Cah. Biol. Mar. (2013) 54 : 359-364

# The rediscovery of *Caulerpa prolifera* in Ria Formosa, Portugal, 60 years after the previous record

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**Abstract:** The westernmost occurrence of *Caulerpa prolifera* on the Atlantic European coast has been accepted in recent decades, to be Huelva province, southern Spain. In April 2011, this species was found in Ria Formosa, southern Portugal, extending its westernmost limit along the Iberian Peninsula coastline. In the course of research into this species it was discovered that this alga had been found in Ria Formosa in the 19th century by the naturalist Welwitsch and subsequently in the 1930s by others but it was never found in the many field studies conducted in Ria Formosa during the past few decades. The species had therefore either become extinct in the area or persisted as a cryptic undetected stage. In order to investigate the source of colonization and to verify the genetic identity, a partial cpDNA region (tufA gene) was sequenced. Comparisons of nucleotide similarity in sequences from the Ria Formosa and from populations of the Atlantic and Mediterranean confirmed the *Caulerpa prolifera* identification and gave clues about a possible origin of this population as deriving from expansion of a Mediterranean source rather than one from the western Atlantic.

**Résumé :** *La redécouverte de* Caulerpa prolifera *dans le Ria Formosa, Portugal, soixante ans après le premier signalement*. La présence la plus occidentale de *Caulerpa prolifera* sur la côte européenne atlantique a été considérée pendant des décennies être la province Huelva au sud de l'Espagne. En avril 2011, cette espèce a été récoltée dans le Ria Formosa, au sud du Portugal, étendant sa limite occidentale le long des côtes de la Péninsule ibérique. Dans le cadre de recherches sur cette espèce, il a été découvert que cette algue avait été trouvée dans le Ria Formosa au 19ème siècle par le naturaliste Welwitsch, puis dans les années 1930 par d'autres, mais elle n'a jamais été retrouvée dans les nombreuses études menées sur le terrain dans le Ria Formosa au cours des dernières décennies. L'espèce a donc soit disparu de la région ou persiste à un stade cryptique non détecté. Afin d'enquêter sur la source de la colonisation et de vérifier l'identité génétique, une région partielle de l'ADNcp (gène tufA) a été séquencée. Les comparaisons de similarité nucléotidique des séquences du Ria Formosa et de populations de l'Atlantique et de la Méditerranée ont confirmé l'identification de *Caulerpa prolifera* et ont donné des indices sur une origine possible de cette population de l'expansion d'une source Méditerranéenne plutôt que de celle de l'Atlantique Ouest.

**Keywords:** Coastal conservation ● Competitive advantage ● Genetic identification ● Ria Formosa ● Seaweed biogeography ● Seaweed distribution limits

Reçu le 1 octobre 2012 ; accepté après révision le 28 janvier 2013.
Received 1 October 2012; accepted in revised form 28 January 2013.

## Introduction

*Caulerpa prolifera* (Forsskål) J. V. Lamouroux (Chlorophyta, Caulerpaceae) is a seaweed species mainly distributed in the Mediterranean Sea, subtropical and tropical Atlantic Ocean (Macaronesia, America). In the Mediterranean and Macaronesian islands, it is the only native species of the genus *Caulerpa* present, co-occurring with congeneric species that are recent introductions. The Caulerpales have a siphonous thallus structure and typically form patches, carpets or meadows over mud and fine sand, but can also grow on rocky substrates colonized by algae (Verlaque & Fritayre, 1994) and in areas previously occupied by seagrasses, such as *Cymodocea nodosa* (Ucria) Ascherson and *Zostera marina* Linnaeus (Terrados & Ros, 1995; de la Rosa et al., 2011). Caulerpales can live down to 30 m depth and below, and are known to require less irradiance than seagrasses (Lloret et al., 2005; Malta et al., 2005). Some Caulerpales can develop into invasive species when growing outside their native ranges. The invasive capacity of the *Caulerpa* genus has been demonstrated for *C. taxifolia* (M. Vahl) C. Agardh in the Mediterranean, accidentally introduced from the Monaco Aquarium in 1984, and for *C. racemosa* (Forsskal) J. Agardh, with different varieties introduced from the Red Sea and Australia. In the Mediterranean, both species have produced large meadows in areas previously colonized by the seagrasses *Posidonia oceanica* (L.) Delile and *Cymodocea nodosa* (Meinesz, 1979). The fast proliferation of these species has been attributed to their competitive advantage over seagrasses (Ceccherelli & Cinelli, 1999; Sánches-Moyano et al., 2001), due to faster growth and the capacity to establish from a small simple fragment (Smith & Walters, 1999). Caulerpa can also withstand herbivory (Boudouresque & Verlaque, 2002), live in association with a large community of symbiotic bacteria (Meusnier et al., 2001 & 2004), and can use organic carbon, strong competitive advantages in degraded marine areas with high levels of organic matter present (Chisholm & Jaubert, 1997).

