UNIVERSITÀ DEGLI STUDI DI PADOVA

UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

SCUOLA DI DOTTORATO DI RICERCA IN BIOSCIENZE E BIOTECNOLOGIE

INDIRIZZO BIOLOGIA EVOLUZIONISTICA

CICLO XXIV

MOLECULAR AND MORPHOLOGICAL INVESTIGATIONS ON SEAWEED BIODIVERSITY AND ALIEN INTRODUCTIONS IN THE ADRIATIC SEA (MEDITERRANEAN, ITALY)

Direttore della Scuola : Ch.mo Prof. Giuseppe Zanotti

Coordinatore d'indirizzo: Ch.mo Prof. Giorgio Casadoro

Supervisore :Ch.mo Prof. Carlo Andreoli

Dottorando: Marion Adelheid Wolf

31 Gennaio 2012

Abstract

Biological diversity is important to the maintenance of healthy ecosystems and also represents an essential component of nations' resources. The algae, macrophytes and phytoplankton, form the base of marine food webs, oxygenate aquatic environments, represent an underutilized resource for harvest and aquaculture, and can be used as biological indicator species. Unfortunately, algal biodiversity can be impacted upon negatively (overall reduction and/or shift in composition) by factors such as global warming, increased environmental stress arising from fisheries and aquaculture activities, and by accidental introductions of non-indigenous species (NIS).

Most introductions of macroalgae into Europe during the last two centuries are related to human activities. Common vectors are shellfish transfers, ship traffic, with algae being carried as fouling organisms, or aquarium escapes. Being usually the sites of aquaculture activities, lagoon environments are particularly subject to these introductions, either accidental or voluntary, which comprises all possible negative effects from cell level to community level. In particular, in the Venice Lagoon, about 20 alien macroalgal species have been introduced since 1983 and more are reported by recent studies.

Seaweeds are difficult to identify due to simply morphologies, phenotypic plasticity and convergent evolution. Molecular analyses are powerful tools for deriving phylogenies independent of the phenotypic characters on which traditional taxonomy is built.

In spite of these considerations, most of the studies dealing with macroalgal biodiversity in Italian waters have been based only on morphological characters. For this reason the first aim of my project was to start molecular surveys on these organisms focusing on the Adriatic Sea (Mediterranean, Italy) and in particular on areas affected by intense shipping traffic and aquaculture activities.

During the three years of this Ph.D. project we identified a series of new alien species using a molecular approach: the red algae *Hypnea flexicaulius, Grateloupia turuturu,* and *Gracilaria vermiculophylla,* and the green species *Ulva pertusa,* and *Ulva californica*. Most of them come from Japan with oysters, mussels and/or the Manila clam *Ruditapes philippinarum* Adams & Reeve.

Two of the molecular marker used for these identifications were the plastid large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L), very commonly employed in the taxonomy of algae, and the mitochondrial *cox*1 gene coding for the subunit I of cytochrome oxidase enzyme and proposed as a barcoding marker in red algae.

Often these introduced entities present cryptic behaviors. To understand if a new reported alien taxon was the result of a recent introduction or was already present in the environment, but erroneously classified because of its cryptic morphology, we compared the sequences of our specimens with those, when available, obtained from old historical material.

This is important, to summarize the occurrence and the distribution of introduced species.

To amplify from old historical material, whose DNA could be partially damaged, we used two non coding regions whose small sizes make them appropriate for this purpose: the plastid *rbc*L-*rbc*S spacer region, proven to be a good marker for species discrimination being essentially invariant within a species, but extremely variable at the interspecific level and, the more variable mitochondrial *cox*2-*cox*3 spacer, useful for population studies.

Some of the identified introduced species are opportunistic, highly competitive seaweeds with high rates of nutrient assimilation and propagule production. This can have negative impacts on the composition of the natural populations of macroalgae and seagrasses leading to environmental alterations and threatening the biodiversity of the Mediterranean Sea flora.

This was the case of the species *Gracilaria vermiculophylla*, noticed by molecular analyses in the Po Delta lagoons (Adriatic Sea) in May 2008, and also found in the Venice Lagoon in March 2009 and along the coasts of the Emilia-Romagna Region in May 2009. Our data showed that this species has high growth rates forming abundant pleustophytic strains with biomasses up to 15 kg fwt m^{-2} that cover completely bottoms overcoming the growth of the local species, including laminar Ulvaceae.

Besides the reports of single alien and potentially invasive species we studied the biodiversity of complex macroalgal genera focusing on those whose members are notoriously components of ships' hulls and ballast waters and, for this reason, result commonly transported and widely introduced macroalgal species.

We studied the green algal genus *Ulva* Linnaeus (Ulvaceae, Chlorophyta) a cosmopolitan taxon, which members show a very simple morphology and a certain degree of phenotypic plasticity, heavily influenced by environmental conditions, making difficult the delineation of species by morphological features alone.

We have focused our attention on three sampling areas: Venice Lagoon, Chioggia inlet, and Lido of Venice. The first two are greatly affected by ship traffics, while the last one is much less influenced by this phenomenon. The molecular analyses, carried out using the *rbc*L gene and plastid elongation factor *tuf*A (proposed as the best candidate for DNA barcoding among green algal species) as molecular markers, revealed the presence of six different species, often with overlapping morphologies: the alien species *U. californica* Wille, and *U. pertusa* Kjellman, beside the species *U. flexuosa* Wulfen, *U. rigida* C. Agardh, *U. compressa* Linnaeus, and one probable new taxon.

Another genus investigated during this project was the genus *Ceramium* (Ceramiaceae, Rhodophyta), a large and systematically complex group due to the high degree of morphological variation. Culture studies, suggesting a strong influence of environment on phenotype, and the use of molecular tools have recently questioned the validity of morphological features used in species recognition. We compared three *Ceramium* taxa from Venice lagoon with samples from northwest Europe using the *rbc*L gene and the *rbc*L-*rbc*S intergenic spacer combined with morphological observations in order to better define the taxonomic position of the Italian species and to make a revision of the taxa really present in this area.

During these surveys on DNA diversity, we discovered specimens that may be flagged as potential new species. Often these findings are not due to extra-Mediterranean introductions but to misidentifications with other species still present in the studied area, that are characterized by a cryptic behaviour.

We characterized a new *Gracilaria* species from the Venice Lagoon, identified using the plastid *rbc*L gene and the intergenic *rbc*L-*rbc*S spacer, combined with morphological observations and pigment composition. This new entity, recorded in the artificial substrata of the Venice Gulf and never found in other localities of the Adriatic Sea, was not described by local or international literature up to now, and adds to the other 11 *Gracilaria* taxa recorded in the Mediterranean Sea.

Probably the recent finding of this new species is the consequence of its misidentification with other specimens whose morphologies are very similar: *Gracilaria gracilis* and *Gracilaria conferta.*

All these studies underline the difficulty in morphological identification of macroalgal species and highlight the current systematic problems: the misidentification of cryptic species, the number of superfluous and synonymised species names recorded and the use of distinct names for a same species sampled from different regions.

Moreover the presence of several morphotypes belonging to the same taxon can lead to other possible errors: assignment of different morphotypes to distinct species or misidentification of species actually different.

These features are great hindrance for the development of a more universal, rather than more restrictive, taxonomy of algae. Systematic studies to solve this kind of problems can involve comparison with type specimens (on which species names are based), typically stored in collections or described in the scientific literature. With the spread of the DNA barcoding method, molecular techniques have developed to extract DNA and to amplify molecular markers also from historical material, making herbaria a precious source of information.

During my PH.D. project we started a systematic study on the type species of the genus *Gracilaria*, a taxonomically challenging group because of structural simplicity, high morphological plasticity, and great species diversity. Classically, the identification of *Gracilaria* species has been based on gross morphological characters and incorrect applications have often led to misidentifications. In this study we analyzed some Mediterranean entities, both preserved in herbarium collection of the University of Padova (Italy) and recently sampled, which morphology was very different and put their belonging to the type species *G. bursa-pastoris* in doubt. The comparison of the *rbc*L-*rbc*S sequences of the different samples considered in this study and that obtained from the lectotype (*Sphaerococcus compressus* Cabrera; herb. alg. Agardh, LD 28984) revealed that the specimens collected in Venice and in Taranto, easily attributable to different species, are actually an example of different morphotypes of the same species, *G. bursa-pastoris*. On the other hand, one herbarium specimen, collected in 1869, at the time misidentified with *G. compressa* (now *G. bursa-pastoris*) on the basis of morphological features, represents, instead, a putative new species never sequenced before.

This project represents the first study on seaweed biodiversity in the Adriatic Sea based on molecular data. The results obtained in this work reveal that DNA barcoding, removing the reliance on morphological features, represents a valid approach to discriminate species and for this reason it is a good way to have an objective survey of the entities present in the different regions.

Abstract

La biodiversità è fondamentale per la conservazione degli ecosistemi e rappresenta una componente essenziale delle risorse di una nazione.
Le alghe sono alla base delle catena alimentare degli ambienti marini, hanno una valenza economica, soprattutto per quanto riguarda le attività di acquacoltura e possono essere usate come specie indicatrici per valutare eventuali impatti ambientali. Sfortunatamente la biodiversità di questi organismi è attualmente minacciata da fattori quali, il riscaldamento globale, l'aumento dello stress dovuto alle attività di pesca e acquacoltura, oltre che all'introduzione di specie aliene potenzialmente invasive.
Ogni anno, in Europa, l'incremento dei traffici navali con paesi extra-Mediterranei, sia per l'importazione di prodotti ittici, che per le attività di acquacoltura porta all'introduzione di nuove specie, di cui la maggior parte macroalghe.
Gli ambienti lagunari sono particolarmente soggetti a questo fenomeno essendo, solitamente, i siti predisposti per gli impianti di allevamento di molluschi di importazione. Ad esempio, per quanto riguarda la laguna di Venezia, 20 macroalghe aliene sono state riportate a partire dal 1983 e altre vengono segnalate ogni anno da recenti studi.
Per quanto riguarda il gruppo delle macroalghe, la discriminazione tra specie è resa particolarmente difficile se basata solo sulle caratteristiche morfologiche sia per l'elevato tasso di variazioni a livello dell'habitus del tallo legato ai diversi parametri ambientali di crescita, che per la presenza di specie criptiche e per lo stile di vita epifita di molte di esse. Negli ultimi anni gli studi effettuati su questi organismi si sono sempre più incentrati sull'uso del *DNA barcoding* come un metodo veloce e affidabile per l'identificazione delle diverse specie sia autoctone che alloctone.
Purtroppo, per quanto riguarda le coste italiane, la maggior parte degli studi effettuati in questo campo sono ancora basati unicamente su caratteri morfologici. Per questo motivo, lo scopo principale del mio progetto di dottorato è stato quello di iniziare un indagine di tipo molecolare sulla biodiversità e distribuzione di questi organismi focalizzandomi sul mar Adriatico (Mediterraneo, Italia) e in particolare su siti affetti da intensi traffici navali e attività di acquacoltura.
Attraverso questo tipo di approccio durante i tre anni di questa ricerca sono state identificate una serie di nuove specie di macroalghe alloctone: *Hypnea flexicaulis*, *Grateloupia turuturu e Gracilaria vermiculophylla* appartenenti al gruppo delle Rhodophyta e *Ulva pertusa e Ulva californica* facenti parte del phylum Chlorophyta. Si tratta soprattutto di specie provenienti dal Giappone dove spesso vengono coltivate in quantità massive per scopi alimentari o industriali.
Due dei marker molecolari usati a questo scopo sono stati il gene plastidiale *rbc*L, codificante per la subunità grande dell'enzima Rubisco, e comunemente utilizzato per lo studio delle macroalghe, assieme al gene mitocondriale *cox*1, codificante per la subunità I dell'enzima citocromo ossidasi e proposto come miglior candidato nell'ambito del DNA barcoding delle Rhodophyta.
Poiché spesso le specie alloctone possono presentare un aspetto morfologico molto simile a quello delle specie autoctone (specie criptiche), sono state effettuate delle comparazioni tra le sequenze dei nuovi campioni con quelle ottenute da campioni conservati in erbari storici. Questo al fine di capire se la specie riportata come nuova introduzione non fosse già precedentemente presente nei nostri mari e erroneamente classificata in base alla sua natura criptica. Ciò è importante per ricostruire la

dinamica temporale delle introduzioni degli organismi rinvenuti oltre a dare indicazioni su quali siano le possibili popolazioni di origine dei nostri campioni e poter quindi identificare il luogo di provenienza delle varie specie.

Per l'amplificazione da materiale storico, il cui DNA potrebbe essere parzialmente deteriorato, sono stati usati come marker degli spaziatori intergenici, che per la loro lunghezza di poche centinaia di basi si prestano efficacemente a questo scopo. In particolare sono stati utilizzati lo spaziatore plastidiale *rbc*L-*rbc*S, che risulta essere un buon marker per la discriminazione a livello inter specifico e il più variabile spaziatore mitocondriale *cox*2-*cox*3 utile negli studi a livello di popolazione.

Molte delle specie alloctone sono caratterizzate da elevati tassi di crescita e dalla capacità di vivere anche in ambienti particolarmente eutrofizzati ed inquinati. Questo spesso causa un rapido rimpiazzo delle popolazioni autoctone di macroalche e fanerogame marine che occupano la stessa nicchia ecologica creando profonde alterazioni ambientali. Le specie alloctone, infatti, a seconda del grado di invasività, possono rappresentare una grave minaccia per la biodiversità della flora del Mediterraneo se non vengono messe in atto appropriate precauzioni.

Questo è stato il caso di una specie di alga rossa, *Gracilaria vermiculophylla*, identificata tramite tecniche molecolari nell'area del delta del Po (Maggio 2008), e rinvenuta durante i campionamenti di questo progetto anche nelle aree della Laguna di Venezia e lungo le coste dell'Emilia-Romagna (Marzo-Maggio 2009). I nostri dati hanno riportato che questa Rhodophyta si è largamente diffusa nelle aree dell'alto Adriatico con biomasse che superano i 15 kg di peso fresco per m^2. Questo ha portato a un parziale rimpiazzo di alcune specie autoctone tra cui anche alcune Ulvaceae.

Oltre all'identificazione di singole specie aliene e di potenziali specie invasive, durante questo progetto, sono stati esaminati anche generi macroalgali particolarmente complessi dal punto di vista tassonomico, i cui membri sono spesso componenti dell'*hull fouling* e delle *ballast waters* delle navi e per questo motivo risultano specie macroalgali comunemente trasportate e potenzialmente introdotte in ambienti diversi dal loro areale di origine.

In particolare ci siamo soffermati sui generi *Ulva* (Ulvaceae, Chlorophyta) e *Ceramium* (Ceramiaceae, Rhodophyta), entrambi taxa cosmopoliti con specie di difficile identificazione a causa dell'elevata variabilità morfologica altamente influenzata dai fattori ambientali.

Sono stati scelti tre siti di campionamento: la laguna di Venezia, il litorale di Chioggia e il Lido di Venezia. I primi due affetti da intensi traffici navali e da un elevata eutrofizzazione delle acque, il terzo meno soggetto a questi fenomeni. Per quanto riguarda il genere *Ulva*, le analisi molecolari condotte utilizzando il gene plastidiale *rbc*L e il fattore di elongazione *tuf*A (proposto come buon marker per il DNA barcoding delle alghe verdi), ha rilevato la presenza di sei diversi taxa di cui due specie aliene *U. californica* e *U. pertusa* oltre a una terza probabile nuova specie.

