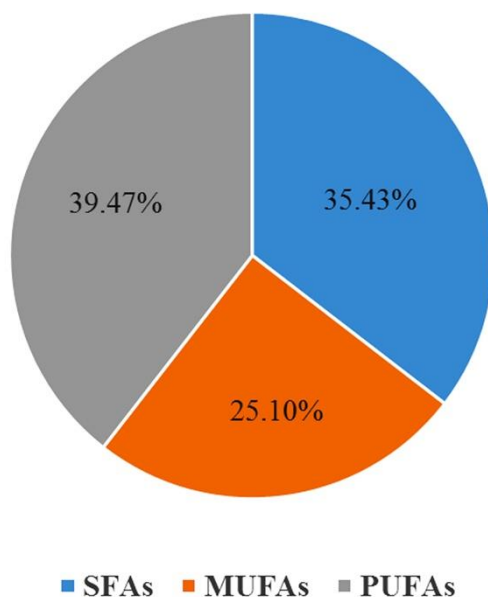
Fatty Acids	RT (min)	Peak area (%) *
Capric acid (C10:0)	17.867	0.05
Undecanoic acid (C11:0)	19.316	0.14
Lauric acid (C12:0)	21.869	0.52
Tridecanoic acid (C13:0)	23.820	0.84
Myristic acid (C14:0)	25.929	0.27
Myristoleic acid (C14:1, $\omega$ –5)	27.124	0.37
Pentadecanoic acid (C15:0)	27.836	0.86
Pentadecenoic acid (C15:1)	29.334	1.44
Palmitic acid (C16:0)	29.907	24.74
Palmitoleic acid (C16:1, $\omega$ –7)	30.825	2.68
Margaric acid (C17:0)	31.678	0.28

Heptadecenoic acid (C17:1) ( <i>cis</i> -10)	32.833	0.07
Stearic acid (C18:0)	33.655	3.88
Elaidic acid (C18:1 <i>t</i> , $\omega$ -9)	34.383	1.46
Oleic acid (C18:1 <i>c</i> , $\omega$ -9)	34.803	19.02
Linolelaidic acid (C18:2 <i>t</i> , $\omega$ -6)	35.221	3.14
Linoleic acid (C18:2 <i>c</i> , $\omega$ -6)	36.414	18.15
Arachidic acid (C20:0)	37.269	0.41
$\gamma$ -Linolenic acid (C18:3, $\omega$ -6)	37.472	1.42
Eicosenoic acid (C20:1, $\omega$ -9)	37.614	0.06
$\alpha$ -Linolenic acid (C18:3, $\omega$ -3)	38.245	16.28
Heneicosanoic acid (C21:0)	39.360	3.12
Behenic acid (C22:0)	40.658	0.31
Lignoceric acid (C24:0)	43.755	0.008
Eicosapentaenoic acid (C20:5, $\omega$ -3)	43.901	0.12
Docosahexaenoic acid (C22:6, $\omega$ -3)	47.043	0.36
<b><math>\Sigma</math> saturated fatty acids (SFAs)</b>		<b>35.43% of the total fatty acids</b>
<b><math>\Sigma</math> monounsaturated fatty acids (MUFAs)</b>		<b>25.10% of the total fatty acids</b>
<b><math>\Sigma</math> polyunsaturated fatty acids (PUFAs)</b>		<b>39.47% of the total fatty acids</b>

\*Peak area represents the proportion of each fatty acid as % of total fatty acids.



**Fig. 11.** GC-MS chromatograph of the fatty acids profile of *Valeriella persica* sp. nov. grown for four weeks (until reaching the end of the late exponential phase) in BG-11 medium.



**Fig. 12.** Percentage distribution of the three classes of fatty acids in *Valeriella persica* sp. nov. grown in BG-11 medium until the late exponential phase (after four weeks). Palmitic acid (C16:0; 24.74%) corresponded to the main fatty acid, followed by oleic (C18:1c,  $\omega$ -9; 19.02%), linoleic (C18:2c,  $\omega$ -6; 18.15%), and  $\alpha$ -linolenic (C18:3n3,  $\omega$ -3; 16.27%) acids.