Until now, the occurrence of *C. prolifera* on European Atlantic coasts was limited to Southern Spain in the Cadiz province (Sánchez-Moyano et al., 2001 & 2007; Rueda & Salas, 2003), and in the Huelva province of Southern Spain. The westernmost limit was a recent record in Isla Cristina marshes, where it was found spreading over approximately 50 ha at 0.5-7 m depth, in two separated meadows (de la Rosa et al., 2011). This study extends this distributional limit into Portugal and analyses its possible origin.

## Methods

### *Field studies and identification*

The Ria Formosa coastal lagoon (Algarve, southern Portugal) is a barrier - island system characterized by shallow channels, mud flats and salt marshes, a similar ecosystem to that at the previous geographical limit of *C. prolifera* in Isla Cristina (southern Spain), which is ca. 50 km to the east.

A seagrass mapping survey in the Fuseta channel was conducted in April 2011 as part of a program of monitoring and mitigating the impacts of channel dredging and inlet opening in Fuseta Island (Fig. 1). This investigation revealed the unexpected presence of *C. prolifera*. Only one patch was found, covering 3 by 4 m, at a depth of 1-4 m (below low tide), despite surveys over all the channels in the vicinity (with a combined length of ca. 500 m). The area where this algal patch was found was previously covered by an extensive meadow of the seagrass *Zostera marina* (now absent) growing along the channel sides. Seagrass cover (intertidal and subtidal) in this area and for all Ria Formosa had been mapped in 2007 and 2009 for the Life Biomares project, but no *Caulerpa* sp. had ever been found (Cunha et al., 2009 & 2011).

A second survey was undertaken in January 2012, when it was confirmed that all the *Z. marina* meadows in the area had disappeared, but the same patch of *C. prolifera* was present and had apparently, increased in area and density. *Z .marina* disappeared due to the increasing amount of sediment in suspension and changes in water currents caused by the dredging and the proximity of inlet relocation. The position of the *Caulerpa* patch was 37°03’13.8’’N-07°44’00.00’’W as measured by a GPS. A sample collected to extract DNA to confirm species identity and to compare with data on Mediterranean, Atlantic and IndoPacific isolates previously sequenced.

The morphology of the alga found was compared with a *Caulerpa prolifera* specimen in the herbarium of Lisbon (LISU, Holmgren et al., 1990) and a recently collected specimen was deposited there referenced as MGC Phyc 5090.

### *DNA extraction, PCR amplification, sequencing and phylogenetic analysis*

DNA was extracted using the CTAB method of Doyle & Doyle (1987) from fresh material from one ramet of *C. prolifera* (a section of stolon bearing several fronds). A partial region of the cpDNA tufA gene was amplified by PCR following Famà et al. (2002). PCR conditions were hot start (95°C, 5 min), 40 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 1 min 30 sec, and final extension of 5 min at 72°C. PCR reactions were performed in a 20 µl volume containing buffer (10X), dNTPs (2 mM), $MgCl_2$ (50 mM), primers (10 mM), 0.3 U AmpliTaq Gold polymerase and approximately 5 to 10 ng of template DNA. Amplified products were visualized on a 1% agarose

