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Research Article

Identification of *Ulva* sp. Grown in Multitrophic Aquaculture Systems

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Abstract

The genus *Ulva* is one of the most numerous of marine and estuarine genera. Traditionally cultivated for human consumption, since the 1990s *Ulva* was integrated into land based Integrated Multi-Trophic Aquacultures (IMTA) for biomass production and bioremediation. A proper taxonomic identification is the first critical step in implementing an algal production program. However, *Ulva* species are difficult to morphological identify due to their phenotypic plasticity. Combined molecular and morphological techniques can lead to better characterization of *Ulva* sp. This study identifies the *Ulva* sp. cultivated in earth ponds facing the Ria Formosa Lagoon (South Portugal) as well as green algae growing spontaneously in the ponds. DNA barcoding with the markers ITS (Internal Transcribed Spacer) identified six species, with *Ulva flexuosa* being the cultivated one. *Ulva flexuosa* was recorded for the first time in South Portugal. However, taxonomic questions were raised because distinct clades were found for this species using published sequences. The 'lettuce-leaf' morphotype observed is not attributable to any of the marine subspecies of *Ulva flexuosa*.

Keywords: DNA-Barcoding; ITS; Species identification; *Ulva flexuosa*

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Introduction

The genus *Ulva* is one of the most numerous of marine and estuarine genera [1]. The cosmopolitan distribution of the genus *Ulva* makes it suitable for cultivation practically everywhere [2]. Traditionally cultivated for human consumption, since the 1990s *Ulva* was integrated into land based Integrated Multi-Trophic Aquacultures (IMTA) for biomass production and bioremediation [3]. *Ulva* spp. withstand the extreme environmental condition of earth ponds and when grown in effluent media, protein content increases (> 40%), resulting in a valuable feed for macroalgivore species with high commercial value [2-5]. The current market for these algae is limited, but could see growth considering the suitability of *Ulva* as a biomass energy resource and its application as a raw material for nutraceuticals, biomaterials and sulphated polysaccharides (Ulvan) [3,5-7]). Given the growing demand for algae, a proper taxonomic identification is necessary in aquaculture [8,9]. Selecting appropriate target species is the critical first step in implementing an algal production programme. Moreover, improper taxonomic identification makes comparing results difficult, inhibiting the consolidation of knowledge about production and other characteristics of cultivated species [9]. An accurate assessment of marine macroalgae is important for conservation, monitoring, and management of biological introductions and invasions [10]. Due to phenotypic plasticity, the morphological characteristics of several *Ulva* species have insufficient taxonomic value [11-13]). The combination of molecular and morphological techniques can lead to better characterization of taxa [14-18]). DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it [19]. The main goal is to identify an unknown sample in terms of a pre-existing classification [20]. The Internal Transcribed Spacer region of ribosomal cistron (ITS) has been used in several studies concerning the *Ulva* species identification [16,21,22]). ITS is proving useful for identification at species level due to its multiple highly variable regions [23-25].

The IPMA aquaculture research station in Olhão (EPPO-EstaçãoPiloto de Piscicultura de Olhão), Portugal, cultivated *Ulva* sp. during an Integrated Multitrophic Aquaculture (IMTA) experiment in earthen ponds. The purpose of this study was to verify the taxonomic identity of the *Ulva* sp. grown and identify the green algae that grew spontaneously in the pond system.

Materials and Methods

The IMTA experiment was conducted at the Aquaculture Research Station in Olhão (EPPO-Estação Piloto de Piscicultura de Olhão), Portugal. The EPPO station is located in the salt marshes of Ria Formosa coastal lagoon, a mesotidal system in the south of Portugal (Figure 1a). *Ulva* spp. were collected in the main discharge channel of EPPO (Figure 1b), a portion was weighted and individually planted in 6 rafts (1 m^2 each), made of horizontal nets stretched between styrofoam floaters (Figure 2a).

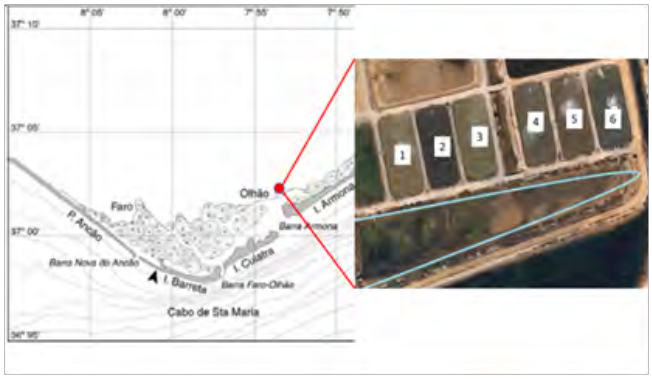

Figure 1: a) Schematic representation of the Ria Formosa lagoon system [26]; b) Satellite image of the ponds used for the IMTA experiment (indicated by numbers); in light blue the discharge channel from which *Ulva* sp. was collected.

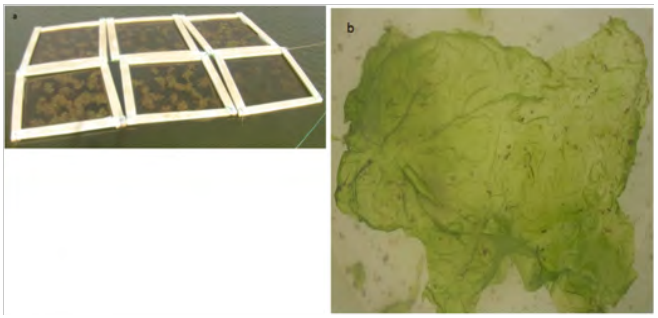

Figure 2: a) The six floating rafts structure used to cultivate b) *Ulva* sp.

Collection and storage of seaweeds

In the beginning of November (3/11/2016), 54 samples of green seaweeds were collected from the earthen ponds, including 17 from the floating rafts. The remaining samples were collected on the perimeter of the ponds or rafts (e.g. ropes). Subsequently, each sample was washed clean with seawater and thoroughly dried with absorbent paper. Of each specimen, a piece of approximately 1 cm2 was preserved in silica. Each bag was labeled with the date of withdrawal, the pond number, letter "f" or "t" (Framework or Pond), and sample number. The remainder of each individual collected was preserved as herbarium voucher. This identification system allowed a visual comparison after the species were identified through Barcoding.

DNA extraction

Silica dried algal biomass was prepared for the DNA extraction through homogenizing the samples by grinding with a tungsten sphere in a mixer mill (Eppendorf A-2-DWP) for 3 minutes at max speed (3,700 rpm). Seaweed DNA was extracted using the NucleoSpin® Plant II Kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) following the manufacture's protocol.