Biodiesel properties derived from the algal FA profile are presented in Table 5. All the theoretical values of biodiesel properties were in the accepted ranges of European standards (CEN 14214) and American Society for Testing and Materials (ASTM D6751-08). However, CFPP and OS came in an accepted range of CEN 14214.

**Table 5.** Comparison of biodiesel properties of *Valeriella persica* sp. nov. and American Society for Testing and Materials (ASTM, 2008), European standards (CEN, 2008); ADU: average degree of unsaturation,  $v_i$ : kinematic viscosity,  $\rho$ : density, CP: cloud point, IV: iodine value, SV: saponification value, CN: cetane number, HHV: higher heating value, LCSF: long-chain saturation factor, CFPP: cold filter plugging point, OS: oxidation stability.



Biodiesel properties	<i>Valeriella persica</i> sp. nov.	Biodiesel standards	
		ASTM D6751-08	CEN 14214
ADU	1.24	–	–
$\nu_i$ (mm <sup>2</sup> s <sup>-1</sup> )	4.43	1.9–6.0	3.5–5.0
$\rho$ (g cm <sup>3</sup> )	0.88	0.88	0.86–0.90
CP (°C)	3.49	– 3 to 12	–
IV (g I <sub>2</sub> .100 g <sup>-1</sup> oil)	104.59	–	Max. 120
SV (mg KOH g <sup>-1</sup> )	117.33	–	–
CN	54.64	Min. 47	51-120
HHV (Mj.kg <sup>-1</sup> )	40.71	–	–
LCSF (wt%)	5.31	–	–
CFPP (°C)	0.19	–13 to –5	–20 to 5
OS (h)	6.02	Min. 3	Min. 6

Nowadays, many studies target valuable species of microalgae with high levels of biomass and lipid productivity to recognize ideal candidates for the third-generation biofuel feedstock. Some species possess high lipid content (20–50% of the cell dry weight) that can even increase under certain environmental conditions [104] such as temperature, light, salinity [105,106] and molecular osmotic pressure [107]. Stated as such, the lipid content of *Valeriella persica* reached 27% of the cell dry weight, interestingly under normal growth conditions, and thus it can be a potential candidate for the production of algal biodiesel.

Based on current knowledge, FAs having 14–20 carbons are suitable for biodiesel production, but PUFAs with more than 20 carbon atoms are used as healthy food [108]. On such basis, the Iranian strain appears to be a potential source to produce viable third-generation biofuels [23,24], because the total content of SAFs and MUFAs was higher than the PUFAs.

In addition, the FA composition of algae has taxonomic value. For instance, most green algae are rich in C16 and C18 FAs, which are fit for biodiesel production [109-111]. With reference to chemotaxonomy, palmitic acid (C16:0) is reported as the main SFA of green

microalgae and the same results have been observed in the present study. The selection of potent biodiesel-producing algae depends on fuel properties derived from FA composition, in addition to the total lipid content [112]. The most important parameters for desired algal biodiesel are the cetane number, viscosity, density, cold filter plugging point, oxidative stability, ignition quality, combustion heat and cold flow [113]. The higher value of CN is preferable for biodiesel production because it is proportionately reported with the oxidative stability, ignition quality and ratio of saturated FAs to unsaturated FAs [114]. CN is one of the most important parameters for combustion quality in biodiesel fuel that is related to the delay time between injection and ignition [115]. On the contrary, the lower value of IV is preferable for being proportionated to the unsaturated FAs [116,117]. CFPP and LCSF are two undesirable indices that should be at low values. FAME profile-derived SV-values, which reflect the highest fatty acid contents, have an inverse relationship with both molecular weight and carbon chain length [118]. According to Table 5, the estimated values of  $v_i$ ,  $\rho$ , CN, IV, and CN in this study, were in accordance with recommended values in international standards (ASTM D6751 and CEN 14214). The values of OS and CFPP were also in agreement with CEN 14214. Overall, the values obtained for *Valeriella persica* comply well with biodiesel standards for commercial biofuel.

