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Research Article

Molecular verification of *Caulerpa racemosa* (Caulerpaceae, Chlorophyta) in the Persian Gulf and Oman Sea (south of Iran)

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Abstract

Caulerpa racemosa is edible marine green algae of the Caulerpaceae family. *C. racemosa* is consumed as a sea vegetable in many countries, especially Southeast Asian countries. In recent years, molecular studies caused some changes in the taxonomic positions of the members of the genus *Caulerpa*. It is necessary to accurately identify species of this genus based on modern molecular studies due to the consumption of some species and also the toxic effect of some other species of this genus. Some species of *Caulerpa* have already been reported from the southern coasts of Iran based on preliminary morphological studies. The purpose of this study was to collect and accurately identify the species of the genus *Caulerpa* in the coastal waters of the Persian Gulf and the Gulf of Oman in the southern coastlines of Iran. This study was based on the combination of molecular and morphological characteristics studies of the collected samples of this genus in this area. In the current article, the results obtained about the *Caulerpa racemosa* are presented. Samples of the genus were collected from different coastal areas of the Persian Gulf and seashores of Oman Gulf along the geographical borders of Iran and transported to the laboratory in cool conditions. After cleaning and washing, some of the cleaned samples were dried in silica gel and used for DNA extraction and thereof PCR process based on *tuf*A gene. In addition, herbarium samples required for morphological studies were prepared and deposited in the herbarium of Agriculture and Natural Resources Research and Education Center of Hormozgan province. The results of this study relatively solve the confusion in *Caulerpa racemosa- peltata* complexity for Iranian members of the complex. In addition to morphological features, the current molecular investigation also helped to accurately identification of the members of this complex in the Iranian seashores of the Persian Gulf and Gulf of Oman. As a highlighted conclusion, the current research on *Caulerpa* species from the Iranian seashores of the Persian Gulf and Gulf of Oman resulted in resolving two species including *Caulerpa sertulariodes* (S. G. Gmelin) Howe forma *farlowii* (Weber-Van Bosse) Børgesen, which was resolved as *Caulerpa selago* (Turner) C. Agardh (Salehi Balashahri *et al*., 2021) and *Caulerpa racemosa* var. *macrophysa* also resolved as *Caulerpa racemosa* (Forsskål) J. Agardh.

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Introduction

Genus *Caulerp*a as a group of family Caulerpaceae in Bryopsidales order comprises around 213 taxa including 104 accepted species, 39 accepted varieties and 70 accepted forma in algaebase (Guiry and Guiry, 2023). Also, a total of 188 taxa including 67 species, 77 variety and 47 forma, currently listed as synonymy on this site. This list of accepted and synonym names shows the high complexity in taxonomy of this genus. Members of this genus are distributed in a wide range of habitats in tropical and subtropical biogeographical marine regions of the world (Meinesz and Boudouresque, 2000; Fama *et al*., 2002), Phenotypic plasticity is a main source of taxonomic complexity in most algal groups, which led to many misidentifications and nomenclatural confusions and many misestimating in biodiversity (Saunders, 2008; Leliaert *et al*., 2009; Tronholm *et al*., 2010), Because of recent expanding of the members of the genus into the temperate marine areas, considerable researches focused on this genus (Meinesz and Hesse, 1991; Piazzi, 2001). Some species such as *caulerpa taxifolia* and *Caulerpa cylindercea* widely spread in the Mediterranean Sea and caused high damage to fish resources in this marine region (Amade *et al*., 1999).

Members of the *Caulerpa* genus show a wide variety of habits, their thallus morphologically consists of stolons, rhizoids, and upright fronds that have various forms of branchlets or ramuli (Fritsch, 1965). These morphological characteristics have been used to determine species delimitation by phycologists. Environmentally conditions cause many phenotypic plasticity, especially in the ramuli, which has led to much confusion, high synonyms, and various taxonomic levels such as subspecies, varieties, forms, and ecads (Peterson, 1972, Ohba and Enomoto, 1987; Coppejans and Prud'homme van Reine, 1992).

Molecular tools as a convenient means have provided valuable devices for researchers to delimit and identify species in taxonomically challenging groups (Verbruggen *et al*., 2009; Saunders, 2008; Leliaert *et al*., 2009), These tools, however, have caused new difficulties for taxonomists such as conflicts between morphological and molecular concepts (Leliaert *et al*., 2009; Sauvage *et al*., 2013) and increasing diversity of cryptic species based on molecular data (De Clerck *et al*., 2013).

