

Article

Settlement and Spreading of the Introduced Seaweed *Caulacanthus okamurae* (Rhodophyta) in the Mediterranean Sea

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Abstract: In this study, we report the first finding of the non-indigenous seaweed *Caulacanthus okamurae* (Rhodophyta) in the Ionian and Adriatic Seas (Mediterranean). Specimens were identified through molecular analyses based on the plastid ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*) marker. The sequences obtained during this study represent the first molecular evidence of the presence of this taxon in the Mediterranean Sea. Stable populations have been detected in some areas of the Mar Piccolo of Taranto (Italy) and in the whole lagoon of Venice, forming dense patches of low turf that reach high biomasses. Turf-forming algae are common in the intertidal zones of tropical regions, but are rare in temperate ones. The particular environmental conditions of transitional water systems, such as the Mar Piccolo of Taranto and the Venice Lagoon, together with the water temperature increase observed in the last years could have favored the settlement and spread of this introduced species.

Keywords: *Caulacanthus okamurae*; Mediterranean Sea; non-indigenous species; *rbcL*; transitional water systems

1. Introduction

Caulacanthus Kützinger is a small genus of the order Gigartinales and includes three taxonomically accepted species [1]. The type species of the genus, *Caulacanthus ustulatus* (Mertens ex Turner) Kützinger, was believed to have a cosmopolitan distribution in the Eastern Atlantic and the Indo-Western Pacific until 2002. In this year, Zuccarello and collaborators [2], through molecular analyses based on the plastid Rubisco spacer and on the mitochondrial *cox2-3* spacer, reported that *C. ustulatus* included two major lineages, an Indo-Western Pacific lineage and an Atlantic lineage. Several Asian authors used the name *Caulacanthus okamurae* Yamada for populations from the Northwestern Pacific (e.g., [3]), but for a long time the validity of the name and the distinctiveness of the species have been in doubt. Observations of the reproductive structures [4,5] and phylogenetic analyses [2] supported the validity of *C. okamurae* as a distinct species, representing the Indo-Western Pacific lineage of *C. ustulatus*.

This small turf-forming seaweed, first described from Japan by Yamada [6], is also native to China, Korea and Taiwan [1]. Introduced populations are known from the North-Eastern Pacific, being found in California, Mexico, British Columbia, Alaska and Washington, where they were probably introduced by hull-fouling, ballast waters and oyster aquaculture [7–11]. In Southern California this species has become invasive in mid-intertidal algal turf communities [9,12,13], where it forms dense patches of low turf exceeding 40% cover. This turf has displaced macroinvertebrates, such as limpets, periwinkles

and barnacles, but has supported an increase of copepods, ostracods, and fleshy seaweeds, including *Ulva* spp., *Gelidium* spp. and *Chondracanthus* spp. [12,13].

In North-Eastern Atlantic, *C. okamurae* was initially reported from Brittany (France) in 2000 under the name *C. ustulatus* [14]. Subsequently, specimens from Brittany were grouped with the Pacific lineage based on the plastid and mitochondrial DNA analyses by Zuccarello et al. [2]. Populations of this species were also found in Belgium [15], the Netherlands [16], the Southern coast of England [17], in Aquitaine (France) [18], in Normandy (France) [19] and in Galicia (North-Western Spain) [20]. In the Mediterranean, *C. okamurae* was reported from two French localities: in Marseille in 2004 and later in Toulon [21].

In this study we analyze populations of *Caulacanthus* sp. from two Italian transitional water systems: the Mar Piccolo of Taranto (Ionian Sea) and the Venice Lagoon (Adriatic Sea). Through morphological and molecular analyses based on the plastid ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL*) gene, we were able to identify the collected specimens as the non-indigenous species (NIS) *C. okamurae*. Distribution and abundance of the introduced *C. okamurae* populations were monitored in both the studied areas.

2. Materials and Methods

Samples of *Caulacanthus* sp. were collected in two Italian transitional water systems: the Mar Piccolo of Taranto and the Venice Lagoon (Figure 1a,b,c).