The quality of the DNA was verified by running 5 µl of the DNA extraction (with 1 µl Gel-Red and 2 µl of loading buffer (5 X Green GoTaq Flexi Buffer)) of six randomly selected samples on a 0.8 % agarose gel.

DNA amplification and sequencing

The nuclear primers ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 3'-CGTATAGTTATTCGCCTCCT-5' were used to amplify nuclear rDNA (Ribosomal DNA) fragment [27]. This fragment contains the Internal Transcribed Spacer 1 (ITS1), the 5.8 S gene, and the Internal Transcribed Spacer 2 (ITS2) [27]. Each pcr reaction consisted of 23.95 µl H2O Milli-Q, 4 µl of 5 X Buffer, 1.6 µl 25 mM Mg, 1.25 µl 2 mM of each dNTP, 2 µl 1.0 µM of each primer, 0,2 µl 5 U/µl Go-Taq, 5.0 µL of diluted (1:100 H2O Milli-Q) genomic DNA extract.

PCR amplification was run a Applied Biosystems 2720 Thermal Cycler (Applied Biosystems™, Foster City, CA) and the profile of the reaction consisted of an initial denature at 95°C for 5 min followed by 35 cycles of 95°C for 30s, 55°C for 30s min and 72°C for 1 min, and a final extension at 72°C for 10 min.

The 54 PCR products were visually checked on a stained electrophoreses gel (2 % agarose). PCR products consisting of a single band with the right size were sequenced. DNA sequencing was performed on an ABI 3130 × l capillary sequencer (Applied Biosystems - CCMAR, Portugal) using the forward primers that were used for PCR.

Molecular analysis

The generated sequences were trimmed and aligned manually using Geneious R7.1.9 [28]. Subsequently identification was based on their DNA sequences by comparing them with sequences present in Genbank [29]. This operation was performed using Nucleotide BLAST web interface [30].

Phylogenetic analyses - alignment

DNA sequence alignment was created using the best quality sequence of each *Ulva* recognized in this study and from respective sequences chosen from BLAST results. Additional sequences for phylogenetic calculation were downloaded from Genbank selecting from other species used in previous papers [11,16,18,22] (Annex, Table 1).

Initial alignment of the nucleotide sets was obtained using Geneious R7.1.9 [28]. Subsequently, the sequences were trimmed to a standard length and the identical sequences removed. The final alignment contained 33 taxa (32 in group taxa plus one outgroup (Ulvaria obscura)), of which five sequences from this study. The alignment was realigned with MAFFT v. 7.310 online applications using Q-INS-I algorithm (with default parameters) [31]. The lasts adjustments of the resulting alignments were carried out using Geneious.

Phylogenetic analyses - construction of phylogenetic tree

The phylogenetic analyses were performed using the Maximum-Likelihood (ML) and Bayesian Inference (BI) methods [32]. The ML tree was obtained using the PhyML online program [33] and the BI tree was constructed using MrBayes present in Geneious R7.1.9. The program jModelTest version 2.1.10 [34] was used to find the model of sequence evolution that best fit the dataset. ML and Bayesian trees were built using the Generalized Time Reversible (GTR) substitution model with discrete gamma distribution in four categories. One thousand bootstrap replications were performed for both methods using default setting to compare relative support of branches.

The phylogenetic analyses, nucleotide homology (%) and sequence divergence (bp) estimates were based on 520 bp, including gaps (Annex, Table 2).

Analysis of morphology and anatomy

Morphology of thalli was assessed for fresh algae using a Nikon SMZ 1000 Stereo microscope whereas for anatomy a Nikon H550S Microscope (© 2017 Nikon Instruments Europe B.V) was used. All photos were captured and prepared using Nis-Elements Software (© 2017 Nikon Instruments Europe B.V).

Results

Molecular analysis

Of the 54 samples used for molecular analysis 24 had the required high quality for analyses. The molecular analysis of the macroalgae collected from the EPPO ponds established that the *Ulva* cultivated in the rafts during the IMTA experiment was *Ulva flexuosa* (Wulfen, 1803). In addition, 5 other *Ulva* and 2 *Cladophora* species were identified from the pond system (Annex, Table 3).

The *Ulva* genus was well represented and consisted of: *Ulva flexuosa* (Wulfen, 1803, xxii,1), *Ulva clathrata* ((Roth) C. Agardh, 1811: 23), *Ulva intestinalis* (Linnaeus, 1753: 1163), *Ulva sapora*[1] [35], *Ulva torta* ((Mertens) Trevisan, 1842: 480) and *Ulva prolifera* (O.F.Müller, 1778: 7). *Ulva sapora* sequence obtained had a bad quality (5.5%) and was omitted from the phylogenetic analysis.

Phylogenetic trees

The phylogenetic analyses performed with the ML (Maximum Likelihood) and BI (Bayesian Inference) methods gave comparable tree topologies with the *Ulva* species coming from the ponds forming four distinct groups (Figures 3 & 4). These four groups, well supported both in the ML and BI trees, consist of: Two monophyletic (C,D) groups, one polyphyletic (A) group and in group B) *U.torta* is paraphyletic with respect to *U.clathrata*. However, the internal nodes are well supported only in the BI tree, with Bayesian Inference Posterior probability (BP) between 56 % and 86 %.

Group A showed that *Ulva flexuosa* present in the pond system formed a monophyletic clade with *Ulva flexuosa* from Hokkaido, Japan, with a nucleotide homology of 99.47 % (2 bp difference) (Table 1). According to this phylogram, either *U.flexuosa* are closely related to monophyletic group of *Ulva californica* (internal node value of 69 %) and the nucleotide homology showed between two species (≈ 97 %)supported a high similarity between these taxa. The *Ulva flexuosa* identified showed a low similarity with other European *U.flexuosa* subspecies with nucleotide homology < 87.9 % (Table 2).

Also the groups C, B and D were well supported and showed that all *Ulva* species sampled were closely related with the species from the North Pacific (nucleotide homology between ≈ 99 % to ≈ 96 %) (Annex, Table 2).

Morphological observations

The gross morphological characteristics (Annex, Table 3) presented a marked homogeneity among the varied species collected, underlining the importance of genetic analysis to identify the different *Ulva* species. The filamentous, herbaceous-like shape was the most common and, with a few exceptions of turf forms (*Ulva sapora* and one *Ulva clathrata*), *Ulva flexuosa* was the only species present with 3 different dominant morphotypes:

a. The lettuce-leaf (Figure 5a-5b).
b. Narrow and broad gregarious thalli (Figure 5c).
c. Filamentous, herbaceous-like shape (Figure 6a-e).