### 3.7. Ecophysiological adaptations

Adaptation is an evolutionary process in which heritable characteristics and genetic-based alterations pass on to the successive generation as a result of environmental changes and selection. The main types of adaptations in algae include cell structures and reproductive

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5 821 strategies [103,119,120]. The latter was described in the previous sub-section. Structural  
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8 822 cellular adaptations in terms of FAs profiling are discussed in the following.  
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10 823 Habitat typology can regulate species composition, diversity of algal assemblages and  
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12 824 metabolism [121]. The occurrence of *Valeriella persica* in desert soils indicates the  
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15 825 adaptations that have been created in the face of harsh conditions. For example, Terlova et  
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17 826 al. [122] observed that terrestrial species of *Tetrademus* are better acclimated to desiccation  
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19 827 stress than the aquatic. The underlying reason is their physiological difference affected by  
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21 828 habitat selection. Consequently, habitat of origin predicts the ability of desiccation tolerance  
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23 829 in the *Tetrademus* species [122]. Beyond doubt, desert algae have specific adaptation  
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25 830 strategies for protection against high levels of UV radiation, high temperatures, low nutrients,  
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27 831 etc. [123]. With respect to these extreme conditions, *V. persica* has the following biological  
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29 832 characterization: (1) cell wall thickening with age; (2) cell wall ornamentation including  
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31 833 hair-like exopolysaccharide appendages; (3) accumulation of orange pigments and lipid  
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33 834 globules in older cells.  
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39 835 The versatility of terrestrial algae can be attributed to the FA composition [5]. It has  
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41 836 been proved that unsaturated lipids are FA-related regulations of membrane fluidity in  
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43 837 extremophilic algae [124]. Our results also confirmed this pattern with 39.47% PUFSs and  
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45 838 25.10% MUFAs of the total FAs (Table 4). FA double bonds play a crucial role in  
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47 839 temperature acclimation. It is an effective mechanism of adaptation to maintain fluidity under  
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49 840 wide temperature fluctuations in desert lands (so hot during the day and cold at night). As  
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51 841 Mironov et al. [125] have remarked, temperature and light are two determinant factors  
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53 842 altering gene expression.  
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The FAs most frequently detected in *Valeriella persica* are palmitic (C16:0; 24.74%), oleic (C18:1c,  $\omega$ -9; 19.02%), linoleic (C18:2c,  $\omega$ -6; 18.15%), and  $\alpha$ -linolenic (C18:3,  $\omega$ -3; 16.28%) acids. This finding is in agreement with data from the soil-dwelling strains *Deasonia* sp. CALU 934 [126] and *Bracteacoccus* sp. [124]. Generally, desert green algae have a lipidome rich in monogalactosyl diacylglycerols (MGDG), digalactosyl diacylglycerols (DGDG), and sulfoquinovosyl diacylglycerols (SQDG) [127], required for the formation and functioning of the cellular membranes. It has also been reported in the hyper-arid desert green algae, like *Pharao desertorum* [5]. SQDG contain palmitic and oleic acids, while MGDG and DGDG encompass hexadecatetraenoic,  $\alpha$ -linolenic, stearidonic and linoleic acids in green algae [127]. Phospholipids yielding a high amount of oleic, palmitic and  $\alpha$ -linolenic acids guarantee the structural integrity and fluidity of the algal membranes [5,127].