The Mar Piccolo of Taranto (40°28'46" N, 17°13'41" E) (Southern Italy, Ionian Sea) belongs to the European network of Long Term Ecological Research (eLTER) as an Italian transitional water system (<https://tinyurl.com/sn3cweu>). This basin, with a total area of about 21 km², is located in the North of Taranto and is divided into two elliptical sub-basins (i.e., the First Inlet, on the West, and the Second Inlet, on the East) by two facing headlands. The Porta Napoli Channel and the Navigabile Canal connect the Mar Piccolo with the near Mar Grande basin and represent the only path for seawater exchange (Figure 1c). Hydrodynamism is weak. Tidal range is minimal, not exceeding 30 cm. Due to the strong anthropogenic pressures, most of the substrata are artificial (e.g., concrete quays, mussel farming equipment). Few natural substrata (i.e., sparse rocks and pebbles) are present only in the Second Inlet. The physical-chemical features of the Mar Piccolo seawater are strongly influenced by 34 submarine freshwater springs, locally named "citri", and a few small rivers flowing into the basin [22]. Temperature ranges between 7.5 °C and 32.3 °C. Salinity ranges between 33.0 and 37.7 [23]. In the Mar Piccolo, seasonal sampling campaigns have been carried out since spring 2011, within the framework of LTER activities. During each season, in five stations (i.e., two in the First Inlet and three in the Second Inlet) three replicates of seaweeds were systematically collected by hand within a 50x50 cm square and brought to the IRSA-CNR laboratory in Taranto. Here the collected biomasses were sorted and each dominant species was weighed through a triple beam balance (Sartorius, Göttingen, Germany). Since spring 2018, thalli of dubious species have been silica gel dried for further molecular analyses. In the Mar Piccolo, *Caulacanthus* sp. represents an understory epiphyte mainly of *Chondracanthus acicularis* and *Ulva* spp., and thus it is not visible to the naked eye.

The Venice Lagoon (45°26'33.9" N, 12°20'36.1" E), located in the North-Western part of the Adriatic Sea, is the largest transitional water system of the Mediterranean Sea with a total surface of about 549 km² (Figure 1b). The basin has a mean depth of about 1 m and a mean tidal excursion of ± 31 cm, accounting for 60% water exchange every 12 hrs throughout three large (600–900 m) and deep (12–15 m) mouths. The trophic level of the lagoon showed strong changes with very high nutrient concentrations both in the water column and surface sediments between the 1960s and the 1990s. In the 1980s, the gross production of nuisance macroalgae, mostly Ulvaceae, was about 18.5 million tons on a fresh weight (fw) basis [24]. In the following years, nutrient concentrations and macroalgal production strongly declined and, currently, they are one order of magnitude lower [25]. Water temperature ranges between −0.5 °C (with glaciation of canals and the inner areas between salt marshes) and over 40.0 °C (July 2019) in very choked areas. Salinity depends on river proximity, but, in general, it is

higher than 25 in most parts of the lagoon. The mean temperature of the lagoon in 88 sites, determined in June–July 2018, was 27.2 ± 1.7 °C with a peak of 33.7 °C. In the same stations, the mean salinity was 28.8 ± 5.4 with a peak of 40.6. In the lagoon of Venice, *Caulacanthus* sp. specimens have been reported since 2002 and cover all the midlittoral substrata; based on the season, they are interspersed with other algal taxa, such as *Ulva* spp., *Ellisolandia elongata* (J. Ellis et Solander) K.R. Hind et G.W. Saunders and *Chaetomorpha aerea* (Dillwyn) Kützinger. At each station, six replicates of 40 × 40 cm were collected and carried to the laboratory for biomass quantification and species identification. The mean value of 6 stations (three in poor quality areas and three in good quality areas), sampled in late spring, was used to estimate the approximate biomass of *Caulacanthus* in the lagoon.