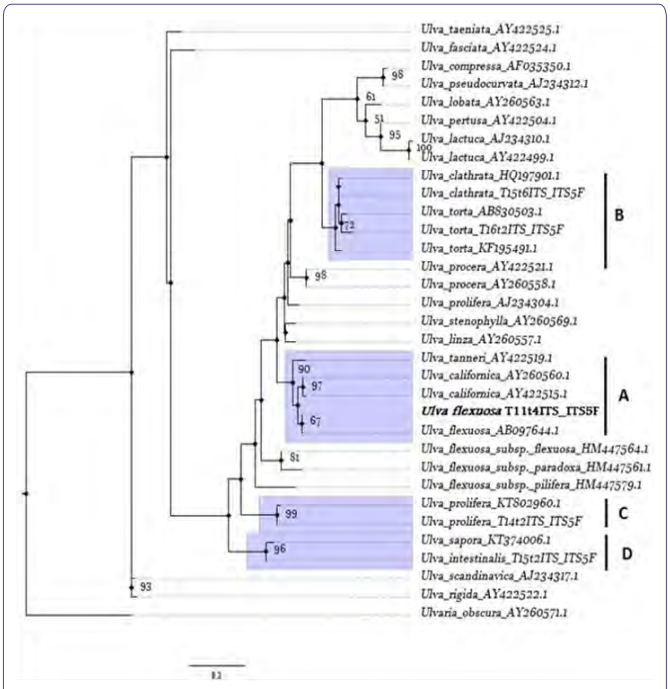

Figure 3: Maximum-Likelihood (ML) tree of ITS sequences calculated using the evolution model GTR+I+G. ML bootstrap values (1,000 replications) are given on the branches. Bootstrap support < 50 % are not shown. Sequences are labelled with taxon name and GenBank accession number of ITS sequence (Annex, Table 1). The tree is rooted using *Ulvaria obscura*; A, B, C and D refer to clades containing *Ulva* collected from EPPO ponds. In bold is stressed the *Ulva flexuosa* identified in this study.

	***U.flexuosa* T11t4ITS**	***U.flexuosa* AB097644**	***U.californica* AY260560**	***U.californica* AY422515**
U.flexuosa T11t4ITS	-			
U.flexuosa AB097644	99.47	-		
U.californica AY260560	97.43	96,81	-	
U.californica AY422515	96.80	96,28	99,47	-

Table 1: Nucleotide homology (in percentage) of ITS region sequences of the four species present in the clade of *Ulva flexuosa* grown within the ponds. In red is stressed the nucleotide homology of *U.flexuosa* grown in the ponds.

The lettuce-like *Ulva flexuosa* was the cultivated one. The specimen had a less rigid structure (thin and papery in texture) than those collected in the drainage channel. Moreover, they lost any anchoring structure present in the wild type. Their thalli had medium to light green, broader than long, flat, irregular contoured with undulated margins and was unbranched (Figure 5a). Under the microscope the

1 *Ulva sapora* is a synonymous name of *Ulva tepida* (Masakiyo, Y. & Shimada, S. (2014) [36]) discovered in Japan for the first time and then reported in Australia (Phillips et al. 2016 [35]) as not indigenous species.

central part of lettuce-like's thallus showed a disordered cell arrangement with 2-4 pyrenoids per cell. Cells were irregularly arranged, polygonal, usually with rounded corners (Figure 5b). Principally measurements are shown in table 3.

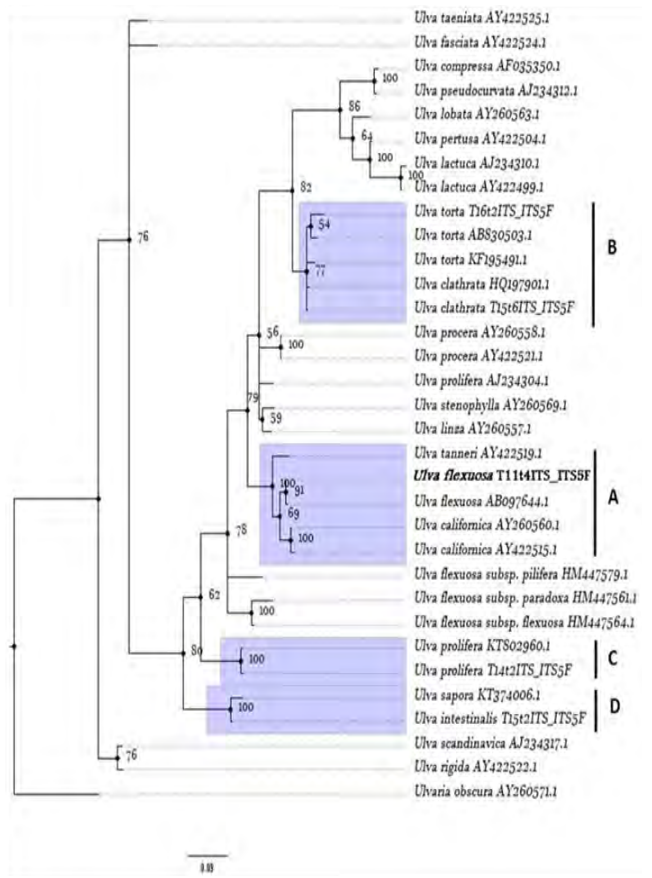

Figure 4: Bayesian tree of ITS sequences. Bayesian Probabilities (%), BP, are given on the branches. Posterior probabilities < 50 % have been omitted. Sequences are labelled with taxon name and GenBank accession number of ITS sequence (Annex, Table 1). The tree is rooted using *Ulvaria obscura*. A, B, C and D refer to class containing *Ulva* collected from EPPO ponds. In bold is stressed the *Ulva flexuosa* identified in this study.

	*U. flexuosa*_ T11t4ITS	*U.flexuosa subsp. flexuosa* HM447564	*U.flexuosa subsp. paradoxa* HM447561	*U.flexuosa subsp. pilifera* HM447579
***U. flexuosa* T11t4ITS**	–			
***U.flexuosa subsp. flexuosa* HM447564**	87.90	–		
***U.flexuosa subsp. paradoxa* HM447561**	84.30	91,71	–	
***U.flexuosa subsp. pilifera* HM447579**	85.75	90.52	85.53	–

Table 2: Nucleotide homology (in percentage) of ITS region sequences between *Ulva flexuosa* grown within the ponds and European *Ulva flexuosa* subspp. In red is stressed the nucleotide homology of *U. flexuosa* grown in the ponds.