Recent molecular studies on the *Caulerpa* genus have further added difficulties in the taxonomy of this genus because caused some mismatch between morphological and molecular species concepts in the genus, most evident in the *C. racemosa-peltata* complex (Fama *et al*., 2002; Sauvage *et al*., 2013; Belton, 2015), All the authors believe that the matching of previously described names with newly obtained molecular data is an extremely difficult task. This research has deposited many misidentified *Caulerpa* specimens on Genbank, which makes it relatively impossible to do accurate taxonomy of the genus. Belton (2015) tried to resolve this problem in the taxonomy of *Caulerpa* genus in Australian waters and provide a framework to integrate traditional nomenclature and molecular-based taxonomies. By using two gene markers

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*rbc*L and *tuf*A, Belton (2016) developed this framework for the most problematic taxons of the genus *C. racemosa* (Forsskål) J. Agardh and *C. peltata* J.V. Lamouroux, known as the *C. racemosa–peltata* complex, which has more than 30 described varieties and forms (Guiry and Guiry, 2023).

C. racemosa is composed of a large cell with a large number of nuclei. Chloroplasts contain free chlorophyll that migrates from one part of the body to other parts, and there is a network of fibrous proteins that help to movement of organs. This species is widely found in temperate and tropical shallow marine areas. In 1926 a new form of algae was reported from Tunisia, possibly migrating from the Red Sea, and later migrated to most of the Mediterranean Sea and became more widespread than the invasive species *Caulerpa taxifolia,* and was known as *C. racemosa* var. cylindracea which molecular investigation resolved it as *Caulerpa cylindracea* (Wirawan *et al*., 2022).

C. racemosa is edible seaweed like *Caulerpa lentillifera* and is widely used as a vegetable in Japan, Fiji, Philippines, and Thailand. It is also eaten by local fishermen in Malaysia and Indonesia. This alga is rich in fiber, protein, minerals (calcium and magnesium), folic acid, ascorbic acid, vitamin A, and vitamin B1, while also being low in fat (4), In addition to the nutritional value, *C. racemosa* also has antibacterial and antioxidant properties (Arfini, 2022; Pangestut *et al*., 2021).

In the Iranian seashores of the Persian Gulf and Gulf of Oman, 11 species and varieties of algae were reported in two reports (Sohrabipour and Rabiei, 1996, 1999) and in a checklist published by Sohrabipour and Rabiei (2007), which included *Caulerpa fastigiata* Montagne , *Caulerpa manorensi*s Nizamuddin, *Caulerpa mexicana* Sonder ex Kützing, *Caulerpa crassifolia* (C. Agardh) J. Agardh, *Caulerpa peltata* J.V. Lamouroux (= C*aulerpa racemosa* var. *peltata* (Lamouroux) Eubank , *Caulerpa racemosa* var. *macrophysa* (Sonder ex Kützing) W.R. Taylor, *Caulerpa sertulariodes* (S.G. Gmelin) Howe forma *farlowii* (Weber-Van Bosse) Børgesen, *Caulerpa sertularioides* (S.G. Gmelin) M. Howe forma *Caulerpa sertulariodes*, *Caulerpa taxifolia* (M. Vahl) C. Agardh , *Caulerpa brachypus* Harvey, *Caulerpa scalpellformis* (Turner) C. Agardh. Based on these published papers, Kokabi and Yousefzadii (2015) reported another checklist including the Caulerpa species (Shams and Amini, 2017) provided short descriptions for some species of the genus Caulerpa collected from southern seashores of Iran.

Materials and methods

Samples of *Caulerpa* were collected from different intertidal regions of the Persian Gulf and Gulf of Oman at the southern coastlines of Iran (Table 1). The collected samples were cleaned and washed thoroughly with seawater to remove sand and other debris then transferred in cool conditions to the laboratory. In the laboratory after precisely cleaning, the samples were washed with tap water and spread on herbarium sheets and small parts of each sample were dried using silica gel and stored at –20°C. The prepared herbarium sheets were deposited in the algal herbarium of the Agriculture and

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Natural Resources Research and Education Center of Hormozgan Province, Bandar Abbas, Iran.