Due to the confusing taxonomy of green coccoids, integrative polyphasic approaches are highly recommended to accurately delineate the species and infraspecific identities [5,19]. In this respect, many terrestrial green coccoids have been misnamed in the older literature. For instance, the strain CALU 934 isolated from the soil sample in Russia has been probably misidentified as *Parietochloris* sp., but re-investigation of this strain placed it into the genus *Deasonia* based on its combined morphotaxonomic and ITS phylogenetic data [126]. Another consideration is the FA composition of this isolate, which is very similar to our novel species *V. persica*. Both yield high amounts of C16 and C18 FAs, including palmitic (C16:0), oleic (C18:1c,  $\omega$ -9), linoleic (C18:2c,  $\omega$ -6) and  $\alpha$ -linolenic (C18:3,  $\omega$ -3) acids. It is worth mentioning that the FA profile is a potential genus-identifying feature [128].

In conclusion, *Valeriella persica* sp. nov. was isolated and characterized from the hyperarid desert soil in Iran based on its diagnostic morphotaxonomic features, peculiar cell wall ultrastructure and life-cycle stages, as well as autecological preferences and 18S rDNA phylogenetic assignment. This study also expanded the so far scarce information about the poorly-known species diversity of cryptic dryland-dwelling green algae in Iran, and, generally, in Asia. Based on its lipid content and fatty acids profiling, *V. persica* is a potential candidate for the biodiesel production on the large scale.

#### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### **Authors' Contributions**

Conceptualization, FS-B, HR, MC and AAS; methodology, FS-B and FH; software, FS-B, ZS and AYN; validation, FS-B, MC, AYN and AAS; formal analysis, FS-B and AYN; investigation, FS-B and AAS; writing—original draft preparation, FS-B; writing—review and editing, MC and AAS; supervision, HR, ZS, MC and AAS; project administration, FS-B; funding acquisition, HR. All authors have read and agreed to the published version of the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages.

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**Supplementary Material**

**Fig. S1.** Additional morphotaxonomic features of *Valeriella persica* sp. nov.

**Fig. S2.** Additional morphotaxonomic features of *Valeriella persica* sp. nov.

**Fig. S3.** Autospore formation in *Valeriella persica* sp. nov.

**Fig. S4.** Progressive cleavage and successive bipartition in *Valeriella persica* sp. nov.

**Fig. S5.** Zygote formation by *Valeriella persica* sp. nov.

**Fig. S6.** Schematic depiction of the cell wall ultrastructure of *Valeriella persica* sp. nov.

**Fig. S7.** Irregular shapes of *Valeriella persica* sp. nov.

**Fig. S8.** *Valeriella persica* sp. nov. showing the outcome of zoospore formation

**Fig. S9.** Additional morphotaxonomic features of *Valeriella persica* sp. nov.

**Video 1.** A 7-cell colony of *Valeriella persica* sp. nov. enclosed in an invisible envelope.

The release of a cell indicates cell independency. Note the way the released cell comes out of the thick gelatinous matrix.

**Video 2.** Cell aggregation of *Valeriella persica* sp. nov. The newly released cells formed short pseudo-filaments.

**Video 3.** Aggregated parent cells of *Valeriella persica* sp. nov. showing two types of cell wall rupture. In the 2<sup>nd</sup> second, all the cell contents were ejected through a pore. In the 36<sup>th</sup> second, a small amount of the cell contents were released through a pore.

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- 1338     **Video 4.** Aggregated parent cells of *Valeriella persica* sp. nov. showing cell wall rupture.
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- 1340     **Video 5.** A ruptured parent cell of *Valeriella persica* sp. nov. showing multimorphic-cells’
- 1341     cloud. Note released cells in different size, shape and color, and also note the largest cell and
- 1342     its motile cells inside.
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- 1344     **Video 6.** A giant parent cell of *Valeriella persica* sp. nov. with the intact wall. Note the motile
- 1345     cells inside.
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- 1347     **Video 7.** A parent cell of *Valeriella persica* sp. nov. Cell wall rupture has occurred and a
- 1348     small number of cells were released. Note that cells are different in size and color.