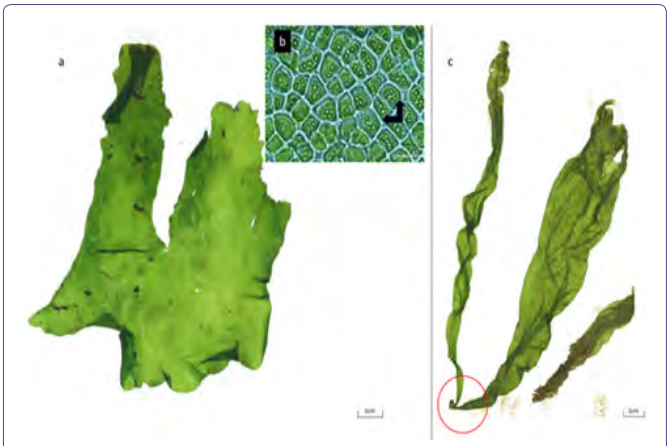

Figure 5: a) Lettuce-shape *Ulva flexuosa*; b) polygonal cells with pyrenoids (black rows); c) Gregarious thalli with discoidal base (red circle). Scale bar a) and c) 1cm. Scale bar for b) is 10µm.

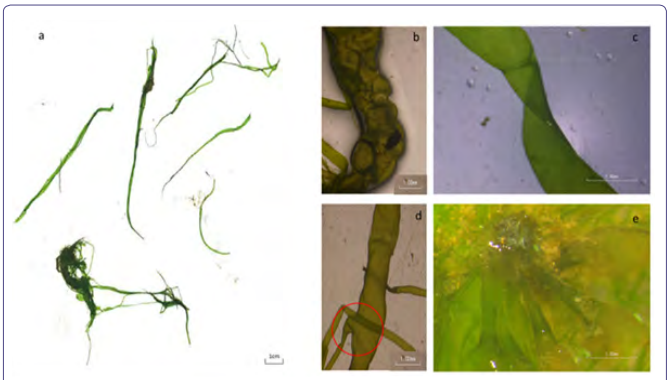

Figure 6: a) *Ulva flexuosa* filamentous morphotype; b) thallus corrugated; c) laminar; d) branch (red circle); e) hollow stipe. Scale bar a) 1cm; scale bars of b), c), d) and e) are 1mm.

U.flexuosa	Length of cells (µm)	Width of cells(µm)	ø of pyrenoids	N° of pyrenoids (in one cells)
Mean	8.04	5.61	1.84	3.5
Min.	5.19	1.99	0.97	1
Max.	11.27	5.87	2.91	4
SD*	1.2	1.08	0.42	
*SD= Standard Deviation				

Table 3: Size of *Ulva flexuosa* cells with wide leaf thalli.

The two remaining morphotypes belong to *Ulva flexuosa* grown within the ponds or attached to the framework. The first of these was characterized by a narrow and broad gregarious thallus attached to substrate by means of a small discoid base and like the cultivated morphotype was unbranched, flat with a thin texture and, started from a narrow base, widening towards the top. The *U.flexuosa* on the framework had a filamentous herbaceous shape and it often presented thalli polyform, slender, tubular compressed or laminar, widening at the top. Observations under the stereoscope revealed the presence of some branches at the base and a stipe that could be hollow. The thalli were fixed by means of a basal disc reinforced by numerous robust rhizoidal filaments. It is worth mentioning the presence of a fourth

morphotype, with lanceolate thallus, although it is represented by a single specimen collected around the 13-pond's perimeter.

Discussion

The identification of *Ulva* spp. present in the EPPO ponds revealed a heterogeneous community, consisting of six taxa of which two were never reported in the Ria Formosa area: *Ulva flexuosa* and *Ulva torta*.

Ulva flexuosa was identified as the species cultivated and its lettuce-leaf morphotype is not attributable to any of the subspecies of this marine species.

The ITS allowed to differentiate *Ulva* taxa among our samples. The huge morphological plasticity of the kind probably would otherwise have led to associate the different phenotypes found with a species already recorded in the Ria Formosa. The presence of multiple bands in PCR products has already been reported in the past [24,37]. Therefore, ITS is commonly associated with rbcL (plastid rubisco large subunit) marker to increase the successes of identification [1,11,15,16,22,23,38].

Ulva flexuosa

U.flexuosa was originally described by Wulfen from the Adriatic Sea in the 1803. Currently, *Ulva flexuosa* species includes 4 subspecies and one variety: *U.flexuosa* ssp. *flexuosa* (Wulfen 1803: xxii, 1), *U.flexuosa* ssp. *paradoxa* (C. Agardh) M.J. Wynne [39], *U.flexuosa* var. *linguiformis* [13], *U.flexuosa* ssp. *biflagellata* (Bliding) A. Sfriso & D. Curiel [40] and *U.flexuosa* ssp. *pilifera* (Kützing) M.J. Wynne [39,41,42]. Among the three morphotype here reported the lettuce-leaf observed is not referable to any of the marine subspecies belonging to *Ulva flexuosa*. However, previous studies recorded a similar phenotype for the freshwater *Ulva flexuosa* ssp. *pilifera* [16,42]). Perhaps this morphotype may be explained considering algae grown in IMTA systems tend to develop leaves larger than wild types [43]. The remaining two morphologies have a taxonomic response. The filamentous one, based on the polymorphism of the thallus and the presence of a tubular stipe, could be associated to *Ulva flexuosa* ssp. *flexuosa* [13]. The gregarious thalli, instead, was similar to the *Ulva flexuosa* morphotype described by Wolf et al. [17] in the Venice lagoon, *Ulva flexuosa* from Busan and Pohang, Korea [44] and with the *U.flexuosa* ssp. *pilifera* identified in a recent study in the Polish freshwater [42]. However, genetic identity discarded the hypothesis of three distinct subspecies confirming instead the enormous plasticity of *Ulva* genus. There are several factors that can explain this phenomenon. *Ulva flexuosa* can 'switch' its thallus morphotype from tubular to foliose along their life and it is more frequent in culture due to stresses unique to artificial systems [22,45]. The place where the thalli develop (e.g. bottom or water surface) and environmental factors such as salinity and temperature can also affect morphological plasticity [25,42]). In our case, the fact of having collected seaweed in November after a week of intense rain may have favoured the finding of different morphotypes due to lowering of the temperature and salinity. Furthermore, in the past the role of bacterial community on morphology variation of *Ulva* genus has been shown [46,47]). The capacity of *Crassostrea gigas* to remove large amounts of bacteria [48] could perhaps have provoked a change in their community promoting change in *Ulva flexuosa* phenotype. All these possibilities need further studies.

Historically this species has been recorded in neighbouring countries along the coastal zone between Tanger (Morocco) and Melilla (Spain) [49] and in the Cadiz Bay [50]. Furthermore, *U.flexuosa* has been included in the list of macroalgae of the North coast of Portugal, along Minho, Douro Litoral, and Beira Litoral regions [51] and in Corunna harbour, Spain [52].