Nel caso del genere *Ceramium*, la cui sistematica è oggigiorno ancora in uno stato di caos, le sequenze ottenute dall'amplificazione del gene *rbc*L e dello spaziatore intergenico *rbc*L-*rbc*S, dei tre gruppi morfologici identificati, sono state comparate con quelle di specie provenienti dal Nord Atlantico. Questo, assieme a un attento esame morfologico, ha permesso una corretta collocazione tassonomica dei campioni Adriatici e una revisione delle specie finora segnalate per quest'area.

Inoltre, le analisi molecolari di alcuni campioni di dubbia identità ha rilevato la presenza di possibili nuove specie. Spesso questi ritrovamenti non sono dovuti a introduzioni da aree extra-Mediterranee

ma possono essere causate dalla natura criptica di questi taxa, che li rende facilmente confondibili dal punto di vista morfologico, con altre specie già descritte per l'area di studio.
E' questo il caso di una nuova specie di *Gracilaria* rinvenuta in Laguna di Venezia che è stata identificata tramite il gene *rbc*L e lo spaziatore *rbc*L-*rbc*S e di cui è stata effettuata una caratterizzazione completa sia dal punto di vista della morfologia che da quello della composizione in pigmenti. Questa nuova entità va ad aggiungersi alle altre 11 specie di *Gracilaria* riportate per il Mar Mediterraneo.
Tutti gli studi effettuati durante questo progetto sottolineano la difficoltà di identificazione degli organismi macroalgali e i problemi tassonomici correlati a questo e tuttora irrisolti. Un esempio è la moltitudine di nomi e/o sinonimi registrati per la stessa specie spesso usati per identificare campioni provenienti da diverse aree del mondo a seconda delle diverse chiavi dicotomiche utilizzate o a causa della presenza di diversi morfotipi.
A tutt'oggi questo rappresenta un ostacolo per lo sviluppo di una tassonomia universale e più oggettiva dei gruppi macroalgali. Gli studi sistematici atti a risolvere questo tipo di problemi spesso si basano sulla comparazione con campioni tipo delle varie specie conservati in collezioni storiche.
Dato lo sviluppo delle tecniche molecolari anche per quanto riguarda il materiale storico, gli erbari sono diventati una preziosa fonte di informazione per i tassonomi moderni.
Durante il mio dottorato ho iniziato uno studio molecolare sulla specie tipo del genere *Gracilaria* (*G. bursa-pastoris*) caratterizzata da un ampia variabilità fenotipica. Infatti i campioni freschi raccolti nelle aree di Venezia e Taranto presentavano morfologie dei talli molto diverse, tali da mettere in dubbio la loro appartenenza alla specie *G. bursa-pastoris*. Per questo motivo sono stati presi in esame anche campioni storici (erbario A. Forti 1927 conservato all'Orto Botanico dell'Università di Padova) oltre che il lectotipo del genere ottenuto dalla collezione storica di Agardh del Museo Botanico dell'Università di Lund (Svezia). La comparazione con il materiale storico e soprattutto con il campione tipo ha rilevato che si trattava di morfotipi attribuibili tutti effettivamente a *G. bursa-pastoris*, tranne che per un campione di algario (Savona, 1869) che era stato al tempo erroneamente classificato su base morfologica e che rappresenta invece una nuova specie mai sequenziata finora.
Questo progetto di Dottorato rappresenta il primo studio sulla biodiversità macroalgale del mar Adriatico basato su un approccio molecolare. I risultati ottenuti hanno rivelato che il DNA barcoding rappresenta un metodo efficace per la discriminazione delle specie e permette di ottenere, così, una visione oggettiva delle entità presenti nelle diverse aree di studio.

TABLE OF CONTENTS

1.1 SEAWEED BIODIVERSITY

The term ‘seaweeds’ traditionally includes macroscopic, multicellular, marine algae. However each group of these organisms can present microscopic representatives. All seaweeds, at some stage in their life cycles, are unicellular, as spores or zygotes, and may be temporary planktonic (Amsler and Searles, 1980). Seaweeds play very important roles in many marine communities. They are the nutritional base of some food webs and provide a three-dimensional space where animals shelter, breed, and deposit eggs.

The macroalgal group presents a great diversity in forms and habitats, they can be found attached to rocks in the intertidal zone, in giant underwater forests, and floating on the ocean’s surface. They can be very tiny, or quite large, growing up to 50 meters (e.g. Giant Kelp: *Macrocystis pyrifera*). These photosynthetic organisms are simpler than ‘land plants’, being their tissues not organized into many distinct organs.

According to W.H. Harvey (1836) seaweeds are divided into three main groups based on their pigmentation. This was the first use of a biochemical criterion in plant systematics.

- **Green Algae (Chlorophyta)** - chlorophylls *a* and *b* dominate giving them a green colour.

- **Brown Algae (Heterokontophyta)** – fucoxanthin, which reflects yellow light, gives them their brownish colour.

- **Red Algae (Rhodophyta)** – phycoerythrin gives a red colour; phycocyanin reflects blue light.

While green algae usually look green and brown algae usually appear brown or yellow-brown, red algae, due to combinations of pigments, can appear coloured from bright red to black, and some may even look brownish (Fig. 1).

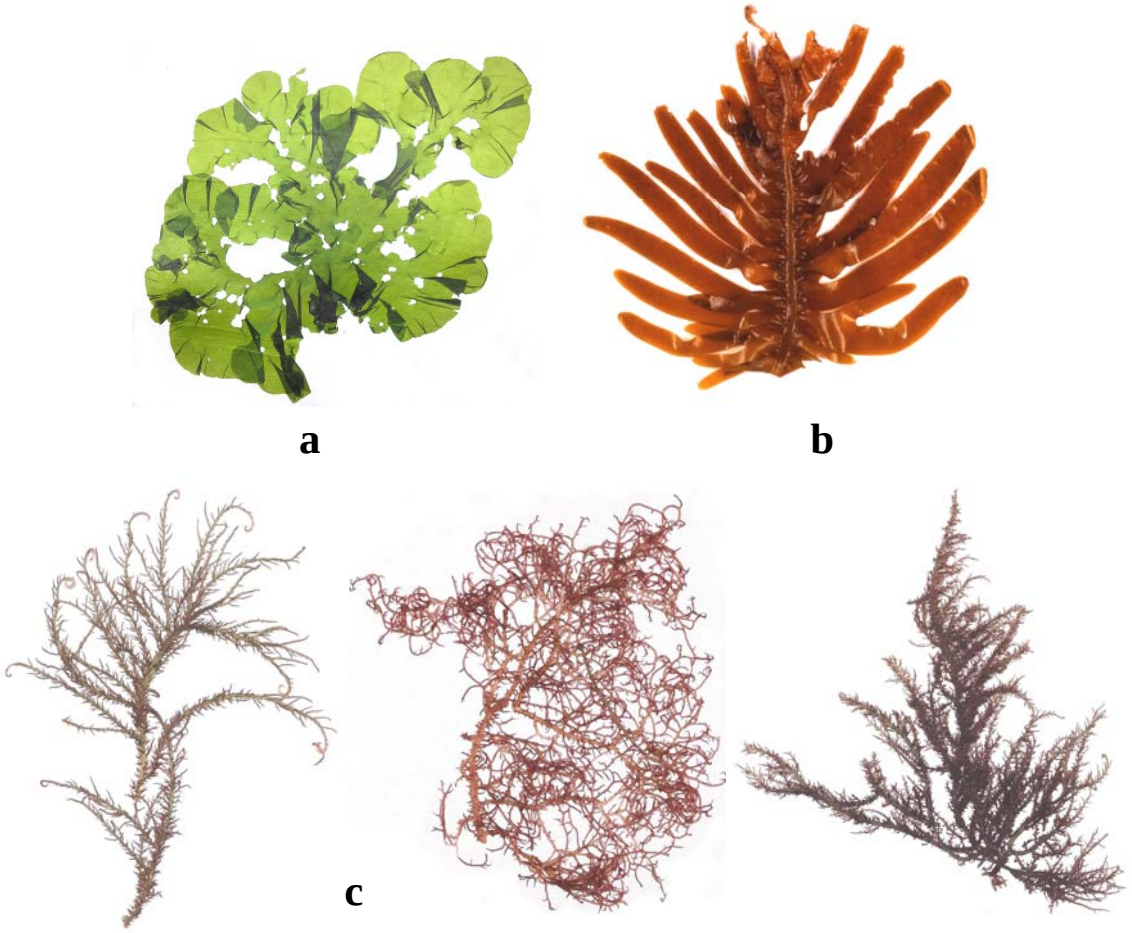


Fig. 1. Examples of habitus in the different macroalgal groups. a) the green alga *Ulva pertusa*; b) the brown alga *Undaria pinnatifida*; c) red algae *Hypnea* spp.

Morphology. As regards structure, in general the macroscopic algae are expected to have a holdfast, a stipe, and a fronde or blade.

- **Holdfast**: attach organ. The holdfast is needed as an anchor and is made up of many fingerlike projections called haptera.

- **Stipe**: the function of this structure is to support the rest of the plant. It is variable among different groups: it can be flexible, stiff, solid, gas-filled, very long (20 m), short, or completely absent.

- **Blade**: the main function of the blade is to provide a large surface for the absorption of light. In some species the blade also support the reproductive structures. Some taxa have only one blade, which may be divided, while other species have numerous blades.

Most seaweeds are organized in this way, but many others lack one or more of these structures, due to morphological modification and adaptation. The three structures of a typical alga may appear to be equivalent to the root, stem, and leaf of a flowering plant (Fig. 2). However this is only a superficial similarity, in fact, algal tissues are not modified internally to assume the specialized structural and translocatory functions of the 'land plants'. (Abbott and Hollenberg, 1976).

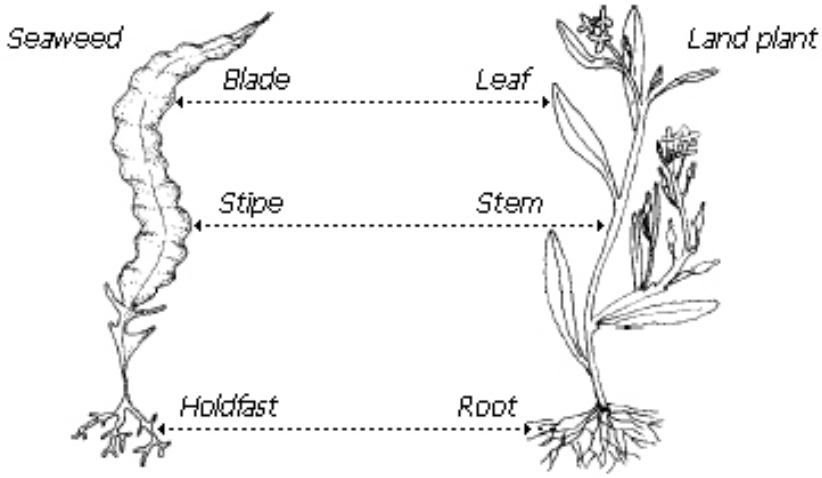


Fig. 2. Structures of a typical alga in comparison with those of a flowering plant. Source: www.dfo-mpo.gc.ca.

Thallus construction. The internal structures of seaweeds are composed of similar cells with simple differentiation. In fact, most of them are filamentous or are built up of united or corticated filaments. Parenchymatous development is found only in kelps, fucoids, Ulvales, Dictyotales, and some others (Lobban and Harrison, 1994).

Filamentous development. The meristematic region can be localized (commonly at the apex, but may be intercalary) or not; in this case, cell division takes place throughout the plant. A simple filament consists of an unbranched chain of cells attached by their end walls and results from cell division only in the plane perpendicular to the axis of the filament. Unbranched filaments are uncommon among seaweeds, except the genera *Ulothrix* and *Chaetomorpha*. Usually, some cell divisions take place parallel to the filament axis to produce branches (Lobban and Harrison, 1994). Branching patterns (Fig. 3) are one of the vegetative features used in seaweed taxonomic identification.

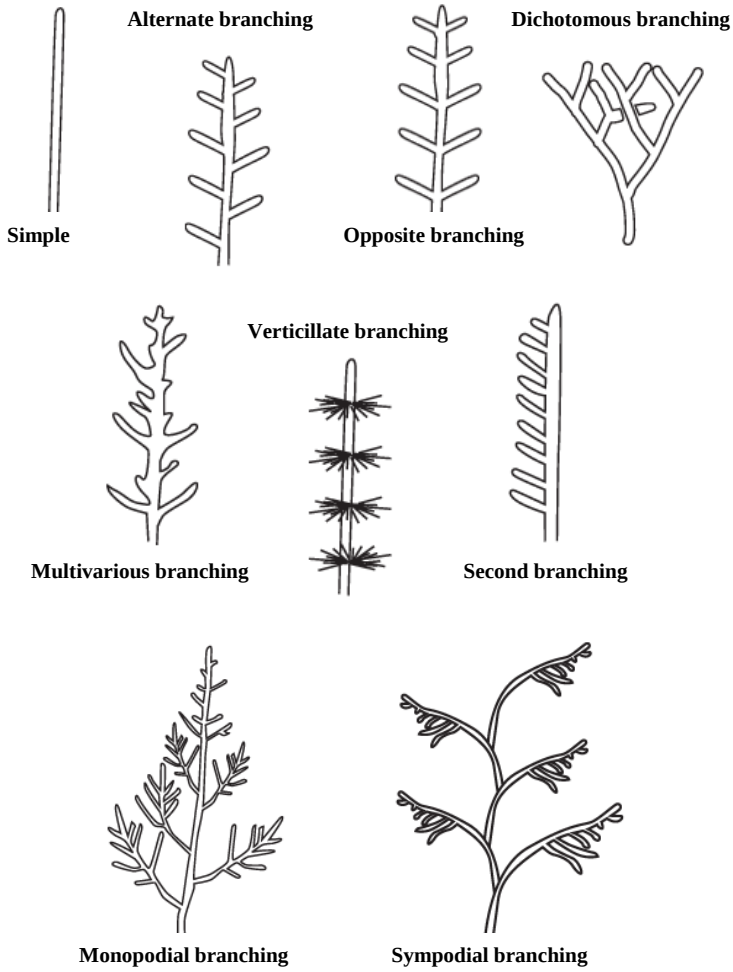


Fig. 3. Examples of different branching patterns.

Filaments consisting of a single row of cells (branched or not) are called uniseriate. When the daughter cells, originated by vertically cell division, do not grow out into branches but remain as compact 'parenchyma', the filament is called pluriseriate (e.g. *Percursaria*, *Bangia*, and *Spacelaria*) (Fig. 4) (Lobban and Harrison, 1994).

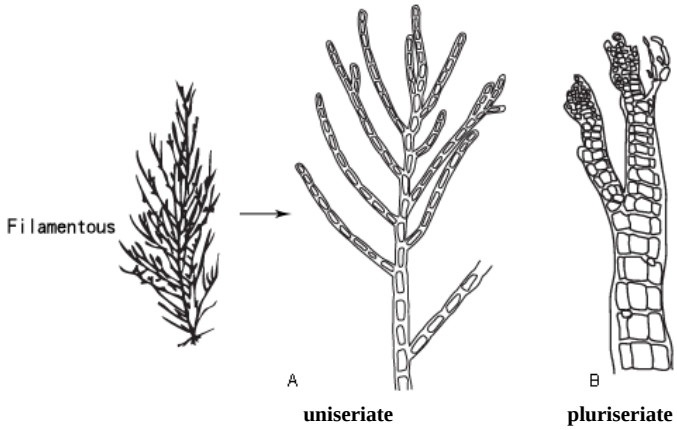


Fig. 4. Internal structure of a filamentous alga. A) uniseriate filament; B) pluriseriate filament.

Branches that do not grow out free can creep down the main filament, forming cortication, such as in *Ceramium* and *Desmarestia*. Cortication can become very extensive like in the case of some of the more massive Rhodomelaceae (such as *Laurencia* and *Acanthophora*).