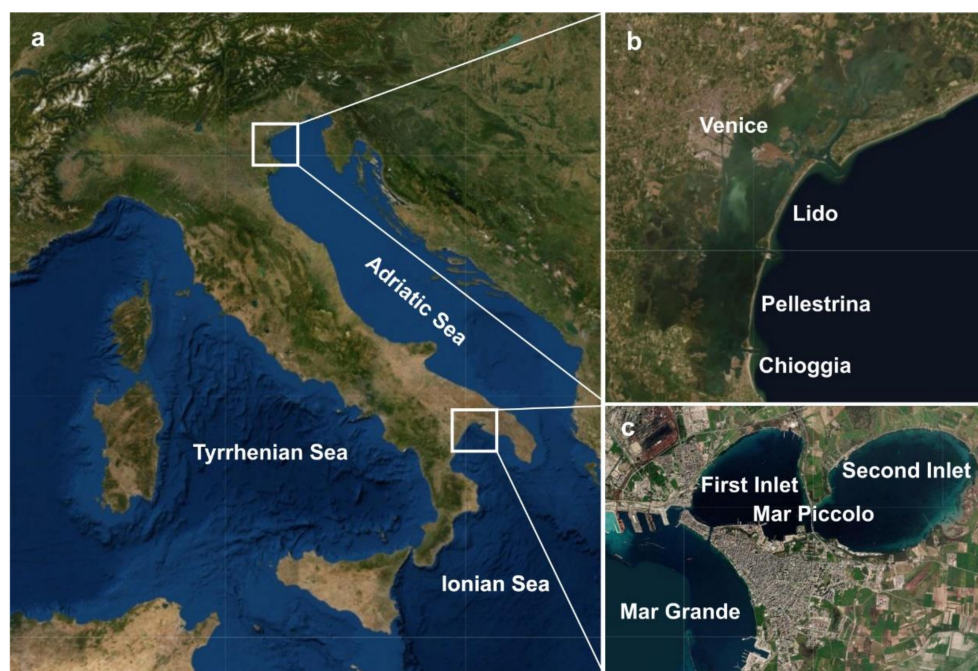


Figure 1. Sampling sites. (a) Map of the Italian peninsula showing the position of the studied areas (squares), (b) map of the Venice Lagoon, (c) map of the Mar Piccolo of Taranto. The maps were created using the National Geographic MapMaker (<https://mapmaker.nationalgeographic.org>).

Caulacanthus sp. specimens for molecular and morphological analyses were collected during April 2018 and January 2020. For each sample, a part was silica gel dried for molecular analyses and a part was preserved in 4% formaldehyde/seawater solution for morphological observations. Specimens were observed using a light microscope (Optika B-510PH, OPTIKA, Italy) equipped with a digital image acquisition system. Final pictures, suitable for publication, were created with GIMP v. 2.8.22 (<https://www.gimp.org>) and Inkscape v. 0.92 (www.inkscape.org).

Genomic DNA was extracted using the Genomic DNA purification kit (Thermo Scientific™, Waltham, MA, USA). Amplification of the *rbcl* gene was carried out with the primer pairs F57-R753 and F577-RrbcSstart [26], following the PCR conditions listed in [27]. The obtained PCR products (about 1200 bp long) were cleaned using the HT ExoSAP-IT (Applied Biosystems™, Waltham, MA, USA) and sequencing was carried out at the Eurofins Genomics Sequencing Service (Germany), with the same primers employed in the amplification reactions. The GeneStudio sequence analysis software (<http://genestudio.com/>) was used to assemble the final consensus sequences. The new sequences (one for each sampling site) were deposited in the International Nucleotide Sequence Database Collaboration (INSDC) repositories, through the European Nucleotide Archive (ENA) platform, with the following GenBank accession numbers: LR761600 (Venice Lagoon) and LR761601 (Mar Piccolo of Taranto).

The identities of the obtained sequences were checked using the BLAST program [28] available at the USA National Center for Biotechnology Information (NCBI) web server (<http://www.ncbi.nlm.nih.gov>) and

the sequence alignments were generated with the ClustalW computer program [29]. A dataset of 21 *rbcL* sequences of specimens belonging to the order Gigartinales was constructed, including the sequences obtained in this study and sequences with the highest identity found through the BLAST search. The species *Gracilaria salicornia* (family Gracilariaceae) was chosen as the outgroup.