Table 1: The geographical information of collecting sites of the samples.

Location	Y	X
Jask	25.636320 N	57.764019 E
	25.640710 N	57.777129 E
Qeshm island	26.981241 N	56.232606 E
	26.969403 N	56.264558E
Chabahar	25.165417 N	61.501707 E
	25.272164 N	60.735824 E

Molecular analysis

DNA extraction was carried out using the modified CTAB method (Promega, Madison, WI, USA) and QiAgen kit. The PCR amplification of the extracted DNA was carried out using *tuf*A gene marker based on the protocols proposed by Kazi *et al.* (2013) or Famà *et al.* (2002), The PCR products were sent to Pioneer Technology Gene Company for sequencing. The obtained sequences were then edited with ChromasPro ver. 1.5 (Technelysium Pty Ltd, Australia), After that the newly obtained sequences were blasted in NCBI and the most similar submitted and published sequences (FASTA format) were extracted and aligned in MEGA 7.0 (Kumar *et al*., 2016) and neighbor-joining phylogenetic tree were constructed.

Results

Morphology of *Caulerpa racemosa* (Forsskål) J. Agardh

The genus of *Caulerpa* is morphologically distinguished by the siphonous stolon which was attached to the substrate by siphonous rhizoids and had upright assimilators (or fronds) that usually bear characteristic different shape of branchlets (ramuli).Based on these basic morphological features, the taxonomic definition of this genus member in taxonomic levels of the species and variety are determined by morphological variation in shape and configuration of the ramulies (Balton *et al*., 2014; Riosmeña-Rodriquez *et al*., 2014).

The samples of the collected *Caulerpa* specimens which were morphologically identified as *C. racemosa* in the current study characterized by short to moderate long erect branches (up to 4 cm) bearing sparse to dense ramulis with short stalks (up to 6 mm height) which, radially, alternately to irregularly were arranged on the erect branch. The distal parts of the ramuli are mainly discoid or truncate shapes, which are up to 8 mm in diameter. The members of the species grow on dead corals covered with sands or on sandy-muddy bottoms at the middle to low intertidal or subtidal zones. In protected or moderately wave-exposed shores they form entangled mats that attach to the sea bottom with densely branched rhizoids. Those growing on rocky-wave exposed reefs possess short erect branches covered with spherical or globose tips ramulies (Fig. 1).

Molecular study

A total set of 50 sequences was obtained from the samples of *Caulerpa spp.* including four sequences from Iran and two sequences of *Caulerpella ambigua* as outgroups were used for phylogenetic analysis. The aligned set composed of 719

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base pairs of *tuf*A gene nucleotides, which of 530 characters were constant, 38 variable characters were parsimony-uninformative and 151 were parsimony-informative characters.