Pseudoparenchymatous development. The adhesion of several filaments produces thalli that are called multiaxial. This type of development is very common among the red algae (Fig. 5) (Coomans and Hommersand, 1990; Lobban and Harrison, 1994).

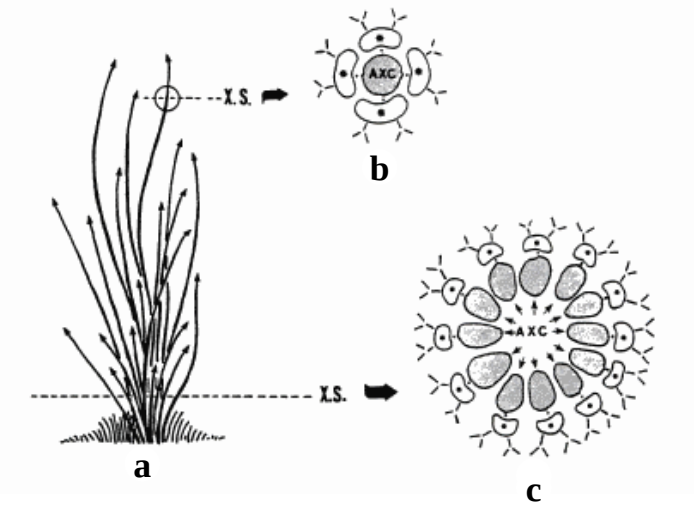


Fig. 5. Filamentous thallus construction . a) Growth of *Dumontia incrassata* showing schematically the axial filaments and apical cells; b) cross-section in the uniaxial part of the thallus near the tip shows a single axial cell (AXC) surrounded by four pericentral cells that have produced cortical cells; c) cross section through base shows multiaxial construction with a core of axial cells, each with one pericentral cell. Source: Lobban and Harrison (1994).

The adhesion of filaments can also produce a pseudoparenchymatous crust (*Peyssonnelia*, *Ralfsia*) or blade (*Anadyomene*) (Fig. 6).

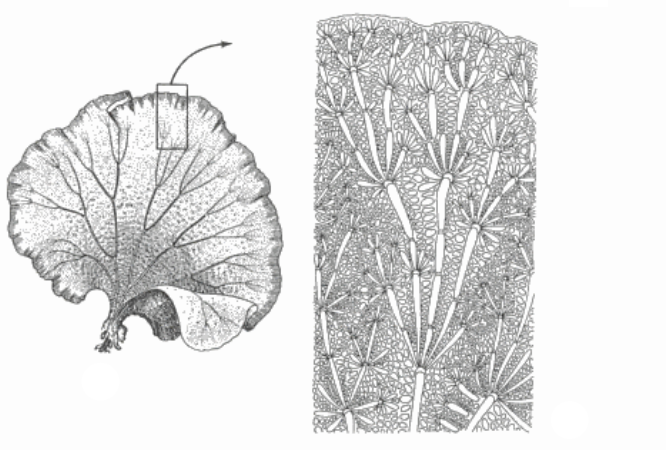


Fig. 6. Formation of blade-like thallus from filaments in *Anadyomene stellata*. Source: Lobban and Harrison (1994).

A potential disadvantage of pseudoparenchymatous growth is lack of cytoplasmatic contact between adjacent cells in different filaments, a problem that red algae overcome through secondary pit connections (Fig. 7) (Raven, 1986; Lobban and Harrison, 1994).

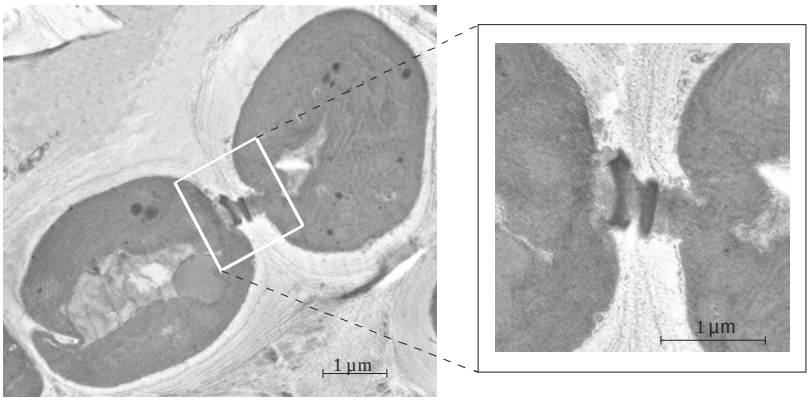


Fig. 7. Example of a pit-connection between two cortical cells in the red alga *Grateloupia turuturu*.

Parenchymateous development. Cell division in two planes can alternatively result in a monostromatic sheet of cells, as in *Monostroma* and some species of *Porphyra*. In Delesseriaceae, marginal meristems produce the wings, while apical cells produce the axial filaments. Such solid tissues are called parenchyma and may become thicker through cell division in a third plane, as in *Ulva*, distromatic *Porphyra*, kelps, and fucoids.

The table below (Tab. A, Littler et al., 1983) summarizes the main morphological groups of macroalgae considering the external habitus of the thallus and the internal structure.

Functional-form group	External morphology	Internal construction	Texture	Sample genera
Sheet	Thin, tubular, and sheetlike (foliose)	Uncorticated, one to several cells thick	Soft	*Ulva*
Filamentous	Delicately branched	Uniseriate, multiseriate, or lightly corticated	Soft	*Polysiphonia*
Coarsely branched	Coarsely branched, upright	Corticated	Fleshy-wiry	*Gracilaria*
Thick, leathery	Thick blades and branches	Differentiated, heavely corticated, thick-walled	Leather, rubbery	*Fucus*
Jointed calcareous	Articulated, calcareous, upright	Calcified genicula, flexible intergenicula with parallel cell rows	Stony	*Corallina*
Crustose group	Prostrate, encrusting	Calcified or uncalcified parallel rows of cells	Stony or tough	*Ralfsia*

Tab. A. Functional-form groups of macroalgae. Modified from Littler et al., 1983.

Habits. Macroalgae often attach to hard surface (rocks, shells) using their holdfasts. Usually they grow vertically away from the substratum, except in the cases of crawling filamentous algae (e.g. *Entocladia*) and crustose plants (e.g. *Ralfsia* and *Porolithon*) that do not grow up into the water column. The vertical habit has some advantages for the macroalgal plants bringing them closer to the light, allowing them to grow extensively without great competition for space, and permitting them to harvest nutrients from a greater volume of water (Lobban and Harrison, 1994).

Small seaweeds are slightly buoyant and, for this reason, support tissues are not necessary for their upward growth, since water provides support. However, strength and resilience are required to withstand water motion. Larger seaweeds developed some adaptations to keep them upright: some have rigid, big stipes (e.g., *Pterygophora*); others adopt flotation (e.g., *Hormosira*); kelps and fucoids have special gas-filled structures (pneumatocysts) (Fig. 8); and other seaweeds, like the erect species of *Codium* (Dromgoole, 1982), have gas trapped among the filaments (Lobban and Harrison, 1994).

Fig. 8. Pneumatocysts of *Fucus vesciculosus*. Source: www.wikipedia.org, author: Anne Burgess.

Environments. Most of the macroalgal species are marine benthonic organisms and grow abundantly on rocky shores in the intertidal zone. However, populations of floating seaweeds are not uncommon (Norton and Mathieson, 1983). Muddy and sandy areas have fewer seaweeds, because most species cannot anchor there, though some

siphonous greens (e.g., some species of *Halimeda* and *Udotea*) produce penetrating, rootlike holdfasts, which may also serve in nutrient uptake (Littler et al., 1988).
There is also a paucity of freshwater macroalgae. Freshwater Rhodophyceae and Phaeophyceae are represented by relatively very few genera and species, and Ulvophyceae are also scarce, with a limited members of genera as *Cladophora*.

Uses. Seaweeds have been used for food, medicine, and as fertilizers for centuries. About red algae, two important substances are found in the cell walls: agar and carrageenan. These gelling compounds are used in food products and scientific research. Carrageenan is an important ingredient in toothpaste and many milk products, such as ice cream and chocolate milk. Agar has many scientific applications in microbiology, biotechnology, and criminology, and it is also used in the packaging of canned meats. One of the most popular seaweeds used as food products is the red alga *Porphyra* (known as 'nori'), which is used in sushi wraps and other Japanese dishes. 'Nori' is grown in commercial seaweed farms on the east coast of North America and in Asia.
Some common species of green algae, belonging to the genus *Ulva* (sea lettuce), are also used as food products.
Brown seaweeds are utilized in numerous ways: for food, cosmetics, pharmaceuticals, and in sciences. Many tasty kelps are harvested from wild populations and also grown in commercial kelp farms. Two unique types of compounds are found in brown algae, algin and fucans, which are used in the manufacturing of consumer goods. Algin is found within the cell walls of brown algae, and is an emulsifier used in food products. Fucans are sulfated polysaccharides and have potential medicinal uses. Brown algae are also collected, treated, and sold as fertilizers for terrestrial agriculture.

Life cycle. Rhodophyta, Chlorophyta and Heterokontophyta, the three main algal phyla, have complex life cycles which show tremendous variation, consisting of separate and alternating phases of free-living individuals with different ploidies and modes of reproduction. Many seaweeds have a biphasic, or what is called a haploid-diploid, life cycle, and these species alternate between a sexual phase where the cells are haploid ('n') and an asexual phase where the cells are diploid ('2n'). By meiosis diploids produce haploid spores, that, subsequently, develop into male and female haploid adults called gametophytes. The adult gametophytes produce female and male

gametes, whose fusion produces diploid adults, the sporophytes, completing the life cycle. This is the generalized life cycle of seaweeds. There are some complexities. Usually, male and female gametophytes are dioecious, but some species, can be monoecious. Normally, gametes are released into the water, where the oocyte is fertilized by the sperm cell and forms a diploid zygote, which then settles to the bottom, attaches to the substrate, and grows into a mature alga. Moreover, red algae have a modified biphasic cycle – actually a triphasic cycle (Fig. 9) - with the addition of a short-living carposporophyte, a diploid stage formed on the surface of the female gametophyte thallus by the union of haploid gametes (Saunders and Hommersand, 2004; Thornber, 2006). This carposporophyte lives on the female thallus, from which it acquires nutrients. It produces diploid spores, called carpospores, which are released into the water, settle, and grow into mature, free-living tetrasporophytes. The tetrasporophytes, by meiosis, produce tetraspores (each spore is formed in a group of four) that are released into the water column, where they settle to become male and female gametophytes.

Another complication is that mature free-living phases of some species have morphologies that are heteromorphic, with independent stages that are distinctly different from each other, and other species are isomorphic, with phases that look almost identical to each other. The algae with isomorphic phases sometimes have different demographics: they are differently distributed as gametophyte or sporophyte populations, depending on seasons, the amount of shelter or wave exposure, and other environmental factors.

Seaweeds can also reproduce asexually through fragmentation or division. This occurs when parts of a plant break off and develop directly into new individuals.

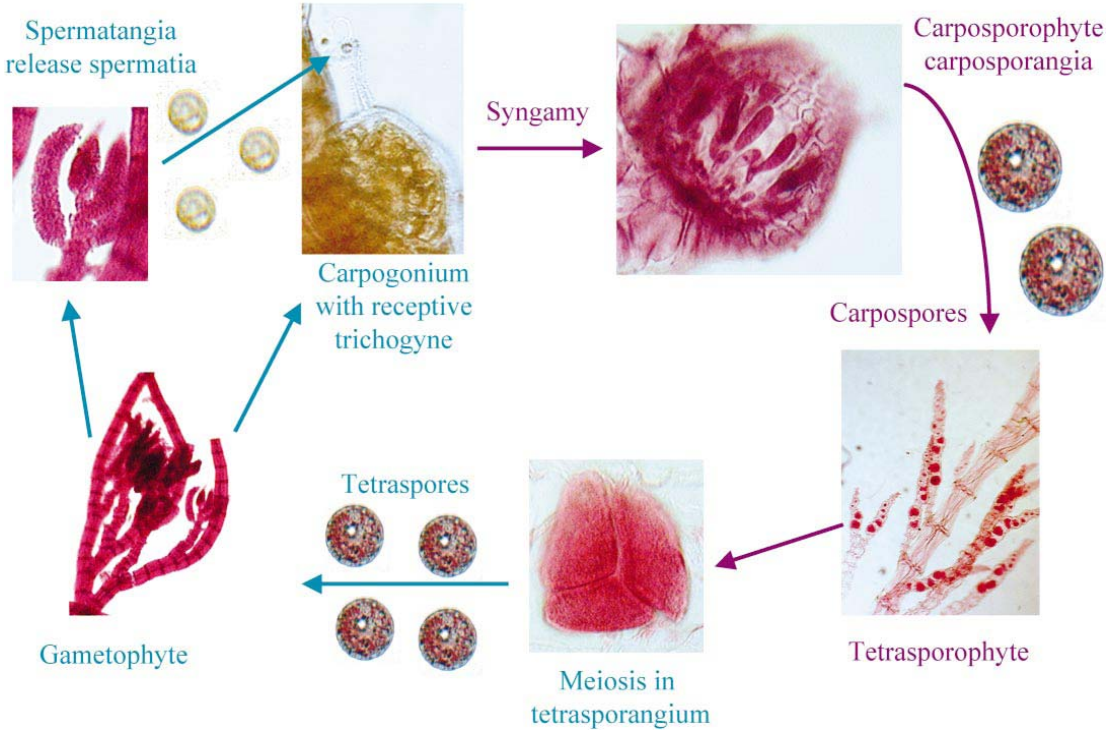


Fig. 9. Typical Florideophyte life cycle. Blue and purple text and arrows represent haploid and diploid stages, respectively. Source: Saunders and Hommersand (2004).

Taxonomy. Macroalgal systematics is very complex and in continuous evolution. Linnaeus (1753) assigned the multitude of diverse species to the subdivision Algae of the class Cryptogamia, which also included ferns, mosses and fungi. This early classification clearly underrepresented macroalgal diversity and substantial taxonomic refinements inevitably followed. Lamouroux (1813) was the first to use colour to separate algal taxa. Harvey (1836) followed, establishing the three major groups of macroalgae that are recognized today (green, brown, and red algae), based on biochemical features.

Numerous taxonomic changes were made in the following decades, but only with the relatively recent advent of ultrastructural and molecular systematic data a more detailed classification of the multitude of species belonging to this group was started.

According to Lewis and McCourt (2004) the chlorophytic line is now represented by two phyla (Chlorophyta and Charophyta), including 10 classes. The brown algae are incorporated in a larger chromophytic lineage, Heterokontophyta, including actually a wide diversity of lineages distributed among some 15 classes (cf. Andersen, 2004). The Rhodophyta systematics is more complex and a debate whether the two principal

lineages, Bangiophyceae and Florideophyceae, should be recognized as distinct classes or included as subclasses within a single class Rhodophyceae (cf. Dixon, 1973) is still open. Nowadays the most supported red algal classification system is that proposed by Saunders and Hommersand (2004), based on molecular and phylogenetic approaches.

Rhodophyta. The majority of seaweed species belong to the division Rhodophyta, or red algae, with estimates of up 7,000 species worldwide (Bunker et al., 2010). They are nearly all marine, with just a few species living in freshwater streams or in extreme environments such as the edges of volcanoes. The red algae are found in a variety of physical forms, including simple and branched filaments, fleshy plants, and sheets. Some red algae are single celled, while others can reach lengths of 2 or 3 meters. The division Rhodophyta is the oldest seaweed group on earth, with fossil records 1.2 billion years ago. In addition to chlorophyll *a*, red algae contain phycobiliproteins as accessory pigments and, in particular, phycoerythrin, which gives the red coloration to this group. These accessory pigments allow them to grow in deeper waters than the other algae. Red algae also grow in the intertidal zone.