The *Ulva flexuosa* T11t4 sequence turned out to be almost identical (2 bp of difference) to that recorded by Shimada in Hokkaido, Japan [11] forming a well-defined clade in both phylogenetic trees. This observation may suggest the origin of these macroalgae could be the North Pacific and other studies suggest a common origin between the *Ulva flexuosa* of South Europe and the Pacific. An investigation about cryptic (species with morphologies identical or similar, although genetically different [17] and new species in the North Adriatic reported an *Ulva flexuosa* quite identical to one reported in British Columbia (Canada) [17]. Moreover, a Greek *Ulva flexuosa* var. *linguiformis* was closer related with a Japanese specimen [11,13,41]).

The *Ulva flexuosa* specimens from the EPPO ponds and South Europe did not match genetically with *Ulva flexuosa* subspecies from North Europe [16,22]), as was already detected by Marês and Shimada [16,23]. Marês et al. [16] proposed to indicate *U.flexuosa* as indigenous species of the inland waters of the Europe proposing a different nomenclature for the Asian, however, no mention was made about marine *Ulva flexuosa*.

Other taxa

Not only *Ulva flexuosa* was recorded for the first time in the Ria Formosa lagoon, also *Ulva torta* was first reported whereas *Ulva intestinalis, Ulva prolifera* and *Ulva clathrata* have been already mentioned in some studies that took place in the lagoon [53,54]. Historically, all these taxa, with sometimes the exception of *Ulva torta*, have shown a similar geographical distribution, jointly with *U.flexuosa*, in Portugal and neighboring countries [49-52]. Moreover, in the port of Corunna they occupied the same environment [52]. Nevertheless, among the studies listed above only Alsufyani et al. [54] provided a molecular identification by means of molecular techniques. This can lead to some doubts about the real distribution of this species.

Conclusion

The presence of *Ulva flexuosa* in the South Portugal broadens its geographic distribution in the country. The use of the molecular marker ITS was successful on macroalgae cultivated but there was low amplification success. For this reason subsequent investigations of green macroalgae would require the use of markers with a higher success rate such as tufA or associating rbcL (plastid rubisco large subunit) with the use of ITS [24]. Two studies in Poland and the USA leaded to the hypothesis that macroalgae previously identified as subspecies of *Ulva flexuosa* may be young species undergoing separation due to isolation and adaptation to different habitats [42,55]). Hence the recommendation to investigate into the phylogenetic relationships between *U.flexuosa* subspecies using more sensitive and specific molecular markers (e.g. ISSR (Inter-Simple Sequence Repeat), or SCAR (Sequence Characterized Amplified Region)) [42,56]). The genetic data collected in this experiment may lead to conclude that the origin of the macroalgae present in EPPO ponds could be the North Pacific. However, the scale of the present study does not allow to state which is the actual distribution area of the *Ulva* spp. identified and their

status of native or introduced species. The presence of several species of *Ulva* suggests they withstand the ponds environment and proposes *Ulva* spp. as excellent candidates for the IMTA land-based systems.

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References

1. Kraft LG, Kraft GT, Waller RF (2010) Investigations into Southern Australian *Ulva* (Ulvophyceae, Chlorophyta) taxonomy and molecular phylogeny indicate both cosmopolitanism and endemic cryptic species†. Journal of phycology 46: 1257-1277.

2. Ben-Ari T, Neori A, Ben-Ezra D, Shauli L, Odintsov V, et al. (2014) Management of *Ulva lactuca* as a biofilter of mariculture effluents in IMTA system. Aquaculture 434: 493-498.

3. Carl C, De Nys R, Paul NA (2014) The seeding and cultivation of a tropical species of filamentous *Ulva* for algal biomass production. PLoS One 9: 98700.

4. Ding L, Teng L, Lu Q, Luan R, Huang B (2014) The morphological comparison, variation and molecular analysis between two green tidal algae Enteromorpha prolifera and *E.clathrata* from China. Biology.

5. Bruhn A, Dahl J, Nielsen HB, Nikolaisen L, Rasmussen MB, et al. (2011) Bioenergy potential of *Ulva lactuca*: Biomass yield, methane production and combustion. Bioresource Technology 102: 2595-2604.

6. Castelar B, Reis RP, dos Santos Calheiros AC (2014) *Ulva* lactuca and *U.flexuosa* (Chlorophyta, Ulvophyceae) cultivation in Brazilian tropical waters: Recruitment, growth, and Ulvan yield. Journal of Applied Phycology 26: 1989-1999.

7. Costea O, Malta E-J, Lópeza JC, Fernández-Díaza C (2015) Production of sulfated oligosaccharides from the seaweed *Ulva* sp. using a new Ulvan-degrading enzymatic bacterial crude extract. Algal Research 10: 224-231.

8. Winberg PC, Skropeta D, Ullrich A (2011) Seaweed cultivation pilot trials-towards culture systems and marketable products. Australian Government Rural Industries Research and Development Corporation, Canberra, Australia.

9. Radulovich R, Neori A, Valderrama D, Reddy CRK, Cronin H, et al. (2015) Farming of seaweeds. Seaweed Sustainability: Food and Non-Food Applications. Pg no: 27-59.

10. Melton JT, Collado-Vides L, Lopez-Bautista JM (2016) Molecular identification and nutrient analysis of the green tide species *Ulvaohnoi* M. Hiraoka& S. Shimada, 2004 (Ulvophyceae, chlorophyta), a new report and likely nonnative species in the Gulf of Mexico and Atlantic Florida, USA. Aquatic Invasions 11: 225-237.

11. Shimada S, Hiraoka M, Nabata S, Iima M, Masuda M (2003) Molecular phylogenetic analyses of the Japanese *Ulva* and *Enteromorpha* (*Ulva*les, Ulvophyceae), with special reference to the free-floating *Ulva*. Phycological Research, 51: 99-108.

12. Hofmann L, Nettleton J, Neefus C, Mathieson AC (2010) Cryptic diversity of *Ulva* (*Ulva*les, Chlorophyta) in the Great Bay Estuarine System (Atlantic USA): Introduced and indigenous distromatic species. European Journal of Phycology 45: 230-239.

13. Cormaci M, Furnari G, Alongi G (2014) Flora marina bentonica del Mediterraneo: Chlorophyta. Bollettinodell'Accademia Gioenia Di Scienze Naturali 47: 11-436.

14. Loughnane CJC, McIvor LLM, Rindi F, Stengel DB, Guiry MD (2008) Morphology, rbcL phylogeny and distribution of distromatic *Ulva* (Ulvophyceae, Chlorophyta) in Ireland and southern Britain. Phycologia 47: 416-429.