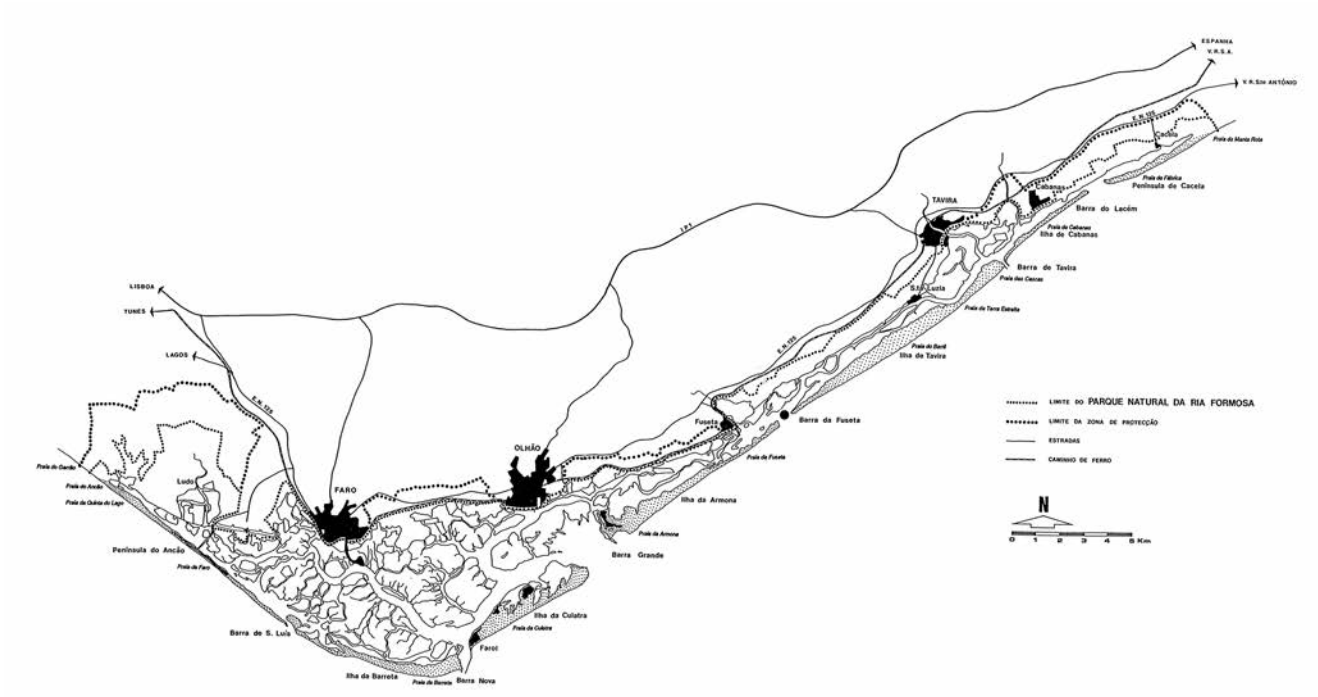


**Figure 1.** Location of the *Caulerpa prolifera* population in Fuseta channel, Ria Formosa, Southern Portugal.

gel and the excess of primers and dNTPs from the PCR product were removed using the Amersham PCR Purification Kit (Qiagen, Hilden, Germany). Cloning was carried out using the pGEM T-Easy Vector system II (Promega, Madison, USA) with JM109 competent cells following the manufacturer's protocol. Eight clones were sent for sequencing to the CCMAR sequencing unit and to MACROGEN (Seoul, Korea) resulting in sequences of 961 bp. After editing the sequences, the resulting sequences (from 884 to 961 bp long) were submitted to GenBank (accession numbers shown in Table 1) and were compared with 25 tufA sequences for *C. prolifera* available in GenBank, with the exception of one (DQ652388) containing ambiguities. Sequence alignments and phylogenetic analyses were conducted with alignment explorer in MEGA4 (Tamura et al., 2007) and the PhyML software (Guindon & Gascuel, 2003) through the PHYLO-WIN graphical interface (Galtier et al., 1996). In all analyses, all positions containing gaps and missing data were eliminated from the dataset. An appropriate evolutionary model for the data set was selected using MODELTEST (Posada & Crandall, 1998). For ML, bootstrap proportion values (> 50%) were calculated following the aLRT (approximate Likelihood Ratio Test). *C. taxifolia* was used as outgroup (Accession number: DQ652360).