Chlorophyta. Only about 10% of green algae are marine species (mainly macroalgae), most live in freshwater (mostly microalgae). There are approximately 1,000 species of green seaweeds worldwide (Bunker et al., 2010). Green algae are more closely related to the green vascular land plants than any other group of algae. They have the same photosynthetic system of vascular plants, which is dominated by the chlorophylls *a* and *b*. The chlorophylls are the pigments that give this group of algae its green coloration. Chlorophylls *a* and *b* absorb red light, which is available in shallow waters, but absent in deeper water. Thus, green seaweeds live most commonly in the shallow intertidal zone. There are more species of green algae in warm tropical oceans than in cooler temperate seas. Some species, notably belonging to the genus *Ulva* can become extremely abundant forming ‘green tides’. The structure of green seaweeds ranges from single-celled forms to multi-cellular sheets, and branched filaments.

Heterokontophyta. Brown algae are all multicellular and are found in a variety of different physical forms, including crusts, filaments, and large elaborate kelps. There are about 1,800 species of Heterokontophyta worldwide, and most of them are marine

(www.seaweed.ie). Brown seaweeds are not closely related to the other groups; in fact they evolved separately and comparatively recently. Like all photosynthetic organisms, brown algae contain the green pigment chlorophyll *a*, but the dominant pigments are fucoxanthin and chlorophyll *c*, which give them the brown or olive-green colour. Most brown algae live in the intertidal or shallow subtidal zone and they are the most abundant in the colder oceanic waters of the Northern Hemisphere, where they can form real forests such the kelp *Laminaria hyperborean* in Ireland (Bunker et al., 2010). Brown algal importance relies in their role of primary producers in the marine environments, as well as because they represent the habitat for a plethora of other organisms.

1.2 ALIEN SEAWEED INTRODUCTION

Biological diversity is important for the maintenance of healthy ecosystems and also represents an essential component of nations' resources. The algae, macrophytes and phytoplankton, form the base of marine food webs, oxygenate aquatic environments, represent an underutilized resource for harvest and aquaculture, and can be used as biological indicators. Unfortunately, algal biodiversity can be impacted upon negatively (overall reduction and/or shift in composition) by factors such as global warming, increased environmental stress arising from fisheries and aquaculture activities, and by accidental introductions of invasive species.

Nowadays, it is proven with certainty that no region of the oceans has remained free of alien (non-indigenous) marine species as reported by several studies all around the world (e.g., Carlton, 1979; Cranfield et al., 1998; Cohen et al., 1998; Coles et al., 1999; Hewitt et al., 1999; 2004; Coles and Eldredge, 2002; Hewitt, 2002; Leppäkoski et al., 2002; Oresanz et al., 2002; Lewis et al., 2003; Castilla et al., 2005; Wyatt et al., 2005). Alien marine species from all major animal, plant and algal phyla have been detected.

Of these, macroalgae represent not only a large component of the globally introduced biota (Fig. 10) (e.g., Ribera and Boudouresque, 1995; Lewis, 1999; Ribera Siguan, 2002; Schaffelke et al., 2006), but also significant economic and environmental risks for which control and management options are limited (e.g., Ribera and Boudouresque, 1995; Thresher, 1999; McEnnulty et al., 2001; Anderson, 2007; Schaffelke and Hewitt, 2007).

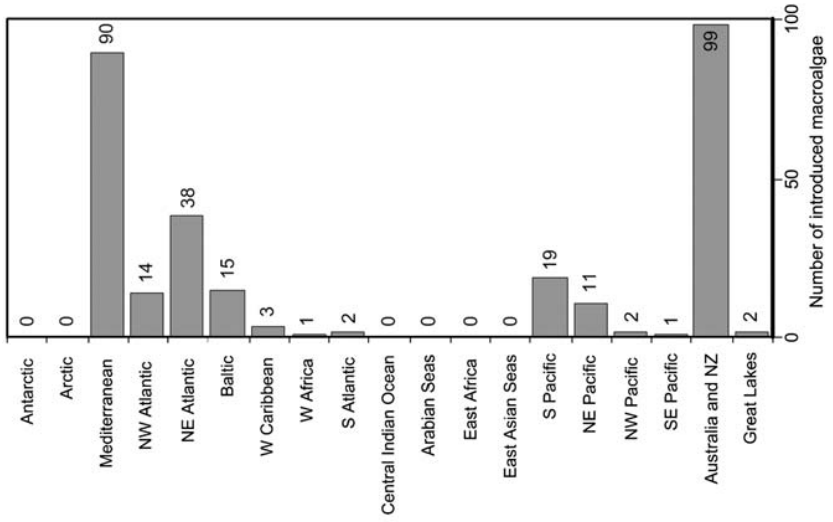


Fig. 10. Number of recorded accidentally introduced taxa of macroalgae for each IUCN (International Union for Conservation of Nature) bioregion. Source: Hewitt et al., 2007.

Over the past five decades, in fact, the Mediterranean Sea has been colonized by several allochthonous macroalgae mainly coming from the Indo-Pacific area. These species were introduced via the Suez or Gibraltar Straits.

Initially, such species colonized warm areas and, successively, in consequence to the recent climate change and temperature increase, they spread in wider regions finding numerous habitats suitable for their growth.

In addition, a negative indirect effect of climate change can occur when some ecosystems become less resistant to invasive species and to their impacts. In extreme cases, climate-driven invasions could lead to completely transformed ecosystems where alien species dominate for function or richness or both, leading to reduced diversity of native species (Mack et al., 2000; Gritti et al., 2006)

Human related introductions and transport mechanisms. Most introductions of alien species into Europe are related to human activities, in particular to the increase of shipping traffic with extra-Mediterranean countries.

The global transfer of marine species by human-mediated means both within and between non-contiguous biotic provinces is of significant concern for biodiversity conservation and the sustainable development of coastal and oceanic resources (e.g., Lubchenco et al., 1991; Carlton, 2001; Ruiz and Carlton, 2003).

Transport mechanisms for marine macroalgae are either connected with intentional introductions or accidental introductions (Fig. 11) (Hewitt et al., 2007).

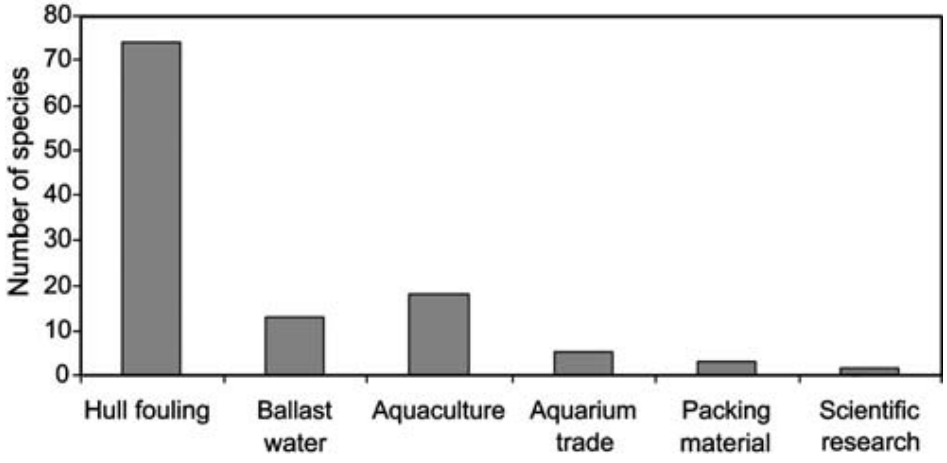


Fig. 11. Number of introduced macroalgal species attributed to specific vectors. Source: Hewitt et al., 2007.

In particular:

- Accidental introductions: woodenhulled vessel boring, fouling, dry and semi-dry ballast; steel-hulled vessel fouling and the transport of planktonic organisms and fragments in ballast water; the unintentional movement of organisms associated with the transfers of mariculture organisms (specifically oyster introductions) (e.g., Elton, 1958; Carlton, 1989; 1996; Ribera and Boudouresque, 1995);

- Intentional introductions: translocations for aquaculture; the transfer of live, frozen and dried food products and the aquarium trade (e.g., Weigle et al., 2005); the use of biological material for packing (e.g., Ribera Siguan, 2002; Miller et al., 2004); and scientific research.

Many of these vectors have not been limited to single species movements but have often resulted in entire assemblages or communities of tens or hundreds of species being transported between disparate bioregions.

From 163 (Ribera Siguan, 2002) to 260 (Smith, Schaffelke and Hewitt, unpublished data) macroalgal species are recognised introductions, with representatives from 7 out of 9 orders in the Chlorophyta, 16 out of 19 orders in the Rhodophyta, and 8 out of 12

orders in the Heterokontophyta (Schaffelke et al., 2006). Of these, less than 3% of macroalgal introductions have been intentional releases.

Mediterranean Sea. The check-list of non-indigenous species (NIS) in the Mediterranean Sea was updated to December 2010 (Zenetos et al., 2010). On the whole, a list of 955 taxa was produced and, out of these, 125 are macrophyte taxa. In the Adriatic Sea alien macrophytes are 49, ca. 39% of the total (Zenetos et al., 2010), and 33 are present in the lagoon of Venice.

In fact, regarding the Italian coasts, the most studied hot spots of marine introductions are the Venice Lagoon and the Mar Piccolo of Taranto basin (Cecere and Petrocelli, 2009; Sfriso et al., 2009).

Being usually sites of aquaculture activities, lagoon environments are particularly subject to these introductions, either accidental or voluntary, with all possible negative effects from cell level to community level (Mc Lusky and Elliot, 2007).

A common vector for these introductions is shellfish transfers. In fact, several seaweed species are known to come from Japan with oysters, mussels, and/or the Manila clam *Ruditapes philippinarum* Adams & Reeve (Farnham, 1980; 1994). In particular, in the lagoons of the Northern Adriatic Sea ca. 6000 ha are used for clam-farming with a production estimated in ca.55,000 tonnes for 2007 (Turolla et al., 2008). In the same basins there are several decontamination plants, important fishing harbours and fish wholesale markets such as: Chioggia and Venice in the Venice Lagoon and Pila and Goro in the Po Delta. In 2003 the markets of Chioggia and Venice had a business volume of ca. 42 and 55 million Euros, respectively, which represented ca. the 61% of the whole North Adriatic activity. Most of the sold products came from extra-Mediterranean countries.

At least 20 alien macroalgal species have been introduced into the Lagoon of Venice since 1983 (Sfriso et al., 2009) and more are reported by recent studies (Cecere et al., 2011; Wolf et al., 2011a). Most of them were first recorded near the fish markets of Chioggia and Venice, where fresh molluscs are imported (Curiel and Marzocchi, 2010).

The Mar Piccolo of Taranto houses the biggest mussel farm in Italy and dry-docks of the Italian Navy Base. 16 of 38 alien seaweed species (i.e., 42%) reported for the Apulian coasts are present in the Mar Piccolo of Taranto, often as first reports for the Mediterranean Sea (Gravili et al., 2010). They have commonly been observed, for the

first time, close to the dock or just settled on the dock where there are fish shops and mollusc import-export enterprises (Cecere et al., 2000; Cecere and Petrocelli, 2009).

Invasive species. In the last years, the massive nutrient enrichment of the seawater has affected coastal and transitional environments, triggering abnormal and extensive growth of nuisance macroalgae.

In fact, some of the introduced species are opportunistic, highly competitive seaweeds, with high rates of nutrient assimilation and propagule production.

In addition to the high growth rates they can show the abilities of growing in dystrophic-hypertrophic environments and of tolerating wide changes of temperature, salinity and light intensity. This can have negative impacts on the composition of the indigenous populations of macroalgae and seagrasses leading to environmental alterations and threatening the biodiversity of the native Sea flora.

Regarding, for example, the Venice lagoon, the species considered invasive or potentially invasive are six: i.e. *Codium fragile* subsp. *fragile* (Suringar) Hariot, *Gracilaria vermiculophylla* (Ohmi) Papenfuss, *Grateloupia turuturu* Yamada, *Heterosiphonia japonica* Yendo, *Sargassum muticum* (Yendo) Fensholt, and *Undaria pinnatifida* (Harvey) Suringar. Out of these, *S. muticum* and *U. pinnatifida* are the most abundant and diffuse species that colonize the hard substrata of the historical centre of Venice, Chioggia and many islands with people concern.

1.3. MACROALGAL TAXONOMIC IDENTIFICATION

Seaweeds are difficult to identify due to simply morphologies, phenotypic plasticity and convergent evolution (Saunders, 2005). Traditional algal classification is based on grass morphological characters such detailed aspects of vegetative and reproductive anatomy.

This system suffers two main problems in species identification: 1) it fails for the many asexual lineages of algae, as well as for species for which life history patterns are not known; 2) it leads to misidentifications in the case of cryptic species (i.e. with morphologies identical or similar also if they represent different taxa), or of environmental influences on morphological characters. In particular, the presence of several morphotypes belonging to the same taxon can lead to two possible errors: assignment of different morphotypes to distinct species or misidentification of species

actually different. These features are a great hindrance for the development of a more universal, rather than more restrictive, taxonomy of algae.

For these reasons molecular analyses are powerful tools for deriving phylogenies independent from the phenotypic characters on which traditional taxonomy is built. They are, in fact, considered more objective and, therefore, more indicative of evolutionary significance, than phenotypes that may be influenced by convergent evolution or by the environment.

DNA barcoding, identifying organisms based on comparisons of short standardized DNA sequences as agreed upon by the Consortium for the Barcode of Life (CBOL; http://barcoding.si.edu/), has been championed as a revolutionary system for the identification and discovery of all of the world's eukaryotic organisms (Hebert et al., 2003a; Hebert et al., 2003b).

About seaweeds, several markers of different DNA regions (nuclear, plastid and mitochondrial) have been used to study phylogenetic relationships at the interspecific and intraspecific level.

The most used markers are the plastid large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) gene, very commonly employed in the taxonomy of algae (Freshwater et al., 1994), the mitochondrial *cox*1 gene, coding for the subunit I of cytochrome oxidase enzyme and proposed as a barcoding gene in red algae (Saunders, 2005); and the nuclear internal transcribed spacer of the ribosomal cistron (ITS), extensively used for investigations of phylogeny, molecular ecology and evolution of marine green macroalgae (Hayden and Waaland, 2004). Other interesting markers are the plastid elongation factor *tuf*A, used to discriminate among green algal species (Fama et al., 2002; Zuccarello et al., 2009; Händeler et al., 2010); the plastid *rbc*L-*rbc*S spacer region, proven to be a good marker for species discrimination being essentially invariant within a species, but extremely variable at the interspecific level (Cho et al., 2003; Skage et al., 2005; Wolf et al., 2011b); and the more variable mitochondrial *cox*2-*cox*3 spacer useful for population studies (Zuccarello et al., 1999). Moreover the small size of the last two non coding regions makes them appropriate to be amplified from old historical material, whose DNA could be partially damnaged (Hughey et al., 2001). Comparison with type specimens (on which species names are based) or other relevant historical material, typically stored in collections, is important. For example this approach can be fundamental, to understand if a new reported alien taxon is the result of

a recent introduction or was already present in the environment, but erroneously classified because of its cryptic morphology, or it can be useful to solve some current systematic problems, such as the great number of superfluous and synonymised species names recorded and the use of distinct names for a same species sampled from different regions.

Moreover the comparison between fresh collected and historical samples can be important to compare seaweed biodiversity patterns and to summarize the occurrence and the distribution of introduced species (Provan et al., 2008; Lister et al., 2010).

The use of these markers also for phylogenetic reconstruction of a particular algal group can be useful to determine which morphological characters are congruent with the molecular data within a specific clade and which not, so that only the ones having a genetic basis will be used for correct species or genus identifications in future studies.