Phylogenetic analyses were performed with MEGA v. X program [30] using the Neighbor Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) methods. For ML, the model that best fit the data according to the ModelTest software implemented in MEGA v. X, under the BIC criterion [31], was GTR + G + I. Non parametric bootstrap re-sampling [32] was performed to test the robustness of the obtained topologies (1000 replicates). The final tree picture was created with Inkscape v. 0.92.

3. Results

3.1. Molecular Analyses

The *rbcL* sequences obtained from the 15 *Caulacanthus* isolates examined in this study were all identical among them. For this reason, only two sequences (one for each sampling site) were included in the phylogenetic reconstruction (Figure 2). The analyses comprised both sequences with the highest identity found through the BLAST search and sequences of species belonging to different taxa of the order Gigartinales. Sequences of the investigated Italian samples were included in a well-supported clade (100NJ/100MP/100ML) with two sequences of *C. okamurai* from South Korea and Atlantic Spain. This clade was sister taxon, with a good bootstrap support (98NJ/97MP/98ML), to a clade including two sequences obtained for the species *C. ustulatus* from Portugal and Namibia. The range of *rbcL* nucleotide divergence found between specimens of *C. okamurai* and *C. ustulatus* was 8.67–8.82%.

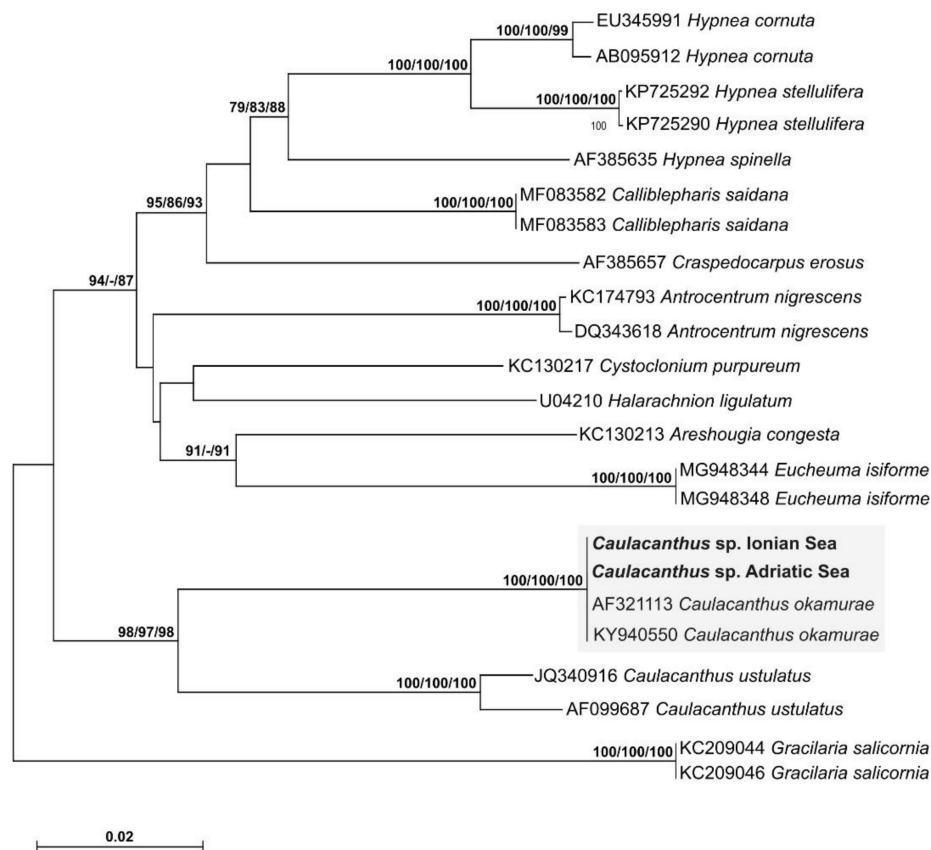


Figure 2. Phylogenetic reconstruction inferred from *rbcL* sequences. The numbers near the nodes indicate bootstrap values (>50) (NJ/MP/ML). The sequences obtained in this study are indicated in boldface font. The clade including *C. okamurai* sequences is highlighted in grey color.