15. Heesch S, Broom JES, Neill KF, Farr TJ, Dalen JL, et al. (2009) *Ulva*, Umbra*Ulva* and *gemina*: Genetic survey of New Zealand taxa reveals diversity and introduced species. European Journal of Phycology 44: 143-154.

16. Mareš J, Leskinen E, Sitkowska M, Skácelová O, Blomster J (2011) True identity of the european freshwater *Ulva* (Chlorophyta, Ulvophyceae) revealed by a combined molecular and morphological approach. Journal of Phycology 47: 1177-1192.

17. Wolf MA, Sciuto K, Andreoli C, Moro I (2012) *Ulva* (Chlorophyta, *Ulva*les) Biodiversity in the north adriatic sea (Mediterranean, Italy): Cryptic species and new introductions. Journal of Phycology 48: 1510-1521.

18. Lawton RJ, Mata L, de Nys R, Paul NA (2013) Algal Bioremediation of waste waters from land-based aquaculture using *Ulva*: Selecting target species and strains. PLoS One 8: 77344.

19. Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc Biol Sci 270: 313-321.

20. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005) Use of DNA barcodes to identify flowering plants. Proc Natl Acad Sci USA 102: 8369-8374.

21. Lin Z, Shen S, Chen W, Li H (2013) Phylogenetic analyses of four species of *Ulva* and *Monostroma grevillei* using ITS, rbc L and 18S rDNA sequence data. Chinese Journal of Oceanology and Limnology 31: 97-105.

22. Rybak A, Czerwoniec A, Gąbka M, Messyasz B (2014) *Ulvaflexuosa* (*Ulva*ceae, Chlorophyta) inhabiting inland aquatic ecosystems: Molecular, morphological and ecological discrimination of subspecies. European Journal of Phycology 49: 471-485.

23. Shimada S, Yokoyama N, Arai S, Hiraoka M (2008) Phylogeography of the genus *Ulva* (Ulvophyceae, Chlorophyta), with special reference to the Japanese freshwater and brackish taxa. In: Borowitzka MA, Critchley AT, Kraan S, Peters A, Sjøtun K, Notoya M (eds.). Nineteenth International Seaweed Symposium. Developments in Applied Phycology, Springer, Dordrecht, Netherlands.

24. Saunders GW, Kucera H (2010) An evaluation of rbcL, tufA, UPA, LSU and ITS as DNA barcode markers for the marine green macroalgae. Cryptogamie Algologie, 31: 487-528.

25. Gao G, Zhong Z, Zhou X, Xu J (2016) Changes in morphological plasticity of *Ulva prolifera* under different environmental conditions: A laboratory experiment. Harmful *Algae* 59: 51-58.

26. Vila-Concejo A, Ferreira Ó, Matias A, Dias JMA (2003) The first two years of an inlet: Sedimentary dynamics. Continental Shelf Research 23: 1425-1445.

27. White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. PCR - Protocols and Applications - A Laboratory Manual, Publisher: Academic Press, Massachusetts, USA. Pg no: 315-322.

28. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, et al. (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647-1649.

29. Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ (2017) GenBank. Nucleic Acids Research 45: 37-42.

30. Madden T (2002) The blast sequence analysis tool. The NCBI Handbook, Maryland, USA.

31. Katoh K, Toh H (2008) Improved accuracy of multiple ncRNA alignment by incorporating structural information into a MAFFT-based framework. BMC Bioinformatics, 9: 212.

32. Mareš J (2011) Combined morphological and molecular approach to the assessment of *Ulva* (Chlorophyta, Ulvophyceae) in the CzechRepublic. University of South Bohemia, Faculty of Science, Department of Botany, Bohemia, Czech Republic. Pg no: 76.

33. Guindon S, Gascuel O, Rannala B (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696-704.

34. Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: More models, new heuristics and parallel computing. Nat Methods 9: 772-772.

35. Phillips JA, Lawton RJ, Denys R, Paul NA, Carl C (2016) *Ulva*sapora*sp.* nov., an abundant tubular species of *Ulva* (*Ulva*les) from the tropical pacific ocean. Phycologia, 55: 55-64.

36. Masakiyo Y, Shimada S (2014) Species diversity of the genus *Ulva* (Ulvophyceae, Chlorophyta) in Japanese waters, with special reference to *Ulva*tepida Masakiyo et S. Shimada *sp.*nov. Bull Natl Mus Nat Sci Ser B 40: 1-13.

37. Couceiro L, Cremades J, Barreiro R (2011) Evidence for multiple introductions of the Pacific green alga *Ulva* australis Areschoug (*Ulvales*, Chlorophyta) to the Iberian Peninsula. Botanica Marina 54: 391-402.

38. O'Kelly CJ, Kurihara A, Shipley TC, Sherwood AR (2010) Molecular assessment of *Ulva* spp. (Ulvophyceae, Chlorophyta) in the Hawaiian islands. Journal of Phycology 46: 728-735.

39. Wynne MJ (2005) A check-list of benthic marine algae of the tropical and subtropical Western Atlantic: Second revision. Beiheftezur Nova Hedwigia, California, USA. Pg no: 152.

40. Sfriso A, Curiel D (2007) Check-list of seaweeds recorded in the last 20 years in Venice lagoon, and a comparison with the previous records. Botanica Marina, Walter de Gruyter, Berlin, Germany. Pg no: 22-58.

41. Guiry MD, Guiry GM (2018) AlgaeBase. World-wide electronic publication. Algaebase, Galway, Ireland.

42. Rybak AS (2018) The *Ulva flexuosa* complex (*Ulva*ceae, Chlorophyta): An updated identification key with special reference to the freshwater and hyperhaline taxa. Phytotaxa 345: 83-103.

43. Neori A, Chopin T, Troell M, Buschmann AH, Kraemer GP, et al. (2004) Integrated aquaculture: Rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture 231: 361-391.

44. Lee SH, Kang PJ, Nam KW (2014) New record of two marine ulvalean species (Chlorophyta) in Korea. Journal of Ecology and Environment 37: 379-385.

45. Hayden HS, Blomster J, Maggs CA, Silva PC, Stanhope MJ, et al. (2003) Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. European Journal of Phycology 38: 277-294.

46. Wichard T, Charrier B, Mineur F, Bothwell JH, De Clerck O, et al. (2015) The green seaweed *Ulva*: a model system to study morphogenesis. Front Plant Sci 7: 6-72.

47. Grueneberg J, Engelen AH, Costa R, Wichard T (2016) Macroalgal morphogenesis induced by waterborne compounds and bacteria in coastal seawater. PLoS One 11: 0146307.