1.4 THESIS GOAL

In general precise species recognition is needed in order to identify invasive organisms, implement biological controls, and preserve and manage the local marine resources (Bickford et al. 2007). For this reason it is important to catalogue the macroalgal biodiversity of these environments to assist in its conservation and utilization.

Regarding seaweeds, up to now, most of the studies dealing with macroalgal biodiversity in Italy have been based only on morphological characters. Thus the first aim of my Ph.D. project was to start molecular surveys on these organisms, focusing on the Adriatic Sea (Mediterranean, Italy) and in particular on sites affected by intense naval traffic and aquaculture activities.

This project represents the first study of seaweed biodiversity in the Adriatic Sea based on molecular data. In fact, DNA barcoding, removing the reliance on morphological features, represents a valid approach to discriminate species and for this reason it is a good way to obtain an objective view of the entities present in the different regions.

REFERENCES

Abbott, I. A., and Hollenberg G. J., 1976. *Marine algae of California*. Stanford University Press, Stanford, California, USA.

Amsler, C. D., and Searles R. B., 1980. Vertical distribution of seaweed spores in a water column offshore of North Carolina. *J. Phycol.* 16:617–619.

Andersen, R.A., 2004. Biology and systematics of heterokont and haptophyte algae. *Am. J. Bot*. 91: 1508-1522.

Anderson, L.W.J., 2007. Control of invasive seaweeds. *Bot. Mar*. 50: 418–437.

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K. L., Meier, R., Winker, K., Ingram, K. K. & Das, I., 2007. Cryptic species as a window on diversity and conservation. *Trends Ecol. Evol.* 22: 148-55.

Bunker, F.S.D, Brodie, J.A., Maggs, C.A. and Bunker, A.R., 2010. *Seasearch guide to seaweeds of Britain and Ireland*. Ross-on-Wye: Marine Conservation Society. pp. 224.

Carlton, J.T., 1979. *History, biogeography and ecology of the introduced marine and estuarine invertebrates of the Pacific Coast of North America.* PhD Dissertation. University of California, Davis, CA, USA. pp. 904.

Carlton, J.T., 1989. Man's role in changing the face of the ocean: biological invasions and implications for conservation of nearshore environments. *Cons. Biol.* 3: 265–273.

Carlton, J.T., 1996. Pattern, process, and prediction in marine invasion ecology. *Biol. Cons. 78*: 97–106.

Carlton, J.T., 2001. *Introduced species in U.S. coastal waters: environmental impacts and management priorities*. Pew Oceans Commission, VA, USA. pp. 28.

Carlton, J.T. & Hodder, J., 1995. Biogeography of coastal marine organisms: Experimental studies on a replica of a sixteenth century sailing vessel. *Mar. Biol.* 121: 721-730.

Castilla, J.C., Uribe, M., Bahamonde, N., Clarke, M., Desqueyroux- Faúndez, R., Kong, I., Moyano, H., Rozbaczylo, N., Santelices, B., Valdovinos, C. and Zavala, P., 2005. Down under the southeastern Pacific: marine non-indigenous species in Chile. *Biol. Inv.* 7: 213–232.

Cecere, E. and Petrocelli, A., 2009. The Mar Piccolo of Taranto. *In*: (E. Cecere, A. Petrocelli, G. Izzo and A. Sfriso, eds) *Flora and vegetation of the Italian transitional water systems*. CORILA, Multigraf Spinea, Venezia. pp. 195–227.

Cecere, E., Petrocelli, A. and Saracino, O.D., 2000. *Undaria pinnatifida* (Fucophyceae, Laminariales) spread in the central Mediterranean: its occurrence in the Mar Piccolo of Taranto (Ionian Sea, southern Italy). *Cryptogamie Algol.* 21: 305–309.

Cecere, E., Moro, I., Wolf, M.A., Petrocelli, A., Verlaque, M. & Sfriso, A., 2011. The introduced seaweed *Grateloupia tururturu* (Rhodophyta, Halymeniales) in two Mediterranean transitional water systems. *Bot. Mar.* 54: 23-33.

Cho, T.O., Fredericq, S. & Boo, S.M., 2003. *Ceramium inkyuii* sp. nov. (Ceramiaceae, Rhodophyta) from Korea; a new species based on morphological and molecular evidence. *J. Phycol*. 39: 237-47.

Cohen, A.N., Mills, C., Berry, H., Wonham, M., Bingham, B., Bookheim, B., Carlton, J., Chapman, J., Cordell, J., Harris, L., Klinger, T., Kohn, A., Lambert, C., Lambert, G., Li, K., Secord, K. and Toft, J., 1998. *Puget sound expedition: a rapid assessment survey of non indigenous species in the shallow waters of puget sound.* Prepared for the Washington State Department of Natural Resources; Olympia, WA. pp. 37.

Coles, S.L., DeFelice, R.C., Eldredge, L.G. and Carlton, J.T., 1999. Historical and recent introductions of non-indigenous marine species into Pearl Harbor, Oahu, Hawaiian Islands. *Mar. Biol.* 135: 147–158.

Coles, S.L. and Eldredge, L.G., 2002. Nonindigenous species introductions on coral reefs: a need for information. *Pac. Sci*. 56: 191–209.

Coomans, R.J. and Hommersand M.H., 1990. Chapter 12: Vegetative growth and organization. *In*: (K. Cole and R. Sheath, eds), *The Biology of the Red Algae*. Cambridge University Press, New York.

Cranfield, H.J., Gordon, D.P., Willan, R.C., Marshall, B.A., Battershill, C.N., Francis, M.P., Nelson, W.A., Glasby, C.J. and Read, G.B., 1998. *Adventive marine species in New Zealand*. NIWA Technical Report 34, National Institute of Water and Atmospheric Research, Wellington, New Zealand. pp. 48.

Dixon, P.S., 1973. *Biology of the Rhodophyta*. New York: Hafner Press. pp. 251.

Elton, C., 1958. *The ecology of invasions by plants and animals*. Methuen and Co., London. pp. 181.

Fama, P., Wysor, B., Kooistra, W. & Zuccarello, G.C., 2002. Molecular phylogeny of the genus *Caulerpa* (Caulerpales, Chlorophyta) inferred from chloroplast *tuf*A gene. *J. Phycol*. 38: 1040-1050.

Freshwater, D.W., Fredericq, S., Butler, B.S., Hommersand, M.H. & Chase, M.W., 1994. A gene phylogeny of the red algae (Rhodophyta) based on plastid *rbc*L. *Proc. Natl. Acad. Sci.* USA 91: 7281-7285.

Gravili, C., Belmonte, G., Cecere, E., Denitto, F., Giangrande, A., Guidetti, P., Longo, C., Mastrototaro, F., Moscatello, S., Petrocelli, A., Piraino, S., Terlizzi, A. and Boero F., 2010. Non-indigenous species (NIS) along the Apulian coast, Italy. *Chem. Ecol. 26*: 121–142.

Gritti, E. Smith, B. and Sykes, M. T., 2006 Vulnerability of Mediterranean basin ecosystems to climate change and invasion by exotic plant species. *J. Biogeogr*. 33: 145–157

Händeler, K., Wägele, H., Wahrmund, U., Rüdinger, M. & Knoop, V., 2010. Slugs'last meals: molecular identification of sequestered chloroplast from different algal origins in Sacoglossa (Opisthobranchia, Gastropoda). *Molecular Ecology Resources* 10: 968-978.

Harvey, W.H., 1836. Algae. *In*: (Mackay, J.T., ed) *Flora hibernica comprising the Flowering Plants Ferns Characeae Musci Hepaticae Lichenes and Algae of Ireland arranged according to the naural system with a synopsis of the genera according to the Linnaean system*.. Dublin, London & Edinburgh: William Curry Jun and Company; Simpkin Marshall and Co; Fraser & Co. pp. 157-254.

Hayden, H.S. & Waaland, J.R., 2004. A molecular systematic study of *Ulva* (Ulvaceae, Ulvales) from the northeast Pacific. *Phycologia*. 43: 364-382.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & Deeward, J.R., 2003a. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B Biology*. 270: 313-321.

Hebert, P.D.N., Ratnasingham, S. & Deeward, J.R., 2003b. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London B Biology*. 270: 96-99.

Hewitt, C.L., 2002. The distribution and diversity of tropical Australian marine bio-invasions. *Pac. Sci. 56*: 213–222.

Hewitt, C.L., Campbell, M.L., Thresher, R.E. and Martin, R.B., 1999. *The introduced species of Port Phillip Bay, Victoria*. Centre for Research on Introduced Marine Pests Technical Report No. 20. CSIRO Marine Research, Hobart. pp. 344.

Hewitt, C.L., Campbell, M.L., Thresher, R.E., Martin, R.B., Boyd, S., Cohen, B.F., Currie, D.R., Gomon, M.F., Keogh, M.J., Lewis, J.A., Lockett, M.M., Mays, N., McArthur, M.A., O'Hara, T.D., Poore, G.C.B., Ross, D.J., Storey, M.J., Watson, J.E. and Wilson, R.S., 2004. Introduced and cryptogenic species in Port Phillip Bay, Victoria, Australia. *Mar. Biol. 144*: 183–202.

Hewitt, C. L., Campbell, M. L. and Schaffelke, B., 2007. Introductions of seaweeds: accidental transfer pathways and mechanisms. *Botanica Marina* 50: 326–337

Hughey J.R., **Silva' P.C. & Hommersand, M.H.,** 2001. Solving taxonomic and nomenclature problems in pacific Gigartinaceae (Rhodophyta) using DNA from type material. *J Phycol* 37:1091-1109.

Lamouroux, J.V.F., 1813. *Essai sur les genres de la famille des Thalassiophytes non articulées*. Paris: C. Dufour. pp. 84.

Leppäkoski, E., Gollasch, S. and Olenin, S., 2002. *Invasive aquatic species of Europe distribution, impact and management.* Kluwer, Dordrecht. pp. 583.

Lewis, J.A., 1999. A review of the occurrence of exotic macroalgae in southern Australia, with emphasis on Port Phillip Bay, Victoria. *In*: (C.L. Hewitt, M.L. Campbell, R.E. Thresher and R.B. Martin, eds). *Marine biological invasions of Port Phillip Bay, Victoria.* Centre for Research on Introduced Marine Pests Technical Report No. 20. CSIRO Marine Research, Hobart, Australia. pp. 61–87.

Lewis, P.N., Hewitt, C.L., Riddle, M. and McMinn, A., 2003. Marine introductions in the Southern Ocean: an unrecognised hazard to biodiversity. *Mar. Poll. Bull*. 46: 213–223.

Lewis, L. A. and McCourt R. M., 2004. Green algae and the origin of land plants. *American Journal of Botany* 91: 1535-1556.

Linnaeus, C., 1753. *Species plantarum, exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas*. Holmiae [Stockholm]: Impensis Laurentii Salvii. Vol. 1 pp. [1-12], 1-560.

Lister D.L., Bower, M.A. & Jones, M.K., 2010. Herbarium specimens expand the geographical and temporal range of germplasm data in phylogeographic studies. *Taxon* 59: 1321-1323

Littler, M. M., Littler, D. S. and Taylor, P. R., 1983. Evolutionary strategies in a tropical barrier reef system: functional form groups of marine macroalgae. *J. Phycol.* 19:229-237.

Littler, M.M., Littler, D.S. and Lapointe, B.E., 1988. A comparison of nutrient and light limited photosynthesis in psammophytic versus epilithic forms of *Halimeda* (Caulerpales, Halimedaceae) from the Bahamas. *Coral Reefs* 6: 219-225.

Lobban, C. S. and Harrison, P. J., 1994. *Seaweed ecology and physiology*. Cambridge University Press, pp. 366.

Lubchenco, J.A., Olson, M., Brubaker, L.B., Carpenter, S.R., Holland, M.M., Hubbell, S.P., Levin, S.A., MacMahon, J.A., Matson, P.A., Melillo, J.M., Mooney, H.A., Peterson, C.H., Pulliam, H.R., Real, L.A., Regal, P.J. and Risser, P.G., 1991. The sustainable biosphere initiative: an ecological research agenda. *Ecology* 72: 371–412.

Mack, R.N. Simberloff, D., Lonsdale, WM, Evans, H., Clout, M. and Bazzaz, FA, 2000 Biotic invasions: causes, epidemiology, global consequences, and control. *Ecol. Appl*. 10: 689–710

McEnnulty, F.R., Bax, N.J., Schaffelke, B. and Campbell, M.L., 2001. *A rapid response toolbox: strategies for the control of ABWMAC listed species and related taxa in Australia*. Centre for Research on Introduced Marine Pests Technical Report No. 23. CSIRO Marine Research, Hobart, Australia. pp. 101.

Mc Lusky, D.S. & Elliot M., 2007. Transitional waters: a new approach, semantics or just muddying the waters? *Estuar. Coast. Shelf Sci.* 71: 359–363.

Miller, A.W, Chang, A.L., Cosentino-Manning, N. and Ruiz, G.M., 2004. A new record and eradication of the northern Atlantic alga *Ascophyllum nodosum* (Phaeophyceae) from San Francisco Bay, California, USA. *J. Phycol*. 40: 1028–1031.

Norton, T.A. and Mathieson A.C., 1983. The biology of unattached seaweeds. *In*: (F. Round and D. Chapman, eds) *Progress in Phycological Research*. Elsevier Sci. Publ., Amsterdam, Vol. 2. pp. 333-386.

Oresanz, J.M., Schwindt, E., Pastorino, G., Bortolus, A., Casas, G., Darrigran, G., Elı´as, R., Lo´ pez Gappa, J.J., Obenat, S., Pascual, M., Penchaszadeh, P., Piriz, M.L., Scarabino, F., Spivak, E.D. and Vallarino, E.A., 2002. No longer the pristine confines of the world ocean: a survey of exotic marine species in the southwestern Atlantic. *Biol. Inv.* 4: 115–143.

Padilla, D. K. & Williams S. L., 2004. Beyond ballast water: aquarium and ornamental trades as sources of invasive species in aquatic ecosystems. *Frontiers in Ecology and the Environment* 2: 131–138.

Provan, J., Booth, D., Todd, N.P., Beatty, G.E. & Maggs, C.A., 2008. Tracking biological invasions in space and time: elucidating the invasive history of the green alga *Codium fragile* using old DNA. *Diversity and Distributions A Journal of Conservation Biogeography* 14: 343-354.

Raven, J.A., 1986: Physiological consequences of extremely small size for autotrophic organisms in the sea. *In*: (T. Platt and W.K.W. Li, eds) *Photosynthetic Picoplankton*. Can. Jour. Bull. Fish. Aquat. Sci., 214, pp. 1-70.

Ribera, M.A. and Boudouresque, C.F., 1995. Introduced marine plants with special reference to macroalgae: mechanisms and impact. *Prog. Phycol. Res*. 11: 187–268.

Ribera Siguan, M.A., 2002. Review of non-native marine plants in the Mediterranean Sea. *In:* (E. Leppa¨ koski, S. Gollasch and S. Olenin, eds), *Invasive aquatic species of Europe distribution, impact and management.* Kluwer Academic Publishers, Dordrecht. pp. 291–310.

Ruiz, G.M. and Carlton, J.T., 2003. *Invasive species: vectors and management strategies.* Island Press, Washington, D.C. pp. 518.

Saunders, G. W. and Hommersand, M. H., 2004. Assessing red algal supraordinal diversity and taxonomy in the context of contemporary systematic data. *American Journal of Botany* 91: 1494–1507

Saunders, G.W., 2005. Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. *Philos. Trans. SerB* 360, 1879-1888.