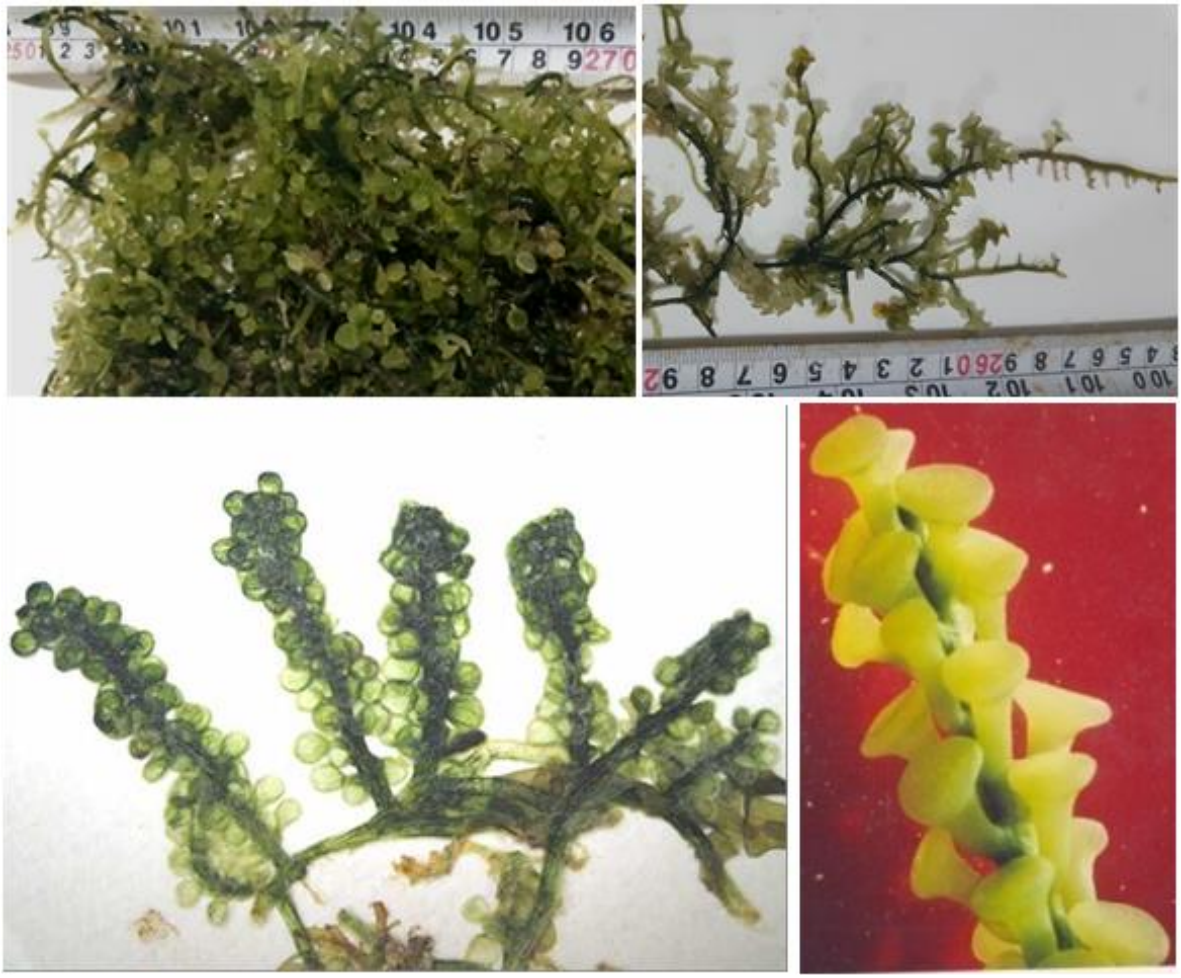

Figure 1: Morphological features of the *Caulerpa racemosa* (a) whole morphology of the collected samples, (b) a detailed image from erect frond shows a discoid distal end of ramuli, (c) a detailed image of erect branches shows ramuli on the herbarium sheet (as *C. racemosa* var. *macrophya* in Sohrabipour and Rabiei , 2007) and (d) a close up image from discoid ends of ramulis of the samples that morphologically already reported as *C. peltata* (Sohrabipour and Rabiei, 2007 and *C. racemosa*, var. *peltata* in Sohrabipour and Rabiei 1999).

Iranian sequences of *Caulerpa racemosa* in phylogenetic analyses grouped in the same clade together with the submitted sequences in GenBank from USA including Florida (DQ652425, DQ652424), New Caledonia (AJ417947, AJ417956), and also, three sequences from Indonesia (FM956052), the Philippines (KT861496), Adelaide (FM956052). The constructed phylogenetic tree was congruent with the previous phylogenetic analyses which were reported by Fama *et al*. (2002) and Sauvage *et al*. (2013) (Fig. 2).

Intraspecific divergence within the sequences of this clade ranged between 0.0 to 0.5% that is accordance with 3 nucleotides, while interspecific divergence between this clade and clade contains the other varieties of *Caulerpa racemosa* ranged between 2.8-4.4% which was more

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than interspecific divergence between sequences of *Cauelrpa racemosa* and *Caulerpa selago* (2.5-2.7%) (Table 2). This range of divergence among the varieties of the *Caulerpa racemosa* may be led to a distinct species in future investigations.