**Table 1**. GenBank Accession numbers of *Caulerpa prolifer*a sequences used in this study.

| Reference | *C. prolifera* localities | GenBank accession numbers |
|---|---|---|
| Stam et al., 2006 | FLORIDA | DQ652380, DQ652381, DQ652372, DQ652373, DQ652374, DQ652375, DQ652376, DQ652377, DQ652378, DQ652379, DQ652382, DQ652383, DQ652384, DQ652385, DQ652386, DQ652387, DQ652388, DQ652389, DQ652390, DQ652391, DQ652392 |
| Famà et al., 2002 | FLORIDA | AJ417943 |
| | ITALY | FM956042 |
| Famà et al., 2002 | BALI | AJ417942 |
| | ISRAEL | GU815499 |
| This study | PORTUGAL | JX206458, JX206459, JX206460, JX206461, JX206462, JX206463, JX206464, JX206465 |

## Results and Discussion

We report here the range extension of *C. prolifera* in Europe, which previously was ca. 50 km eastwards in southwestern Spain (de la Rosa et al., 2011). This is the only *Caulerpa* species native to the Mediterranean Sea and its rare occurrence in the Atlantic has been restricted to warmer locations such as Cadiz Bay, warmer Macaronesian islands (Madeira, Canaries and Cape Verde) and Florida. The species had been previously recorded in southern Portugal in the 19th century, by the naturalist Frederick Welwitsch (specimens at Welwitsch collection at LISU) in Cape Santa Maria, another section of the Ria Formosa, in April 1845 and 1847 (Fig. 2). It had also been collected by two other researchers in September 1931 (Mendonça) and October 1932 and 1933 (Lami), with herbarium material deposited at the University of Coimbra Herbarium (COI). The presence of this alga was also reported for Ria Formosa by Palminha in 1951, where he describes that "there are important meadows of *C. prolifera* in the muddy sides of the channels, never uncovered by the tides..." (Palminha, 1951). Later, a review of the distribution of the seaweeds of Portugal (Ardré, 1970 & 1971) reported this species for Ria Formosa, but there is no indication if this was a personal observation or based on the older records. The Ria Formosa has been extensively surveyed by marine biologists since the 1980's, with the implementation of many marine biology field studies and surveys from the University of Algarve. It thus seems unlikely that the alga could have been present in such a conspicuous way as reported by Palminha not least because there have been extensive intertidal and subtidal seagrass mapping and monitoring surveys during the last 20 years (e.g. Cunha, 1991; Billingham et al., 2003; Cunha et al., 2009, 2011 & 2013; Guimarães et al., 2012; SeagrassWatch program; Adopt-a-seagrass program). Although it cannot be completely ruled out that it could have persisted unnoticed, it is not known whether *C. prolifera* entirely disappeared from the Ria Formosa or persisted in a cryptic undetected stage or location for the last 60 years.

*Caulerpa prolifera* is considered an opportunistic species, whose development may be highly favored by increased nutrients and suspended sediment load that reduce water column transparency, allowing it to out compete seagrasses. This may be due to its lower light compensation point for photosynthesis, in addition to the possibility of heterotrophy (e.g. Chisholm & Jaubert, 1997) possibly favoured by the presence of many symbiotic bacteria. In Ria Formosa, the recent observations coincide with dredging activities which lowered water transparency considerably, causing loss of seagrass meadows; this might have provided a local competitive opportunity (Cunha & Serrão, 2011). It is interesting to ask why is this meadow not spreading nor colonizing new locations in the Ria Formosa, but to date there is no information to answer this question. The algae did not show marks of herbivory on the fronds. There is also no explanation as to why this alga was so conspicuous previously but was never found again until now. Species at or near their limits of distribution may occur ephemerally; Price et al. (1979) showed this for *Padina pavonica* in northern Europe.