Saunders, G.W., 2009. Routine DNA barcoding of Canadian Gracilariales (Rhodophyta) reveals the invasive species *Gracilaria vermiculophilla* in British Columbia. *Molecular Ecology Resources*. 9: 140-150.

Schaffelke, B., Smith, J.E. and Hewitt, C.L., 2006. Introduced macroalgae – a growing concern. *J. Appl. Phycol.* 18: 529–541.

Schaffelke, B. and Hewitt, C.L., 2007. Impacts of introduced seaweeds. *Bot. Mar*. 50: 397–417.

Sfriso, A. & Curiel, D., 2007. Check-list of marine seaweeds recorded in the last 20 years in Venice lagoon and a comparison with the previous. *Bot. Mar*. 50, 22-58.

Sfriso, A., Curiel, D. & Rismondo, **A.,** 2009. The Lagoon of Venice. *In*: (E. Cecere, A. Petrocelli, G. Izzo and A. Sfriso, eds) *Flora and vegetation of the Italian transitional water systems*. CORILA, Multigraf Spinea, Venezia. pp. 195–227.

Skage, M., Gabrielsen, T.M. & Rueness, J. 2005. A molecular approach to investigate the phylogenetic basis of three widely used species groups in the red algal genus *Ceramium* (Ceramiales, Rhodophyta). *Phycologia* 44: 353-360.

Thornber, C.S., 2006. Functional properties of the isomorphic biphasic algal life cycle. *Integrative and Comparative Biology*. 46: 605-614.

Thresher, R.E., 1999. Key threats from marine bioinvasions: a review of current and future issues. *In:* (J. Pederson, ed) *Marine bioinvasions: proceedings of the first national conference, January 24–27, 1999.* Massachussetts Institute of Technology, Sea Grant College Program, Boston, MA. pp. 24–36.

Wolf, M.A., Sfriso, A., Andreoli, C. & Moro, I., 2011a. The presence of exotic *Hypnea flexicaulis* (Rhodophyta) in the Mediterranean Sea as indicated by morphology, *rbc*L and *cox*1 analyses. *Aquatic Botany*. 95: 55-58.

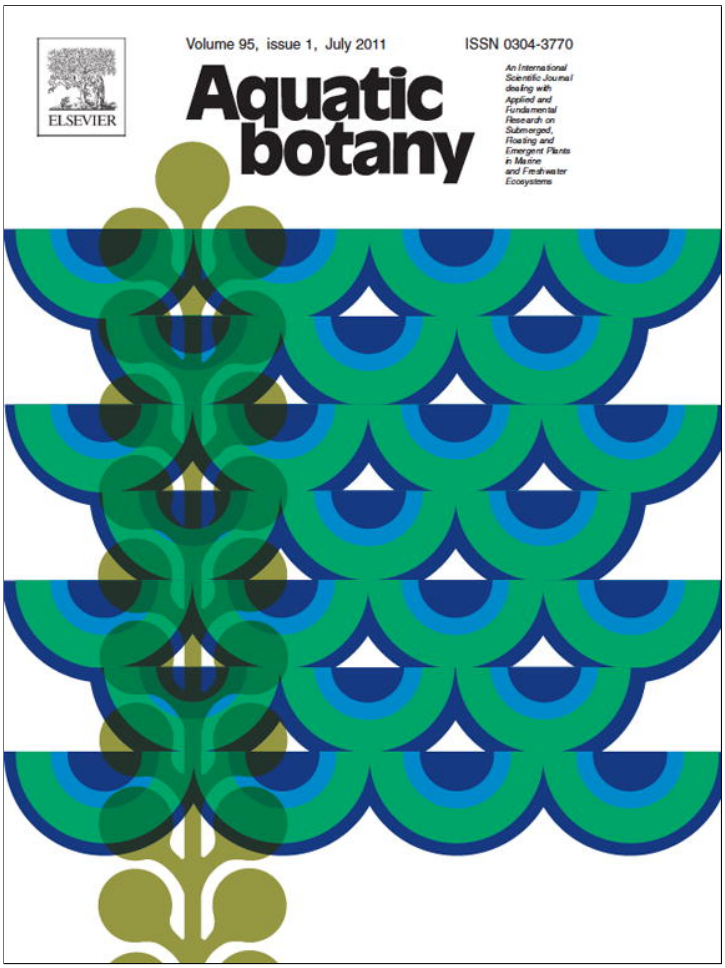
Wolf, M. A., Sciuto, K., Maggs, C. A., Barros-Barreto, M.B.B., Andreoli, C. & Moro, I., 2011b. *Ceramium* Roth (Ceramiales, Rhodophyta) from Venice lagoon (Adriatic Sea, Italy): comparative studies of Mediterranean and Atlantic taxa. *Taxon* 60: 1584-1595.

Weigle, S.M., Smith, L.D., Carlton, J.T. and Pederson, J., 2005. Assessing the risk of introducing exotic species via the live marine species trade. *Cons. Biol.* 19: 213–223.

Wyatt, A.S.J., Hewitt, C.L., Walker, D.I. and Ward, T.J., 2005. Marine introductions in the Shark Bay World Heritage Property, Western Australia: a preliminary assessment. *Div. Dist.* 11: 33–44.

Zuccarello, G.C., Burger, G., West, J.A. & King, R.J., 1999. A mitochondrial marker for red algal intraspecific relationships. *Mol. Ecol.* 8: 1443-1447.

Zuccarello, G., Price, N., Verbruggen, H. & Leliaert, F., 2009. Analysis of plastid multigene dataset and the phylogenetic position of the marine macrolaga *Caulerpa filiformis* (Chlorophyta). *J. Phycol*. 45: 1206-1212.



Aquatic Botany 95 (2011) 55–58

Contents lists available at ScienceDirect

Aquatic Botany

journal homepage: www.elsevier.com/locate/aquabot

Short communication

The presence of exotic *Hypnea flexicaulis* (Rhodophyta) in the Mediterranean Sea as indicated by morphology, *rbc*L and *cox*1 analyses

Marion Adelheid Wolf[a], Adriano Sfriso[b], Carlo Andreoli[a], Isabella Moro[a,*]

[a] Department of Biology, University of Padova, Via U. Bassi, 58/B 35131 Padova, Italy
[b] Department of Environmental Sciences, University of Venice, Calle Larga 2137, 30123 Venice, Italy

ARTICLE INFO

Article history:
Received 26 November 2010
Received in revised form 18 February 2011
Accepted 24 February 2011
Available online 4 March 2011

Keywords:
Exotic macroalgae
Hypnea flexicaulis
Mediterranean Sea
Venice Lagoon
*cox*1
*rbc*L

ABSTRACT

Here we report the first finding of *Hypnea flexicaulis* Yamagishi and Masuda in the Mediterranean Sea (Lagoon of Venice, Italy), identified through molecular analyses using the plastid ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) and the mitochondrial protein-coding cytochrome *c* oxidase subunit I (*cox*1) genes. The phylogenetic reconstruction, based on *rbc*L + *cox*1 multiple alignment, showed that all specimens of *H. flexicaulis* from Venice, Korea, Philippines and Taiwan were included in a monophyletic group supported by a bootstrap value of 100%.

It is highly probable that *H. flexicaulis* has been introduced from Indo-Pacific populations, in particular the Korean one, probably via ship traffic or shellfish transfers.

The use of DNA barcoding combined with morphological observations was, in this case, a rapid way to identify this allochthonous species.

1. Introduction

The genus *Hypnea* Lamouroux, now including about 54 species, is abundant in intertidal and subtidal zones of tropical and warm temperate waters (Masuda et al., 1997). This taxon is economically important as food and for the production of kappa carrageenan (Mshigeni and Chapman, 1994).

Species discrimination in *Hypnea* is complicated by the high degree of morphological plasticity, influenced by environmental factors, that often leads to evident differences also among individuals of the same species (Masuda et al., 1997). Progress in molecular systematics has led to the use of DNA barcoding as a way to identify species without clear morphological diagnostic criteria (Robba et al., 2006). Plastid large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) has been very commonly used in the taxonomy of red algae (Freshwater et al., 1994). The mitochondrial protein-coding cytochrome *c* oxidase subunit I (*cox*1) gene has recently proven to be useful for the identification of red algal species (Saunders, 2005). Basing on the analyses of *cox*1 sequences from several samples of Rhodophyta, Robba et al. (2006) demonstrated that it is a sensitive marker for revealing population structure and the hidden diversity of red algal species.

Hypnea flexicaulis was described for the first time by Yamagishi and Masuda (2000) who studied samples collected in Japan. This species is characterized by flexuous percurrent main axes and antler-like branches with wide branching angles showing abrupt abaxial bending (Yamagishi and Masuda, 2000). The same organism was also identified in Korea, Taiwan and Philippines by Geraldino et al. (2006) using morphological and molecular markers.

Here we report the first finding of *H. flexicaulis* in the Mediterranean Sea. To confirm the identification of the strain collected in the Venice Lagoon (Mediterranean Sea), based on morphology, molecular analyses were carried out using the plastid *rbc*L and the mitochondrial *cox*1 genes. The sequences from our voucher were compared with sequences from specimens from Taiwan, Philippines and Korea.

2. Materials and methods

2.1. Sampling

Samples were collected in July 2009 in some localities of the Venice Lagoon (geographic coordinates: St. 1 (Sant'Angelo shallow bottoms: 45°24′490″N, 12°16′384″E); St. 2 (Malamocco sea-side breakwater: 45°22′141″N, 12°20′376″E); St. 3 (San Leonardo breakwater: 45°20′559″N, 12°14′535″E).

Morphological observations were carried out on samples fixed in 4% formaldehyde-seawater solution and for molecular analyses freeze dried samples were used.

* Corresponding author. Tel.: +39 049 8276255; fax: +39 049 8276260.
E-mail address: isabella.moro@unipd.it (I. Moro).

0304-3770/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquabot.2011.02.009

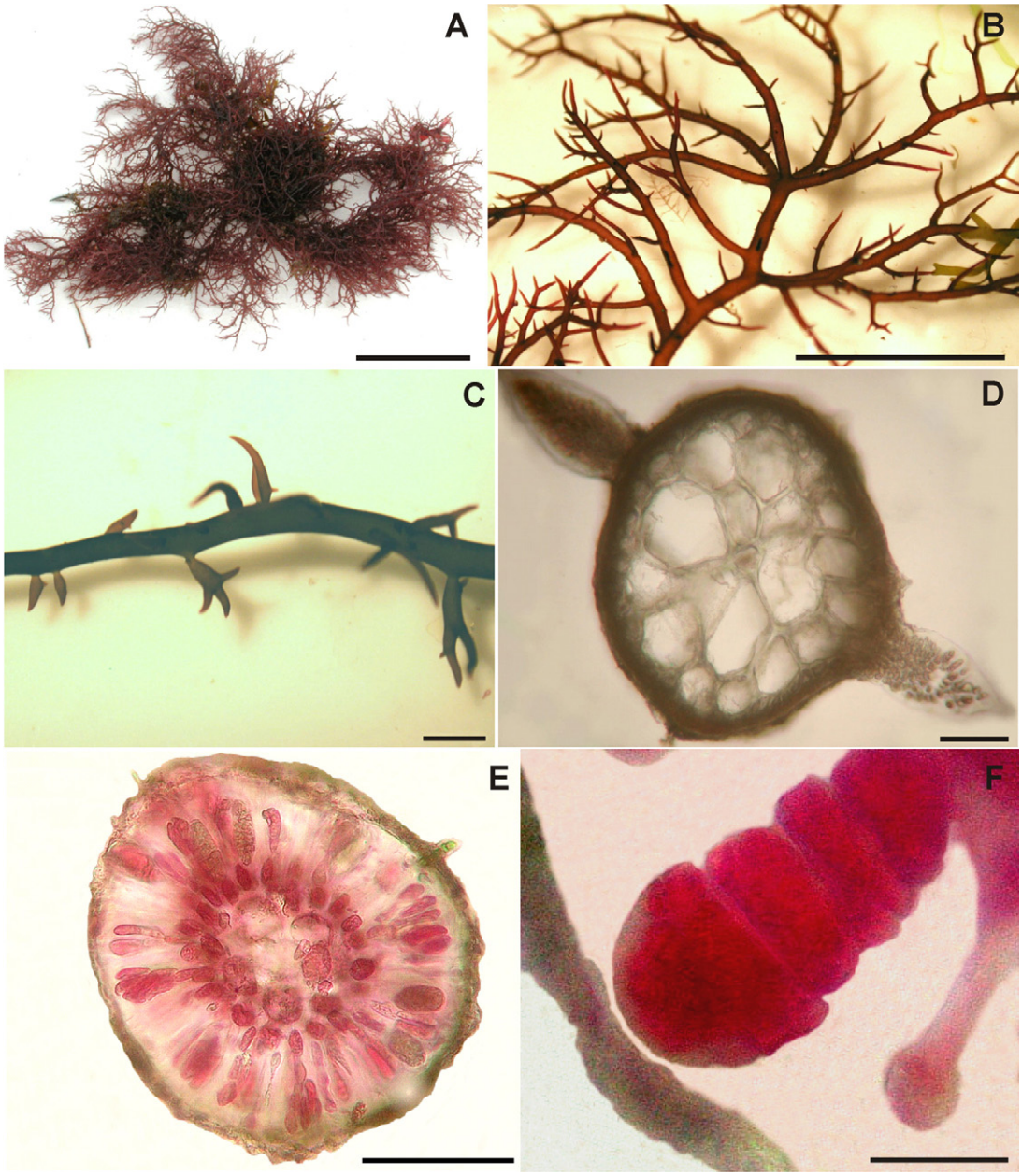


Fig. 1. Morphology of *Hypnea flexicaulis* from the Venice Lagoon. (A) Habitus of the thallus; scale bar = 5 cm. (B) Branching spiralate habitus of the thalli; scale bar = 1 cm. (C) Saddle-shaped adventitious branchlets. Some of them show nemathecia in the lower side; scale bar = 1 mm. (D) Cross sections with five large medullar cells; scale bar = 150 μm. (E) Cross section of a nemathecium on a branchlet lower side; scale bar = 100 μm. (F) Tetrasporangium zonately divided; scale bar = 20 μm.

2.2. Molecular procedures

Total DNA was extracted using the Genomic DNA purification kit (Fermentas International Inc., Burlington, Ontario, Canada). The plastid *rbc*L and the mitochondrial *cox*1 genes were amplified according to Freshwater and Rueness (1994) and Geraldino et al. (2006), respectively. In order to obtain the complete sequence of the *cox*1 gene other two more specific primers (COXIF1, 5′-GTATTTTAGGAGGGTGTATG-3′ and COXIR486, 5′-CGATCTGTTAGAAGCATTG -3′) were constructed by using a *cox*1 sequence of the Korean haplotype of *H. flexicaulis* reported by Geraldino et al. (2006) (GenBank EF136606). The PCR reaction was performed following Geraldino et al. (2009). The amplification and DNA sequencing products were performed according to Moro et al. (2010). Final consensus sequences were assembled using the SeqMan II program from the Lasergene software package (DNAStar©, Madison, WI, USA). Identity of new sequences was checked by using the BLAST program (Altschul et al., 1990) available at the USA National Center for Biotechnology Information (NCBI) web server (http://www.ncbi.nlm.nih.gov) and the sequence alignments were obtained by the ClustalW computer program (Thompson et al., 1994).

The *rbc*L and *cox*1 gene sequence datasets were used for phylogenetic analyses. The analyses were performed according to maximum likelihood (ML) method with the PHYML 2.4.4 program (Guindon and Gascuel, 2003) by applying the GTR+I+G evolutionary model (Lanave et al., 1984). Non parametric bootstrap re-sampling (Felsenstein, 1985) was performed to test the robustness of the tree topology (1000 replicates).