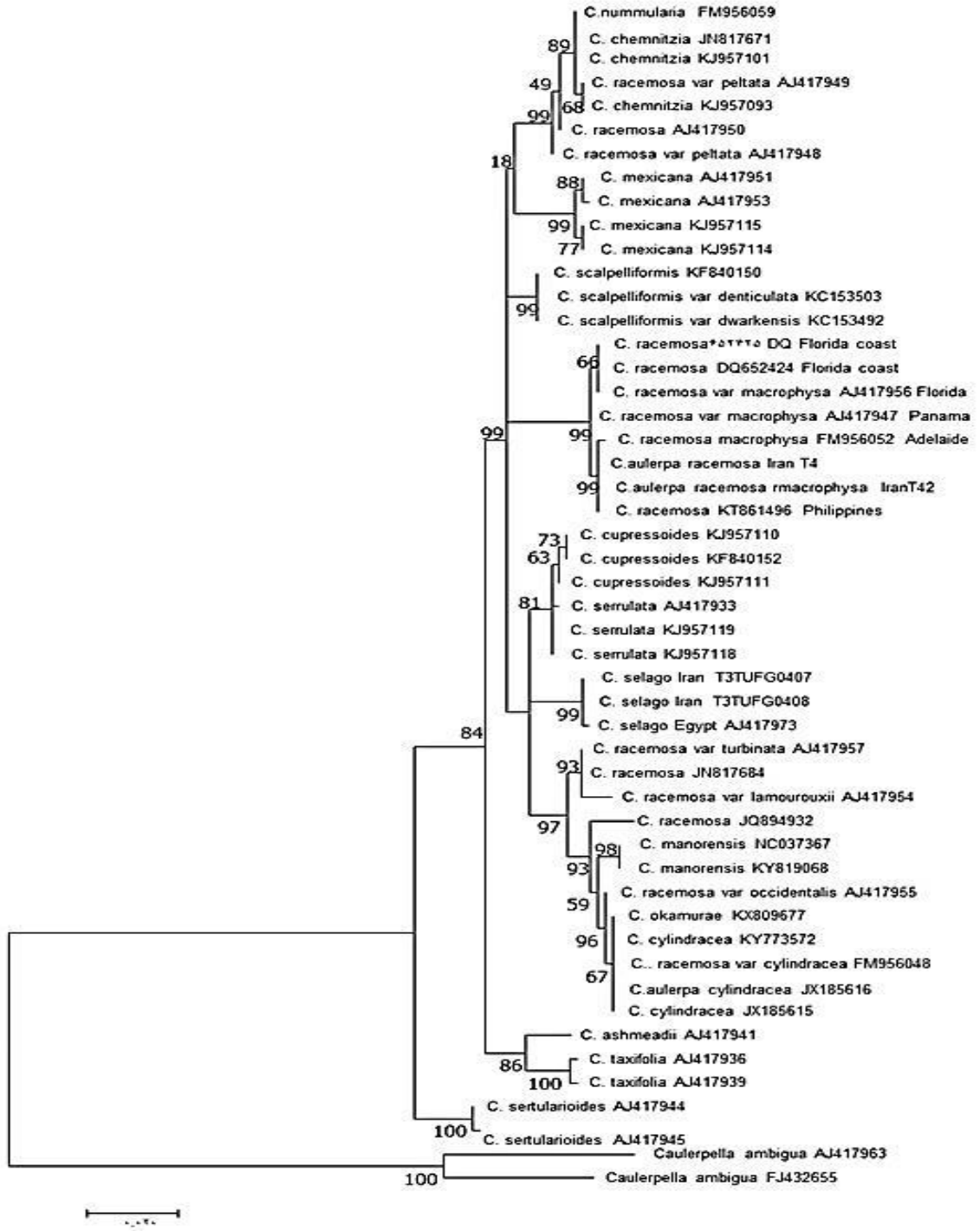

Figure 2: Phylogenetic tree based on *tuf*A gene sequences. Numbers above nodes indicate bootstrap support. Support values (>50 is not shown).