3.2. Morphological Observations

In the Mar Piccolo of Taranto, *Caulacanthus okamurae* made a dense turf at the base of *Chondracanthus acicularis* (Roth) Fredericq and *Ulva* spp., at a maximum depth of 0.7 m and along a narrow part of the coast.

In the Venice Lagoon, *C. okamurae* formed irregular mats on the substratum, appearing as tangled pom-poms interspersed with few other algal taxa (Figure 3).



Figure 3. Tufts of *Caulacanthus okamurae* covering hard substrata in the Venice Lagoon.

Morphological observations carried out on specimens from both the Italian basins showed that thalli grew on hard substrata, including bivalve shells (Figure 4a), and reached a maximum height of about 20 mm (Figure 4b). They were cartilaginous, from dark red to brownish in color. Main axes were cylindrical (Figure 4c) and branching was irregular to sub-dichotomous, with the smallest branchlets, both intercalary and terminal, showing a spine-like shape (Figure 4c,d). The terminal part of thalli was often constituted by two spine-like branchlets curved to form an obtuse angle (Figure 4c). The main axes were 200–250 μm in diameter (Figure 4e). In the cross section, *C. okamurae* did not present rhizoids (Figure 4e), unlike *C. ustulatus*, and this character was present in all the investigated thalli, facilitating the morphological distinction between the two species. Diploid tetrasporophytic thalli (Figure 4f) had tetrasporangia, which occurred as swellings (Figure 4g), mainly in the basal part of the spine-like branchlets (Figure 4f).

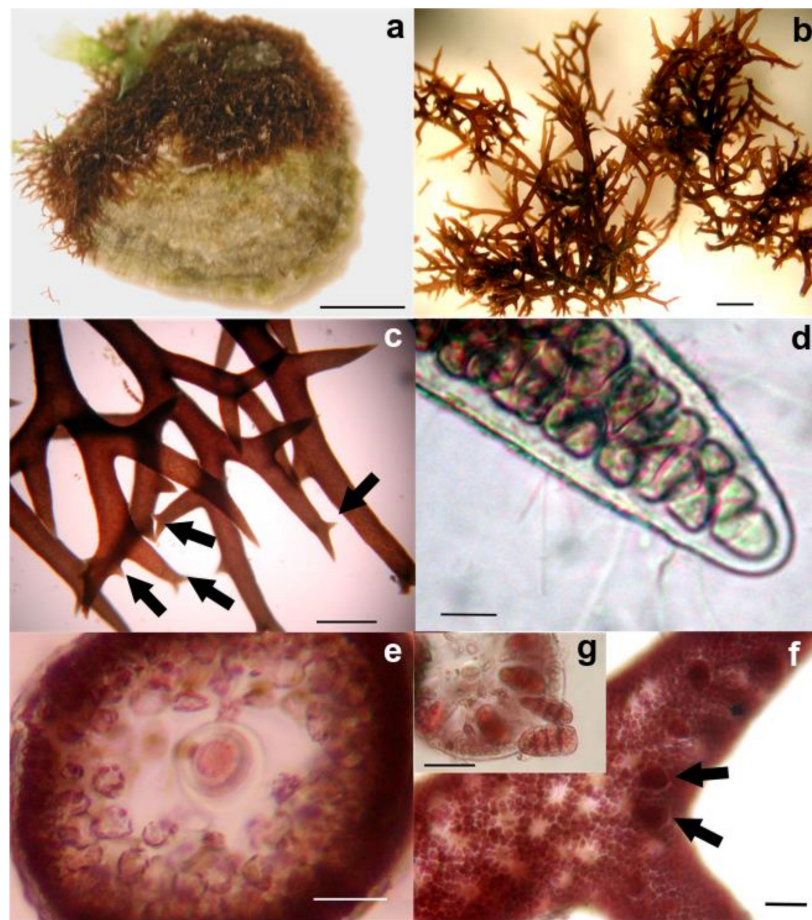


Figure 4. *Caulacanthus okamurae* from Italy. (a) Specimen growing attached on a bivalve shell. (b) General view of thalli. (c) Detail of a thallus showing the branching and the apices (arrows). (d) Magnification of an apex. (e) Cross section. (f) Surface view of a tetrasporophytic thallus, showing tetrasporangia (arrows) formed mainly in the basal part of branches. (g) Cross section in proximity of tetrasporangia. Scale bars: (a): 1 cm; (b): 5 mm; (c): 500 μ m; (d): 10 μ m; (e-g): 50 μ m.