**Figure 2.** Herbarium sheet from the University of Lisbon LISU showing the *C. prolifera* sampled by F. Welwitsch in 1845.

The 544 positions common to the available GenBank sequences used in the final dataset included one single sequence from Portugal, because all the eight replicates were identical in this region. All Atlantic replicates were also identical for this region. Sequences from the Mediterranean versus the Atlantic differed in one bp (Table 2). The sequence from Portugal was identical to the Mediterranean ones, confirming species identity and revealing a Mediterranean origin (see also phylogenetic

**Table 2.** Alignment showing the Single Nucleotide Polymorphism among isolates of *C. prolifera*. (**) Represents 2 identical sequences from Italy and Israel. (*) Represents 22 equal sequences from isolates from Florida, USA.

| |
|---|
| *C. prolifera* Atlantic * |
| …...TTTTTAATGGCTGTCGAAAATGTTGTTTCCAT**T**ACAGGTAGAGGCACTGTAGCAACTGGTGAGTTGAAC… |
| *C. prolifera* Bali |
| …...TTTTTAATGGCTGTCGAAAATGTTGTTTCCAT**A**ACAGGTAGAGGCACTGTAGCAACTGGTCGAGTTGAAC… |
| *C. prolifera* Mediterranean……. ** |
| …..TTTTTAATGGCTGTCGAAAATGTTGTTTCCAT**A**ACAGGTAGAGGCACTGTAGCAACTGGTCGAGTTGAAC… |
| *C. prolifera* Portugal |
| …..TTTTTAATGGCTGTCGAAAATGTTGTTTCCAT**A**ACAGGTAGAGGCACTGTAGCAACTGGTCGAGTTGAAC… |

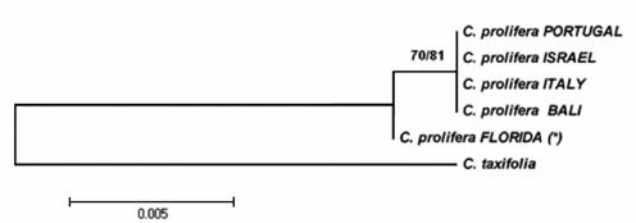


**Figure 3.** Phylogenetic tree inferred using NJ and ML analyses, which gave the same topology. Branch length indicates Tamura-Nei evolutionary distances in number of substitutions per site. Bootstrap support for NJ (left) and ML (right) is shown above the branches. * represent identical sequences from the isolates from Florida.

tree in Figure 3).

Several questions are raised by this discovery. Why did it disappear or at least remain unreported for decades? Alternative hypotheses are that it might be a chance event of rare dispersal or instead caused by environmental changes such as warmer water and/or disturbance regimes that have created habitat conditions more suitable for the *Caulerpa*, but less favorable for seagrasses. A chance dispersal event could have taken place by propagules carried on dredging boats and barge transportation. Notably, the machinery used during the dredging works in Fuseta channel had previously worked in the Huelva province (Sociedade POLIS pers. com.)

### Acknowledgements

Funding was provided by ”Sociedade Polis Litoral Ria Formosa S.A” and the Portuguese Science Foundation FCT (E.V-A, PTDC/MAR/70921/2006 and a postdoctoral fellowship, SFRH/BPD/17206/2004). Particular recognition is given to E. Berecíbar and R. Santos for the updated information on records of *C. prolifera* in Portugal. We thank Ana Balau and Marta Valente for the sequencing work.

Herbaria information

Lisbon Faro, Cabo Sta. Maria 4/1847; 4/1845 leg. Welwitsch LISU.

Lisbon Canal da Fuseta, Ria Formosa 4/2011; 4/2012 leg. Cunha LISU251052.

Coimbra Faro 10/1932 e 1933 leg. Lami COI., Faro 9/1931 leg. Mendoça COI.

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