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Table 2: Divergence ranges within and between the sequences of *Caulerpa* members from Iran and the sequences of *Caulerpa racemosa* from other regions of the world.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1. T4 *Caulerpa racemosa* Iran																						
2. T4 *Caulerpa racemosa* Iran	0.000																					
3. AJ417947*C. racemosa* var *macrophysa*	0.002	0.002																				
4. FM956052 *C. racemosa macrophysa*	0.002	0.002	0.003																			
5. DQ652425 *C. racemosa*	0.003	0.003	0.002	0.005																		
6. DQ 652424 *C. racemosa*	0.003	0.003	0.002	0.005	0.000																	
7. KT 861496 *C. racemosa*	0.000	0.000	0.002	0.002	0.003	0.003																
8. AJ417956 *C. racemosa* var *macrophysa*	0.003	0.003	0.002	0.005	0.000	0.000	0.003															
9. T3TUFGO407 *C. selago* Iran	0.027	0.027	0.025	0.025	0.027	0.027	0.027	0.027														
10. T3TUFGO408 *C. selago* Iran	0.027	0.027	0.025	0.025	0.027	0.027	0.027	0.027	0.000													
11. AJ417973 *C. selago* Egypt	0.028	0.028	0.027	0.027	0.028	0.028	0.028	0.028	0.002	0.002												
12. AJ417948 *C. racemosa* var *pelteta*	0.028	0.028	0.027	0.027	0.029	0.028	0.028	0.028	0.025	0.025	0.027											
13.AJ417957 *C. racemosa* var *lurbinata*	0.030	0.030	0.028	0.028	0.030	0.030	0.030	0.030	0.023	0.023	0.025	0.027										
14. JN817684 *C. racemosa*	0.030	0.030	0.028	0.028	0.030	0.030	0.030	0.030	0.023	0.023	0.025	0.027	0.000									
15. AJ417950 *C. racemosa*	0.030	0.030	0.028	0.028	0.030	0.030	0.030	0.030	0.027	0.027	0.028	0.002	0.028	0.028								

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Table 2 (continued):

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
16. AJ417954 *C. racemosa* var *lamourouxii*	0.035	0.035	0.033	0.033	0.035	0.035	0.035	0.035	0.028	0.028	0.030	0.033	0.007	0.007	0.035							
17. AJ417955 *C. racemosa* var *occidentalis*	0.035	0.035	0.033	0.033	0.035	0.035	0.035	0.035	0.028	0.028	0.030	0.028	0.012	0.012	0.030	0.018						
18. AJ417949 *C. racemosa* var *peltata*	0.032	0.032	0.030	0.030	0.032	0.032	0.032	0.032	0.028	0.028	0.030	0.007	0.030	0.030	0.005	0.037	0.032					
19. FM956048 *C. racemosa* var *cylindracea*	0.037	0.037	0.035	0.035	0.037	0.037	0.037	0.037	0.030	0.030	0.032	0.030	0.013	0.013	0.032	0.020	0.002	0.033				
20. KJ957101 *C. chemnitzia*	0.030	0.030	0.028	0.028	0.030	0.030	0.030	0.030	0.027	0.027	0.028	0.005	0.028	0.028	0.003	0.035	0.030	0.002	0.032			
21. KJ957093 *C. chemnitzia*	0.032	0.032	0.030	0.030	0.032	0.032	0.032	0.032	0.028	0.028	0.030	0.007	0.030	0.030	0.005	0.037	0.032	0.000	0.033	0.002		
22. JN817671 *C. chemnitzia*	0.030	0.030	0.028	0.028	0.030	0.030	0.030	0.030	0.027	0.027	0.028	0.005	0.028	0.028	0.003	0.035	0.030	0.002	0.032	0.000	0.002	
23. JQ894932 *C. racemosa*	0.044	0.044	0.024	0.042	0.044	0.044	0.044	0.044	0.033	0.033	0.035	0.037	0.018	0.018	0.038	0.025	0.013	0.040	0.015	0.038	0.040	0.038

Discussion

The previously reported species of the *Caulerpa* genus from the Persian Gulf and the Gulf of Oman indicated the presence of 11 species of the genus from the southern coastline of Iran (Sohrabipour and Rabiei, 2007, 1996, 1999), In the two checklists published by John and Al-Thahni (2015) and Kokabi and Yusefzaadi (2015), totally 13 species of *Caulerpa* were listed by increasing one species *Caulerpa faridii* Nizamuddin from Chabahar at Iranian seashores of Oman gulf (Kokabi and Yusefzaadi, 2015) and one variety *Caulerpa racemosa* [var. *lamourouxii*] f. *requienii* **(**John and Al-Thahni, 2015) from Abu Dabi. The results of this study relatively solve the confusion in *Caulerpa racemosa-peltata* complex for Iranian members of the complex. Environmental conditions caused by unstable morphological features have led to confusing nomenclature in taxonomic levels of varieties and forma, of *Caulerpa racemosa-peltata* complex (Sauvage *et al*., 2013).