3.3. Distribution and Standing Crop Estimation

In the Mar Piccolo of Taranto very few thalli of *C. okamurae* were collected for the first time at the station Cimino, in the Second Inlet, in summer 2012 as a turf species. Since winter 2013, this species was observed in the same station with increasing biomass values every season until 2016, when the highest annual mean value was recorded (534 ± 272 g fw m^{-2}) as well as a spring peak (2980 g fw m^{-2}). Since 2017, biomass values have fluctuated within seasons and within years. Tetrasporophytic thalli were observed for the first time in summer and autumn 2013; since 2014, they were found mainly in spring, but never in winter. Gametophytic thalli have never been observed. Very few sterile thalli were observed also at the other two stations in the Second Inlet (Battendieri and San Pietro) in spring 2016 and in winter 2017.

In the Venice Lagoon *C. okamurae* grows strongly attached to the midlittoral docks and stones of jetties and breakwater barriers, which extend for about 100 km^2 in the lagoon. It often completely covers oyster and mussel shells, forming a continuous belt that is present all year round, but with the highest biomass in late spring (Figure 3). The species, previously misidentified with *Caulacanthus ustulatus* (Mertens ex Turner) Kützinger, is present everywhere, both in areas of bad or high ecological status. Since the early 2000s (first record September 2002), *C. okamurae* has almost completely replaced *Gelidium spathulatum* (Kützinger) Bornet, *Gelidium pusillum* (Stackhouse) Le Jolis and *Gymnogongrus griffithsiae* (Turner) C. Martius; this last being the dominant species of the midlittoral

in the past [33]. Tetrasporophytic thalli are common, whereas gametophytic thalli have never been observed. The mean biomass recorded in six stations, representing the different distribution of the species on the Venice midlittoral, was 1359 ± 897 g fw m^{-2} , with values ranging from 218 to 2398 g fw m^{-2} . A biomass peak of 3.110 kg fw m^{-2} was recorded, in a single replicate, in late May 2019. The estimation of the total biomass in the lagoon was about 272 tons fw.

4. Discussion

Identification of non-indigenous species (NIS) can be very difficult in the case of macroalgae, particularly for taxa showing cryptic and/or overlapping morphologies. This is the case of *Caulacanthus okamurae* and *C. ustulatus*. Indeed, these two red algal species have a very similar thallus morphology and are distinguishable only for the presence/absence of internal rhizoids and for the shape of carposporangia [21]. As pointed out by Choi and collaborators [4,5], sexual reproduction of those species is very rare and this makes it more difficult to distinguish between the two taxa. In this study, we analyzed several specimens of *Caulacanthus* sp. sampled from two transitional water systems in the Ionian and Adriatic Sea (Mediterranean) and, through molecular analyses based on the plastid *rbcL* marker, we were able to identify them as *C. okamurae*, recording this species for the first time in the investigated areas. As shown in the phylogenetic reconstruction (Figure 2), the Italian samples are genetically identical to specimens of *C. okamurae* from South Korea and are clearly distinct from the cryptic species *C. ustulatus*. The sequences obtained during this study represent the first molecular evidence of the presence of *C. okamurae* in the Mediterranean Sea.

In the Mar Piccolo of Taranto, since the first record, *C. okamurae* was observed closely associated with *Chondracanthus acicularis*, *Gelidium crinale* (Hare ex Turner) Gaillon and *Ulva laetevirens* Areschoug. However, the number of associated species has increased over the years and, among them, several other NIS were identified (e.g., *Grateloupia minima* P. Crouan et H. Crouan, *Hypnea cornuta* (Kützinger) J. Agardh, *Polysiphonia morrowii* Harvey). This seems in accordance with the observations made in Southern California, where *C. okamurae* was considered a good shelter for macroalgae [13]. At the same time, the high number of NIS could be linked to the processing of mollusks performed in the area. Indeed, these organisms are considered the main vectors for NIS introduction in the basin [34].