48. Jones AB, Dennison WC, Preston NP (2001) Integrated treatment of shrimp effluent by sedimentation, oyster filtration and macroalgal absorption: A laboratory scale study. Aquaculture, 193: 155-178.

49. Benhissoune S, Boudouresque CF, Verlaque M (2001) A check-list of marine seaweeds of the Mediterranean and Atlantic coasts of Morocco. I. Chlorophyceaewille s. l. Botanica Marina 44: 171-182.

50. Hernández I, Bermejo R, Lucas J, Juan P, Vergara J, et al. (2010) Contribucion al conocimiento de los macrófitos marinos de saco interno y caños adyacentes de la bahia de Cadiz. Algas 43: 11-16.

51. Araújo R, Bárbara I, Tibaldo M, Berecibar E, Tapia PD, et al. (2009) Checklist of benthic marine algae and cyanobacteria of northern Portugal. Botanica Marina 52: 24-46.

52. Peña V, Barbara I (2002) Caracterización florística y zonación de las algas bentónicas marinas del Puerto de a coruña (N.O. Península Ibérica). Nova Acta Cientifica Compostelana (Bioloxía)12: 35-66.

53. Aníbal J, Madeira HT, Carvalho LF, Esteves E, Veiga-Pires C, et al. (2014) Macroalgae mitigation potential for fish aquaculture effluents: An approach coupling nitrogen uptake and metabolic pathways using Ulvarigida and Enteromorpha clathrata. Environmental Science and Pollution Research 21: 13324-13334.

54. Alsufyani T, Engelen AH, Diekmann OE, Kuegler S, Wichard T (2014) Prevalence and mechanism of polyunsaturated aldehydes production in the green tide forming macroalgal genus *Ulva* (*Ulvales*, Chlorophyta). Chemistry and Physics of Lipids 183: 100-109.

55. Fleming T (2016) Analysis of freshwater and marine *Ulva flexuosa* in Southern California. Pg No: 1-28.

56. Zhao J, Jiang P, Qin S, Liu X, Liu Z, et al. (2015) Genetic analyses of floating *Ulva* prolifera in the Yellow Sea suggest a unique ecotype. Estuarine, Coastal and Shelf Science 163: 96-102.

57. Zhang Q (2015) *Porphyra* aquaculture rafts is the major source of floating green *algae* in the yellow sea: Evidence of intraspecific genetic analysis on *Ulva prolifera*. National Center for Biotechnology Information, Maryland, USA.

58. Ogawa T, Ohki K, Kamiya M (2013) Differences of spatial distribution and seasonal succession among *Ulva* species (Ulvophyceae) across salinity gradients. Phycologia 52: 637-651.

ANNEX

Taxa	Collection sites	Source	Accession Number ITS
***Ulvaria obscura* spp. *Blytii* ((Areschoug) Bliding, 1969)**	Padilla Bay, WA, USA	Hayden et al. [45]	AY260571
***Ulva californica* (Wille in Collins, Holden et Setchell, 1899)**	La Jolla, CA, USA	Hayden et al. [45]	AY260560
***Ulva californica* (Wille in Collins, Holden et Setchell, 1899)**	Northeast Pacific	Lawton et al. [18]	AY422515
***Ulva clathrata* ((Roth) C. Agardh, 1811)**	Yellow Sea, China	Teng et al. 2010	HQ197901
***Ulva flexuosa* (Wulfen,1803)**	Oshoro, Hokkaido, Japan	Shimada et al. [11] Lawton et al. [18]	AB097644
***Ulva flexuosa* spp. *pilifera* (Kützing), M.J.Wynne 2005**	Poland	Mareš et al. [16] Rybak et al. [22]	HM447579
***Ulva flexuosa* spp. *paradoxa* ((C.Agardh) M.J.Wynne, 2005)**	Czech Republic	Mareš et al. [16] Rybak et al. [22]	HM447561
***Ulva flexuosa* spp. *flexuosa* (Wulfen, 1803)**	Sweden	Mareš et al. [16] Rybak et al. [22]	HM447564
***Ulva lactuca* (Linneus, 1753)**	N.A.*	Mareš et al. [16] Rybak et al. [22]	AJ234310
***Ulva lactuca* (Linneus, 1753)**	Northeast Pacific	Mareš et al. [16] Rybak et al. [22]	AY422499
***Ulva linza* (Linneus, 1753)**	Humboldt Bay, CA, USA	Hayden et al. [45]	AY260557
***Ulva procera* (K.Ahlner) Hayde, et al. [45]**	N.A.	Hayden et al. [45]	AY260558
Ulva procera	Northeast Pacific	Mareš et al. [16] Rybak et al. [22]	AY422521
Ulva prolifera	Yellow Sea (China)	Zhang [57]	KT802960
***Ulva pseudocurvata* (Koeman et van den Hoek, 1981)**	N.A.	Mareš et al. [16] Rybak et al. [22]	AJ234312
Ulva rigida	Northeast Pacific	Mareš et al. [16] Rybak et al. [22]	AY422522
Ulva sapora	Shelly Beach, Caloundra Australia	Phillips et al. [35]	KT374006
Ulva scandinavica	N.A.	Mareš et al. [16] Rybak et al. [22]	AJ234317
***Ulva taeniata* ((Setchell) Setchell et Gardner, 1920)**	Monterey, CA, USA	Mareš et al. [16] Rybak et al. [22]	AY422525
Ulva tanneri	Northeast Pacific	Mareš et al. [16] Rybak et al. [22]	AY422519
Ulva torta	Fukui (Japan)	Ogawa et al. [58]	AB830503

Ulva torta	Clovelly, NSW (Australia)	Lawton et al. [18]	KF195491
*N.A.: Not available			

Table 1: Sources of taxa used to create the phylogenetic trees.

Clade	Species	Collection sites	Accession Number ITS	Homology %	D.B.S (bp)*
A	*Ulva flexuosa* T11t4	EPPO pond			
	Ulva flexuosa	Oshoro, Hokkaido, (Japan)	AB097644	99.47	2
	Ulva californica	La Jolla, California (U.S.A.)	AY260560	97.33	12
	Ulva californica	Northeast Pacific	AY422515	96.8	14
B	*Ulva torta* T16t2	EPPO pond			
	Ulva torta	Fukui (Japan)	AB830503	95.65	17
	Ulva torta	Clovelly, NSW (Australia)	KF195491	94.39	20
	Ulva clathrata T15t6	EPPO pond		95.17	19
	Ulva clathrata	Yellow Sea (China)	HQ197901	94.91	22
B	*Ulva clathrata* T15t6	EPPO pond			
	Ulva clathrata	Yellow Sea, (China)	HQ197901	99.49	2
	Ulva torta	Fukui (Japan)	AB830503	97.71	9
	Ulva torta	Clovelly, NSW (Australia)	KF195491	95.69	17
	Ulva torta T16t2	EPPO pond pond		95.17	19
C	*Ulva prolifera*	EPPO pond			
	Ulva prolifera	Yellow Sea (China)	KT802960	98.6	5
D	*Ulva intestinalis*	EPPO pond			
	Ulva sapora	Shelly Beach, Caloundra (Australia)	KT374006	96.48	14
*Distance between sequences (base-pair)					

Table 2: Nucleotide homology (%) of ITS region sequences of the EPPO samples and other Ulva specimens available in GenBank, that grouped in the ITS phylogenetic tree.*Distance between sequences (base-pair).