Fama *et al*. (2002) in their first molecular analyses based on *tuf*A gene data combined with detailed anatomical studies on pyrenoid size, chloroplast size, number of thylakoids in granum, and also number and length of starch grains, showed that the members of clade of *Caulerpa racemosa* have similar detailed morphological features.

The most important visible primary criteria for morphological distinction of the species are the branching pattern of the

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assimilator, ramuli shapes, and configuration. These morphological features, however, can shows high variation under environmental conditions changes (Peterson, 1972; Calvert, 1976; Ohba and Enomoto, 1987; Ohba *et al.*, 1992; Riosmena-Rodríguez *et al.*, 2014) can a lat of to considerable taxonomic confusion (Calvert., 1976**),** therefore using new molecular tools may resolve this complexity.

Some Iranian speciemens of the *Caulerpa, Caulerpa sertulariodes* (S. G. Gmelin) Howe forma *farlowii* (Weber-Van Bosse) Børgesen, which were already reported from various regions of the Iranian and Arabian shores based on the first report of the taxa in this area (Nizamuddin and Gessner, 1970) was resolved as *Caulerpa selago* (Turner)C. Agardh (Salehi Balashahri *et al*., 2021) and *Caulerpa racemosa* var. *macrophysa* also resolved as *Caulerpa racemosa* (Forsskål) J. Agardh (current study).

The complexity of *Caulerpa racemosa-peltata* complex has already led to many molecular studies based on molecular data focused on *tuf*A gene. So the *tuf*A (encoding elongation factor TU) of the chloroplast gene as the most common partial gene, generally has been used for the species distinction in the *Caulerpa* genus by almost algal taxonomist in recent years (Famà *et al.*, 2002; Senerpont Domis *et al.*, 2003; Verlaque *et al*., 2000, 2003, 2004; Durand *et al*., 2003; Stam *et al.*, 2006; Kazi *et al.*, 2013; Sauvage *et al.*, 2013; Belton *et al.*, 2014, 2015, 2019; Draisma *et al.*, 2014; Fernández- García *et al.*, 2016; Dumilag *et al*., 2016).

In the current study, based on the molecular data of *tuf*A gene, we approved the presence of *Caulerpa racemosa* (Forsskål) J. Agardh that had already been reported as *Caulerpa racemosa* by Sohrabipour and Rabiei (1999, 2007) and also as *Caulerpa racemosa* var. *macrophysa* (Sohrabipour and Rabiei., 2007) from the Iranian coasts of the Persian Gulf and Gulf of Oman. Phylogentic analysis of *tuf*A gene resolved *Caulerpa racemosa* var. *macrophysa* as *Caulerpa racemosa* (Forsskål) J. Agardh (Fig. 2).

Conspecificy of *Caulerpa racemosa* var. *macrophysa* with *Caulerpa racemosa* was already been approved in the previous studies based on *tuf*A gene data analyses which combined with morphological studies (Fam`a *et al*., 2000, 2002; Verlaque *et al*., 2000; Stam *et al*., 2006; Draisma *et al*., 2014; Sauvage *et al*., 2013) and phytochemical investigations (Wirawan *et al*., 2022). Altamirano *et al*. (2014) in their study based on ITS1-5.8S-ITS2 sequences also showed this conspecificy in *Caulerpa racemosa*.

In the recently published paper (Salehi Balashahri *et al*., 2021) a new record, *Caulerpa selago* (Turner) C. Agardh, also was reported based on the *tuf*A data obtained from the gene information of the collected samples of *Caulerpa* specimens that all have already been reported as *Caulerpa sertulariodes* (S. G. Gmelin) Howe forma *farlowii* (Weber-Van Bosse) Børgesen from Iranian and Arabian regions of the Persian Gulf (Sohrabipour and Rabiei, 2007, 1996; Kokabi and Yusefzaadi, 2015; John and Al-Thahni, 2015), which all of them followed Nizamuddin and Gessner (1970).

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The results of this study showed that modern molecular studies are good tools for the best classification of seaweed species which help to better program planning for economic exploitation of the seaweed resources or environmental issues in marine macroalgae ecological studies concerning the climate change problems that may affect the species richness in marine ecosystems.

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Conflicts of interest

The authors have no conflicts of interest to declrae. All co-authors have seen and agree with the contents of the manuscript. We certify that the submission is original work and is not under review at any other publication.

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