In the Mar Piccolo, *C. okamurae* showed a considerable increase in biomass during the first three years of settlement, decreasing in the following years, and up to now it has not spread to the rest of the basin from the station of first finding, except for two insignificant records in other stations. A similar behavior was observed in the Mar Piccolo for other two NIS native from Japan: *Undaria pinnatifida* (Harvey) Suringar and *Grateloupia turuturu* Yamada [23,35]. *Undaria pinnatifida* spread over a limited area and has completely disappeared in ten years. *Grateloupia turuturu* settled down without spreading in the basin or outside, and the level of settlement was considerably reduced after ten years. Temperature is considered the main environmental factor controlling both species spreading [23,35].

The optimal temperatures for sexual reproduction of *C. okamurae* are between 19 °C and 27 °C, as assessed in lab-cultures by Choi and collaborators [4,5]). This range is within the mean spring-summer temperature values measured in the Mar Piccolo seawater [23]. However, since 2012, the average values of water temperature, measured in the basin in the warmest week, have been considerably higher than those evaluated in lab-cultures for *C. okamurae* optimal growth (at the time identified as *C. ustulatus*) [36]. These hot conditions have very probably limited the sexual reproduction capability of this species, leaving asexual reproduction as the only way to maintain the population, but not enough to support *C. okamurae* spreading. Therefore, on the basis of eight-year observations, *C. okamurae* can be considered settled down in the Mar Piccolo, but not invasive. For the moment, this turf species seems to support biodiversity rather than representing a threat.

The lagoon of Venice is a hotspot of non-indigenous species (NIS) [37], with over 30 macroalgal species whose extra-Mediterranean origin has been verified [38,39]. Out of the total 323 seaweed taxa reported for the lagoon, in 2018 the contribution of NIS to the total biomass was approximately 32% (146,521 tons fw). Among them, three taxa (*Agarophyton vermiculophyllum* (Ohmi) Gurgel, J.N. Norris et Fredericq, *Agardhiella subulata* (C. Agardh) Kraft et M.J. Wynne and *Hypnea cervicornis* J. Agardh)

accounted for ca. 89.7% of the total NIS. *A. vermiculophyllum* and *H. cervicornis* are species that grow mainly free-floating in choked areas, whereas *A. subulata* can grow also in mud sediments, attached to bivalve and gastropod shells. Almost all the other NIS are attached to hard substrata, resulting therefore in lower biomasses. This is the case of *C. okamurae*, of which stable populations have been reported since the early 2000s [40], misidentified with *C. ustulatus*. Temperatures above 19 °C are measured from May to October, but gametophytes have never been observed. In spite of this, this species has rapidly colonized all the hard substrata of the midlittoral zone, forming a dense belt below the *Blidingia* belt, in association with *Chaetomorpha aerea*, many *Ulvaceae* and seasonal species belonging to Phaeophyceae. In the lagoon of Venice, *C. okamurae* can be considered invasive, because it colonizes every suitable substratum of the area. However, even though it reduces the presence of other species, it does not replace them, contributing to increase biodiversity, as shown by Sfriso and collaborators [39] for the other macroalgal NIS.

Turf-forming algae are common in the intertidal zones of tropical regions but are rare in temperate ones. In the last years, the particular environmental conditions of transitional water systems, such as the Mar Piccolo of Taranto and the Venice Lagoon, together with the water temperature increase could have favored the settlement and spreading of turf-forming NIS. In the light of this, and due to the resemblance with *C. ustulatus* in the gross morphology, a molecular screening of *Caulacanthus* specimens is advisable to assess the real distribution of *C. okamurae* in the Mediterranean Sea.

Author Contributions: Field work and specimen collections were carried out by A.P. and A.S. Morphological analyses were performed by A.P. and A.S. Molecular and phylogenetic analyses were carried out by M.A.W. and K.S. The research design was accomplished by A.P., E.C., M.A.W. and A.S. Manuscript drafting and editing was performed by A.P., M.A.W., K.S. and A.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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