The *rbc*L and the *cox*1 gene sequences of the *H. flexicaulis* collected in the Venice Lagoon were deposited in DDBJ/GenBank™/EBI

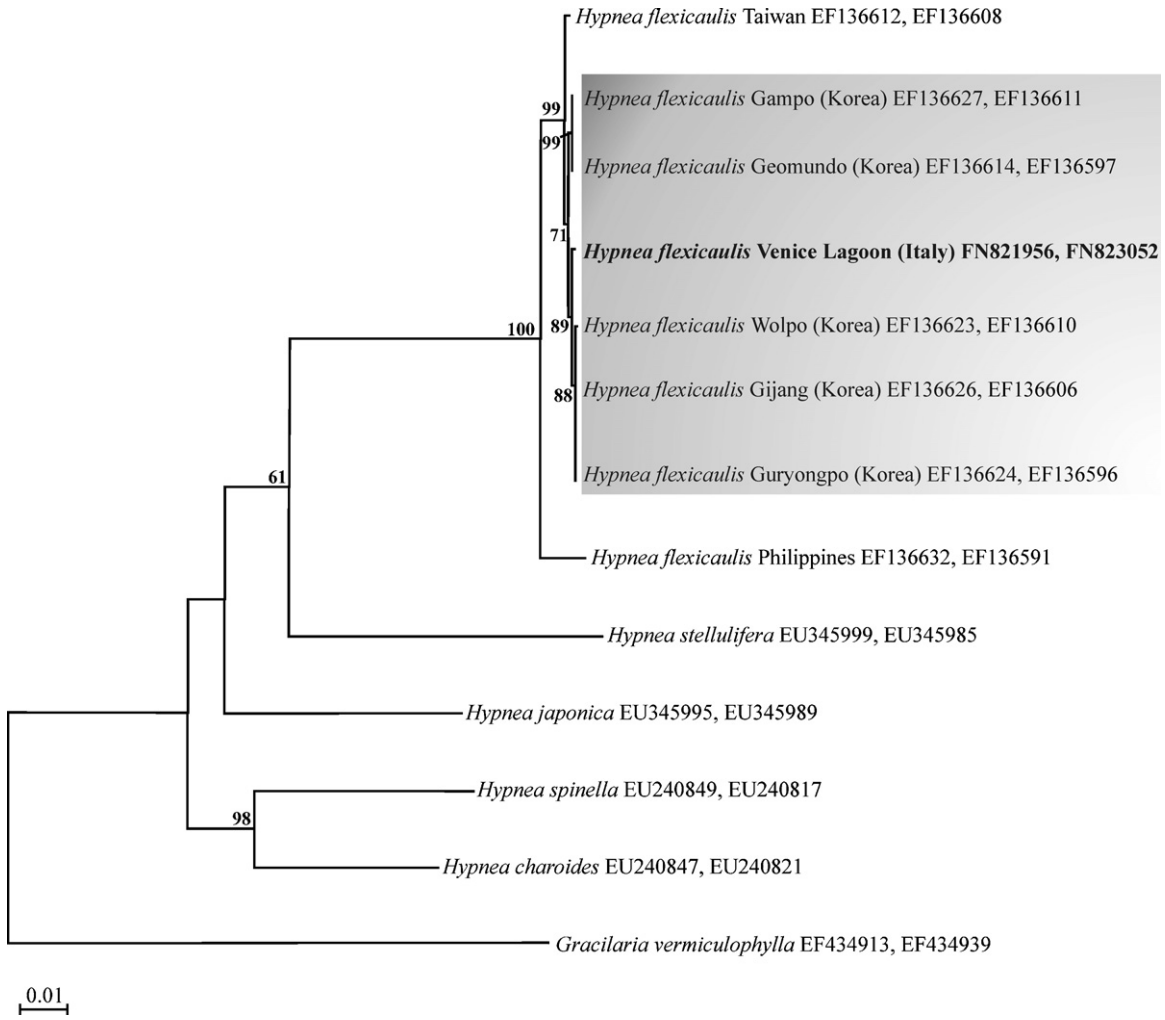


Fig. 2. Maximum likelihood tree inferred from *rbc*L + *cox*1 sequences calculated using the GTR + I + G evolutionary model. Numbers above each node refer to bootstrap values (1000 replications). Bar represents 0.01 nucleotide substitutions per site.

Data Bank with the accession numbers FN821956 and FN823052, respectively.

3. Results

3.1. Morphological results

Our samples matched the morphological description of *H. flexicaulis* reported by Yamagishi and Masuda (2000). Thalli are brownish–red or greenish–yellowish, fleshy or subcartilaginous, 5–35 cm high (Fig. 1A). The flexuous percurrent main axis (0.7–2.5 mm in diameter) is terete throughout branching in an alternate-spiral manner at angles of 45–150° (Fig. 1B). However, axes grow in the opposite direction to the branches, turning away from them so that the branching angles become wider. Abundant adventitious branchlets 0.5–2 mm high are curved, hooked or saddle-shaped showing abrupt abaxial bending (Fig. 1C). The cross section shows 4–6 big pyriform radially elongated medullar cells, 150–200 μm large and 200–300 μm high, that surround a small axial cell of 40–60 μm in diameter (Fig. 1D).

The tetrasporophytic phase is very common whereas sexual stages have not been recorded. Tetrasporangia are produced in nemathecia (Fig. 1E) confined in the lower side of the ultimate branchlets. They are pyriform, zonately divided and interspersed with sterile paraphyses (Fig. 1F).

3.2. Molecular results

The *rbc*L and *cox*1 sequences, obtained for the nine isolates sampled from the different collection sites, were all identical among them.

Identification of the strain from the Venice Lagoon as *H. flexicaulis* was obtained comparing its *rbc*L gene sequence with published data available in GenBank. The partial plastid gene sequence (741 bp) showed 100% identity with the sequences of 18 specimens collected by Geraldino et al. (2006) in five different locations in Korea. There was only one base pair difference between the Mediterranean voucher and the specimens from Taiwan, Philippines (Geraldino et al., 2006) and Japan (Yamagishi and Masuda, 2000).

The mitochondrial *cox*1 gene was also analyzed to better define the taxonomic position of the Mediterranean strain. The comparison of the *cox*1 sequence (1470 bp) of our *H. flexicaulis* strain with other ones reported in GenBank as the same species showed a percentage of similarity of 99% with the sequences of 20 specimens of *H. flexicaulis* recovered from Korea and Taiwan (Geraldino et al., 2006). The sequence of the specimens from Philippine (GenBank EF136591), instead, differed from the Mediterranean voucher in 33 bp (similarity percentage of 97%).

The phylogenetic reconstruction based on *rbc*L + *cox*1 multiple alignment and obtained from ML analyses is shown in Fig. 2.

All specimens of *H. flexicaulis* from Venice, Korea, Philippines and Taiwan are included in a monophyletic group supported by a bootstrap value of 100%.

4. Discussion

The molecular results combined with the morphological features strongly suggest that our strain is identifiable as *H. flexicaulis,* a species never recovered before in the Mediterranean Sea.

However, it is interesting highlighted that the *cox*1 sequence from the Mediterranean strain differed in 33 bp from the specimen of Philippines. Geraldino et al. (2006) hypothesized that, basing on a higher intraspecific divergence of the *cox*1 gene in the phylum Rhodophyta, the Philippines population might be biologically different from *H. flexicaulis* from Korea and Taiwan due to the different environmental conditions (the Philippines is more tropical than Taiwan). We can also hypothesize that our strain has been introduced by the Korean populations (99% identity between *cox*1 sequences). This is also supported by the 100% identity of our *rbc*L sequences with those obtained from the five localities in Korea and by the phylogenetic tree in which the Venice isolates of *H. flexicaulis* matched with all Korean specimens forming a separate clade.

It is highly probable that *H. flexicaulis* has been introduced by Indo-Pacific populations, in particular the Korean one. Two pathways are responsible of marine algae dispersal around the world: recent anthropogenic transport mechanism and natural dispersal events.

Most introductions of macroalgae into Europe during the last two centuries are related to human activities. A common vector is shellfish transfers, in fact several seaweed species are known to come from Japan with oysters, mussels and/or the Manila clam *Tapes philippinarum* Adams & Reeve (Farnham, 1980, 1994). Other transport mechanisms are ship traffic, with algae being carried as fouling organisms (Carlton and Hodder, 1995), or aquarium escapes (Padilla and Williams, 2004). In particular, in the Venice Lagoon, every year the increasing of naval traffic with extra-Mediterranean countries for both the import of fish products and aquaculture activities introduces new species, mainly macroalgae (Sfriso and Curiel, 2007; Cecere et al., 2009).

The other opportunities for dispersal of marine benthic organisms include prehistoric introductions through the Tethyan Seaway (especially for species also extending into warm temperate waters) and through the Arctic Ocean. In fact, after the opening of the Bering Strait in the Pliocene or Pleistocene, a connection was established between the Pacific and Atlantic Oceans (Lindstrom, 2001). For example, the seaweed *Acrosiphonia arcta* (Dillwyn) Gain studied by van Oppen (1995) is distributed from Chile, through the western United States, Greenland, and Iceland to northern Europe. Through molecular analyses (ITS sequences, RFLPs and RAPDs) van Oppen (1995) revealed clearly separated populations that, although currently recognized as a single species, may represent cryptic or incipient species.

This study highlights the importance of using DNA barcoding to discriminate between species of macroalgae, in particular red algae, which are extremely difficult to identify and classify on morphological grounds alone. The employment of molecular analyses, indeed, could represent an easier and rapid way to identify allochthonous species threatening the biodiversity of the Mediterranean Sea flora, so that appropriate precautions can be taken before they become invasive.

Finally, this research led us to document the first occurrence of the exotic species *H. flexicaulis* in the Mediterranean Sea.

Acknowledgments

This work was supported by grants provided by Progetto di Ateneo of the University of Padova.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Carlton, J.T., Hodder, J., 1995. Biogeography of coastal marine organisms: experimental studies on a replica of a sixteenth century sailing vessel. Mar. Biol. 121, 721–730.
Cecere, E., Petrocelli, A., Izzo, G., Sfriso, A., 2009. Flora and Vegetation of the Italian Transitional Water Systems. CoRiLa, Multigraf, Spinea.
Farnham, W.F., 1980. Studies on aliens in the marine flora of southern England. In: Price, J.H., Irvine, D.E.G., Farnham, W.F. (Eds.), The shore environment, Vol. 2: Ecosystems. Academic Press, London, pp. 875–914.
Farnham, W.F., 1994. Introductions of marine benthic algae into Atlantic European waters. In: Boudouresque, C.F., Briand, F., Nolan, C. (Eds.), Introduced species in European coastal waters. European Commission. Office for Official Publications of the European Union, Luxembourg, pp. 28–36.
Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using bootstrap. Evolution 39, 783–879.
Freshwater, D.W., Rueness, J., 1994. Phylogenetic relationships of some European *Gelidium* (Gelidiales, Rhodophyta) species, based on *rbc*L nucleotide sequence analysis. Phycologia 33, 187–194.
Freshwater, D.W., Fredericq, S., Butler, B.S., Hommersand, M.H., Chase, M.W., 1994. A gene phylogeny of the red algae (Rhodophyta) based on plastid *rbc*L. Proc. Natl. Acad. Sci. U.S.A. 91, 7281–7285.
Geraldino, P.J.L., Yang, E.C., Boo, S.M., 2006. Morphology and molecular phylogeny of *Hypnea flexicaulis* (Gigartinales, Rhodophyta) from Korea. Algae 21, 417–423.
Geraldino, P.J.L., Yang, E.C., Kim, M.S., Boo, S.M., 2009. Systematics of *Hypnea asiatica* sp. nov. (Hypneaceae, Rhodophyta) based on morphology and nrDNA SSU, plastid *rbc*L, and mitochondrial *cox*1. Taxon 58, 606–616.
Guindon, S., Gascuel, O., 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.
Lanave, C., Preparata, G., Saccone, C., Serio, G., 1984. A new method for calculating evolutionary substitution rates. J. Mol. Evol. 20, 86–93.
Lindstrom, S.C., 2001. The Bering Strait connection: dispersal and speciation in boreal macroalgae. J. Biogeogr. 28, 243–251.
Masuda, M., Yamagishi, Y., Chiang Lewmanomont, K., Xia, B., 1997. Overview of Hypnea (Rhodophyta, Hypneaceae). In: Abbott, I.A. (Ed.), Taxonomy of Economic Seaweeds, Vol. 6. California Sea Grant College System, La Jolla, pp. 127–133.
Moro, I., Rascio, N., La Rocca, N., Sciuto, K., Albertano, P., Bruno, L., Andreoli, C., 2010. Polyphasic characterization of a thermo-tolerant filamentous cyanobacterium isolated from the Euganean thermal muds (Padua, Italy). Eur. J. Phycol. 45, 143–154.
Mshigeni, K.E., Chapman, D.J., 1994. Hypnea (Gigartinales, Rhodophyta). In: Akatsuka, I. (Ed.), Biology of Economic Algae. SPB Academic Publishing, The Hague, pp. 245–281.
Padilla, D.K., Williams, S.L., 2004. Beyond ballast water: aquarium and ornamental trades as sources of invasive species in aquatic ecosystems. Front. Ecol. Environ. 2, 131–138.
Robba, L., Russel, S.J., Barker, G.L., Brodie, J., 2006. Assessing the use of mitochondrial *cox*1 marker for use in DNA barcoding of red algae (Rhodophyta). Am. J. Bot. 93, 1101–1108.
Saunders, G.W., 2005. Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. Philos. Trans. Ser. B 360, 1879–1888.
Sfriso, A., Curiel, D., 2007. Check-list of marine seaweeds recorded in the last 20 years in Venice lagoon and a comparison with the previous. Bot. Mar. 50, 22–58.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: improving the sensitivity of progressive weighting position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
van Oppen, M.J.H., 1995. Phylogenetic relationships among some Ulotrichalean (Ulvophyceae, Chlorophyta) algae, as inferred from rDNA ITS and partial 18S sequences, 59–76. Tracking trails by cracking codes, PhD Dissertation, University of Groningen, Haren.
Yamagishi, Y., Masuda, M., 2000. A taxonomic revision of *Hypnea charoides-valentiae* complex (Rhodophyta, Gigartinales) in Japan, with a description of *Hypnea flexicaulis* sp. nov. Phycol. Res. 48, 27–35.

Botanica Marina 54 (2011): 23–33 © 2011 by Walter de Gruyter • Berlin • New York. DOI 10.1515/BOT.2011.009

The introduced seaweed *Grateloupia turuturu* (Rhodophyta, Halymeniales) in two Mediterranean transitional water systems

Ester Cecere[1], Isabella Moro[2], Marion A. Wolf[2], Antonella Petrocelli[1,*], Marc Verlaque[3] and Adriano Sfriso[4]

[1] Institute for Marine Coastal Environment C.N.R., v. Roma 3, 74123 Taranto, Italy, e-mail: antonella.petrocelli@iamc.cnr.it
[2] Department of Biology, University of Padova, v. U. Bassi 58b, 35131 Padova, Italy
[3] UMR 6540, CNRS, Université de la Méditerranée, Parc scientifique et Technologique de Luminy, F13288 Marseille cedex 9, France
[4] Department of Environmental Sciences, University of Venice, Dorsoduro, 2137 Venice, Italy

* Corresponding author

Abstract

Lagoon environments are particularly subject to the introduction, either accidental or voluntary, of alien species that may cause biological pollution. On the basis of morphological data and molecular analyses (*rbc*L and mitochondrial *cox*2-*cox*3 spacer sequences) we confirmed the taxonomic identity (previously only hypothesised) of *Grateloupia turuturu* in both the Lagoon of Venice and the Mar Piccolo basin of Taranto, where it was most probably introduced by shellfish transfers. A detailed morphological description of vegetative and reproductive thalli is given, focusing particularly on the encrusting bases. The bases act as resting structures that are able to survive during unfavourable environmental conditions. In both the Lagoon of Venice and the Mar Piccolo of Taranto, *G. turuturu* does not yet exhibit invasive behaviour.