Sample	Description	Morphological assessment
11-t3	*Ulva flexuosa* (Wulfen,1803)	Filamentous, herbaceous shape
11-t4	*Ulva flexuosa* (Wulfen,1803)	Filamentous, herbaceous shape
11-f2	*Ulva flexuosa* (Wulfen,1803)	Lettuce-leaf, flat, rounded undulate margins.
16-t1	*Ulva flexuosa* (Wulfen,1803)	Lettuce-leaf, flat, rounded undulate margins.
16-t2	*Ulva torta* ((Mertens) Trevisan, 1842)	Narrow small leaf, rounded on top.
16-t5	*Ulva flexuosa* (Wulfen,1803)	Linear compress thalli, tapering toward the base.
16-t6	*Ulva sapora* (Phillips et al. [35])*	Turf form, Thin-short filamentous
16-f3	*Ulva flexuosa* (Wulfen,1803)	Filamentous, tubular and linziformis.
16-f4	*Ulva flexuosa* (Wulfen,1803)	Filamentous, herbaceous shape
12-t2	*Cladophora albida* ((Nees) Kutzing, 1843)	Dark green, musk form
12-t3	*Cladophora vagabunda* ((Linnaeus) Hoek, 1963)	Narrow liner flat leaf
12-t5	*Ulva flexuosa* (Wulfen,1803)	Filamentous, herbaceous shape

14-t2	*Ulva prolifera* (O.F.Müller, 1778)	Filamentous, herbaceous shape
14-t3	*Ulva flexuosa* (Wulfen,1803)	Filamentous, herbaceous shape
13-t2	*Ulva flexuosa* (Wulfen,1803)	Linear compress thalli, herbaceous shape.
13-t5	*Ulva flexuosa* (Wulfen,1803)	Lettuce-Leaf, flat, rounded edges, undulate margin
13-t6	*Ulva flexuosa* (Wulfen,1803)	Lanceolate Leaf.
13-t8	*Ulva clathrata* ((Roth) C.Agardh, 1811)	Filamentous, herbaceous shape
13-f2	*Ulva flexuosa* (Wulfen,1803)	Filamentous, herbaceous shape
13-f3	*Ulva flexuosa* (Wulfen,1803)	Lettuce-leaf present some perforation
15-t2	*Ulva intestinalis* (Linnaeus, 1753)	Tubular, herbaceous shape
15-t3	*Ulva flexuosa* (Wulfen,1803)	Narrow and broad gregarious thalli, small discoid base
15-t4	*Ulva flexuosa* (Wulfen,1803)	Linear compress thalli, round on top.
15-t6	*Ulva clathrata* ((Roth) C.Agardh, 1811)	Turf form, Thin-short filamentus
*This name is currently regarded as a synonym of *Ulva tepida* (Masakiyo and S.Shimada, 2014) (Algaedatabased).		

Table 3: *Ulva* taxa identified with short morphological description.

Journal of Anesthesia & Clinical Care

Journal of Addiction & Addictive Disorders

Advances in Microbiology Research

Advances in Industrial Biotechnology

Journal of Agronomy & Agricultural Science

Journal of AIDS Clinical Research & STDs

Journal of Alcoholism, Drug Abuse & Substance Dependence

Journal of Allergy Disorders & Therapy

Journal of Alternative, Complementary & Integrative Medicine

Journal of Alzheimer's & Neurodegenerative Diseases

Journal of Angiology & Vascular Surgery

Journal of Animal Research & Veterinary Science

Archives of Zoological Studies

Archives of Urology

Journal of Atmospheric & Earth-Sciences

Journal of Aquaculture & Fisheries

Journal of Biotech Research & Biochemistry

Journal of Brain & Neuroscience Research

Journal of Cancer Biology & Treatment

Journal of Cardiology: Study & Research

Journal of Cell Biology & Cell Metabolism

Journal of Clinical Dermatology & Therapy

Journal of Clinical Immunology & Immunotherapy

Journal of Clinical Studies & Medical Case Reports

Journal of Community Medicine & Public Health Care

Current Trends: Medical & Biological Engineering

Journal of Cytology & Tissue Biology

Journal of Dentistry: Oral Health & Cosmesis

Journal of Diabetes & Metabolic Disorders

Journal of Dairy Research & Technology

Journal of Emergency Medicine Trauma & Surgical Care

Journal of Environmental Science: Current Research

Journal of Food Science & Nutrition

Journal of Forensic, Legal & Investigative Sciences

Journal of Gastroenterology & Hepatology Research

Journal of Gerontology & Geriatric Medicine

Journal of Genetics & Genomic Sciences

Journal of Hematology, Blood Transfusion & Disorders

Journal of Human Endocrinology

Journal of Hospice & Palliative Medical Care

Journal of Internal Medicine & Primary Healthcare

Journal of Infectious & Non Infectious Diseases

Journal of Light & Laser: Current Trends

Journal of Modern Chemical Sciences

Journal of Medicine: Study & Research

Journal of Nanotechnology: Nanomedicine & Nanobiotechnology

Journal of Neonatology & Clinical Pediatrics

Journal of Nephrology & Renal Therapy

Journal of Non Invasive Vascular Investigation

Journal of Nuclear Medicine, Radiology & Radiation Therapy

Journal of Obesity & Weight Loss

Journal of Orthopedic Research & Physiotherapy

Journal of Otolaryngology, Head & Neck Surgery

Journal of Protein Research & Bioinformatics

Journal of Pathology Clinical & Medical Research

Journal of Pharmacology, Pharmaceutics & Pharmacovigilance

Journal of Physical Medicine, Rehabilitation & Disabilities

Journal of Plant Science: Current Research

Journal of Psychiatry, Depression & Anxiety

Journal of Pulmonary Medicine & Respiratory Research

Journal of Practical & Professional Nursing

Journal of Reproductive Medicine, Gynaecology & Obstetrics

Journal of Stem Cells Research, Development & Therapy

Journal of Surgery: Current Trends & Innovations

Journal of Toxicology: Current Research

Journal of Translational Science and Research

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