Keywords: alien species; *Grateloupia turuturu*; Lagoon of Venice; Mar Piccolo of Taranto; Mediterranean Sea.

Introduction

One of the major anthropogenic stresses on coastal marine environments is so-called ''biological pollution'', which comprises all possible negative effects from cell level to community level linked to the introduction of alien species (Elliot 2003, Olenin et al. 2007). Recent means of transportation have increased the speed of shellfish transfers for aquaculture and direct sale, facilitating the introduction of alien species (Minchin 2007). As a consequence, coastal and especially transitional water systems (e.g., coastal lagoons, estuaries) (Mc Lusky and Elliot 2007), which are usually the sites of aquaculture activities, are among environments most threatened by biological pollution.

In the Mediterranean Sea, the Thau Lagoon on the French coast represents the main hot spot for the introduction of alien species (Verlaque 2001), with 58 species of seaweeds to date (Verlaque et al. 2007). In Italy, the hot spots of marine introductions are the Lagoon of Venice and the Mar Piccolo of Taranto basin, two transitional water systems continuously monitored to detect alien species as quickly as possible (Cecere and Petrocelli 2009, Sfriso et al. 2009).

Grateloupia turuturu Yamada is reportedly the largest red alga in the world [Simon et al. 2001 as *G. doryphora* (Montagne) Howe] and among the most widespread alien and invasive seaweed species (Marston and Villalard-Bohnsack 2002, Mathieson et al. 2008). In the Mediterranean Sea, several native species of the genus *Grateloupia* C. Agardh have been described, of which only *Grateloupia filicina* (J.V. Lamouroux) C. Agardh and *G. dichotoma* J. Agardh have unquestioned status (Verlaque et al. 2005). In addition, three profusely branched and two foliose alien *Grateloupia* species, all native to Japan, are also present (Verlaque et al. 2005):

- *Grateloupia asiatica* Kawaguchi *et* Wang, previously misidentified as *G. filicina* in Japan;
- *Grateloupia patens* (Okamura) Kawaguchi *et* Wang;
- *Grateloupia subpectinata* Holmes, previously identified in Europe as *G. filicina* var. *luxurians* A. Gepp *et* E.S. Gepp;
- *Grateloupia turuturu*, a taxon introduced in numerous countries worldwide and often misidentified as *G. doryphora,* a southeastern Pacific endemic species that is restricted to Peru and perhaps Chile (Gavio and Fredericq 2002);
- *Grateloupia lanceolata* (Okamura) Kawaguchi, a species introduced with *G. turuturu* into the Thau Lagoon (France) where it was also misidentified as *G. doryphora*.

In Italy, a foliose *Grateloupia* species was reported for the first time (from the Strait of Messina) in 1981 as *Grateloupia doryphora* (De Masi and Gargiulo 1982) and in 1993 from the Lagoon of Venice (Tolomio 1993), although it had been present there for the previous five years (A. Sfriso unpublished data). Wilkes et al. (2006) demonstrated in a molecular study (*rbc*L sequence) that the Sicilian species is neither *G. doryphora* nor *G. turuturu*, but a taxon closely related to the second introduced foliose species, *G. lanceolata*. However, Gavio and Fredericq (2002) and Sfriso et al. (2006) hypo-

thesised that specimens from the Lagoon of Venice belonged to *G. turuturu*.

In February 2007, several thalli of a foliose *Grateloupia* species were collected for the first time in the Mar Piccolo basin in Taranto, and identified as *G. turuturu* on the basis of their morphological characteristics (Cecere and Petrocelli 2007).

The aim of the present morphological and molecular study was to confirm the identity of *G. turuturu* as the foliose species introduced into the Mar Piccolo basin and the Lagoon of Venice. We also present information on seasonal dynamics and recruitment of the species in both localities, which are examples of Mediterranean northern and central lagoons, respectively. The possible vectors of introduction in both localities are also discussed.

Materials and methods

Study sites

The Mar Piccolo of Taranto The Mar Piccolo of Taranto (40°28′N; 17°15′E) is a coastal, nearly-enclosed coastal basin located to the north of Taranto (southern Italy, Ionian Sea) (Figure 1). It has a surface area of ca. 20 km^2 and is divided into two smaller basins (the First and Second Inlets) by a two facing promontories of land. The lagoon features of the basin are explained mainly by the presence of 34 submarine freshwater springs and several small river outflows. The maximum depth is 12 m in the First Inlet and 8 m in the Second Inlet. Salinity ranges from 33 to 38. Seawater temperature varies from 7°C to 32°C (G. Alabiso pers. comm.). In 2009, in the area where a *Grateloupia* population was present, seawater temperature reached the lowest value (10°C) in January and February and the highest (28°C) in August. Tidal range is minimal, not exceeding 30 cm. The Mar Piccolo of Taranto houses the biggest mussel farm in Italy and dry-docks of the Italian Navy Base.

The Lagoon of Venice The Lagoon of Venice, located in the northern Adriatic Sea (45°24′N; 12°17′E) (Figure 1), has a surface area of ca. 549 km^2 and is the largest Mediterranean transitional environment. It receives the inputs of 24 freshwater tributaries. The lagoon, divided into three main hydrological basins (southern, central and northern basins), is a very diversified environment; its mean depth is about 1–1.2 m. However, in the main canals, depths can reach 10–20 m. Mean salinity ranges from 28 to 33, with the lowest values near river outflows and the highest (ca. 43) in tidal marshes during summer. The lagoon is also characterized by wide temperature variations throughout the year; the usual range is 5–30°C. However, values lower than 0°C and higher than 32°C are sometimes recorded. Mean tidal amplitude is ±31 cm, but under certain meteorological conditions it can range from -80 cm to +160 cm. The main recent anthropogenic impact is the free-harvesting of the mollusc *Tapes philippinarum* Adams *et* Reeve, which was introduced for aquaculture purposes (Sfriso et al. 2009).

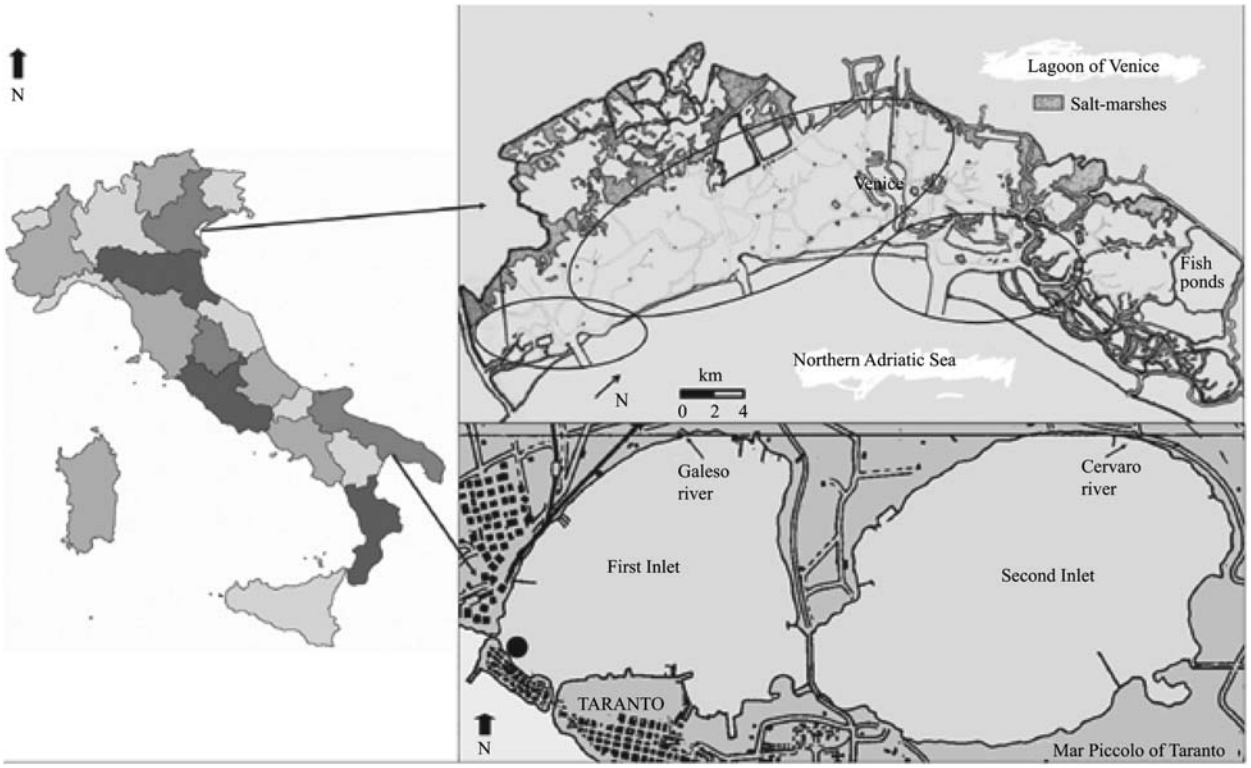


Figure 1 *Grateloupia turuturu*: locations of the Mar Piccolo of Taranto and the Lagoon of Venice, Italy.
The black circle in the Mar Piccolo and the ellipses in the Lagoon of Venice indicate the species distribution.

Sampling and observations

In both the Mar Piccolo of Taranto and the Lagoon of Venice, specimens were hand-collected. The thalli were brought to the laboratory and preserved in buffered 4% formaldehyde-seawater. Observations were made on both fresh and fixed material. Thalli were sectioned manually with a razor blade. Sections were observed either unstained or stained with 1% aqueous Aniline Blue acidified with a drop of HCl (0.5%) with both stereo and compound microscopes (Leica Microsystems®, Wetzlar, Germany). Photomicrographs were captured using a Nikon Coolpix 4500 (Nikon, Tokyo, Japan).

Dried and liquid preserved samples from Taranto and Venice have been deposited in the Herbarium of the Istituto Sperimentale Talassografico ''A. Cerruti'' of Taranto (TAR).

Molecular analyses

DNA analyses were carried out both on fresh thalli from the Mar Piccolo of Taranto and the Lagoon of Venice, and on one dried specimen (not previously formalin-treated) collected in the Lagoon of Venice and identified as *Grateloupia doryphora* by Tolomio (1993).

Genomic DNA was extracted from small fragments of thalli using a Genomic DNA purification kit (Fermentas International Inc., Burlington, ON, Canada), following the manufacturer's instructions.

The plastid large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) was amplified using the primer combination F57-R753 and F753-R1381, as described by Freshwater and Rueness (1994). PCR reactions were performed in the following sequence: initial denaturation of 2 min at 94°C followed by 30 cycles of 40 s at 94°C, 40 s at 50°C and 40 s at 72°C, with a final 5 min extension at 72°C. The amplification products were cleaned with an ExoSAP-ITTM kit (GE Healthcare, Uppsala, Sweden), following the manufacturer's protocol.

The mitochondrial *cox*2-3 intergenic spacer region was amplified using two degenerate primers (*cox*2-for and *cox*3-rev), and PCR conditions followed Zuccarello et al. (1999).

Because of the presence of multiple bands, DNA extraction from agarose gels (2%) was necessary to isolate the amplified *cox*2-3 spacer region; to do this, we used a DNA gel extraction kit (Millipore, Bedford, MA, USA). PCR products were sequenced with an automated ABI DNA sequencer (Applied Biosystems, Foster City, CA, USA) at the BMR Genomics Sequencing Center (Padova University).

The *rbc*L gene and the intergenic spacer *cox*2-3 consensus sequences were assembled using the program SeqMan II in the Lasergene software package (DNAStar©, Madison, WI, USA) and analysed by similarity search using the BLAST program (Altschul et al. 1990) available at the NCBI web server (www.ncbi.nlm.nih.gov/blast). The *rbc*L and *cox*2-3 sequences of different *Grateloupia turuturu* strains were deposited in DDBJ/GenBank™/EBI Data Bank with accession numbers reported in Table 1.

The taxonomic identification of each specimen collected in the Lagoon of Venice and in the Mar Piccolo of Taranto was made by comparing *rbc*L and *cox*2-3 gene sequences with published data available in GenBank (Table 1).

Finally, *rbc*L and *cox*2-3 datasets were constructed from the sequences obtained plus suitable sequences found in the DDBJ/GenBank™/EBI Data Bank. Alignments for phylogenetic analyses were generated with ClustalW software

Table 1 *Grateloupia turuturu*: list of sequences used in *rbc*L and *cox*2-3 comparisons (with Genbank accession numbers).

Molecular marker	Collection locality	Reference	GenBank accession no.
*cox*2-3	Hokkaido, Japan	Verlaque et al. (2005)	AY775371
	Brittany, France		AF414922, AF414923
	Thau Lagoon, France		AF414926, AF414927
	Tholen I, The Netherlands	Marston and Villalard-Bohnsack (2002) as *G. doryphora*	AF414924, AF414925
	Rhode Island, USA		AF414913, AF414916
	Hampshire, UK		AF414917, AF414918
	New Zealand	D'Archino et al. (2007)	EF091852
	Mar Piccolo, Italy	Present study	FN821954
	Lagoon of Venice, Italy	Present study, coll. C. Tolomio	FN821955
		Present study	FN821953
*rbc*L	Hokkaido, Japan		AF488820
	East Korea		AF488821, AY083215
	Brittany, France		AY100003, AY083216
	Hampshire, UK	Gavio and Fredericq (2002)	AY100002
	Rhode Island, USA		AF488818, AY100004
	New York, USA		AF488819
	Mar Piccolo, Italy	Present study	FN821951
	Lagoon of Venice, Italy	Present study, coll. C. Tolomio	FN811952
		Present study	FN821950

(Thompson et al. 1994). Phylogenetic analyses were performed following the maximum likelihood (ML) method in PHYML 2.4.4 software (Guindon and Gascuel 2003) with the GTR+I+G evolutionary model (Lanave et al. 1984). Non-parametric bootstrap re-sampling (Felsenstein 1985) was performed to test the robustness of the tree topology (1000 replicates).

Seasonal dynamics

Studies were performed in 2009 at both localities. Monthly observations were carried out on both natural and artificial substrata across each basin in order to evaluate the invasion capability of the species.

Results

Molecular analyses

The mitochondrial *cox*2-3 intergenic spacer region sequences of specimens collected in Taranto and Venice, and the herbarium sample from Venice identified as *Grateloupia doryphora* by Tolomio (1993) did not show any variation. This region ranged from 300 to 317 bp in length and included the 3′ end of the *cox*2 gene, the intergenic spacer region (144 bp) and the 5′ end of the *cox*3 gene.

Our sequences had an identity value of 100% with other *cox*2-3 sequences of *Grateloupia turuturu* available in GenBank (EF091852, AF414916, AF414915, AF414914) for specimens collected in New Zealand and USA (Rhode Island), and differed by one base-pair (0.33%) from other sequences of vouchers sampled in Japan, The Netherlands, France, and UK.

The *rbc*L sequences of the samples collected during the present study in the Lagoon of Venice (1364 bp long) and in the Mar Piccolo of Taranto (1360 bp long) had 100% identity with *rbc*L sequences of *Grateloupia turuturu* specimens collected from New York and Rhode Island (USA), and had only one nucleotide difference (<1% of divergence) from other sequences of vouchers sampled in Japan, Korea, France, and UK. From the herbarium specimen collected in 1991 in the Lagoon of Venice and identified as *G. doryphora*, we obtained a partial *rbc*L gene sequence of only 655 bp, due to the DNA degradation during the long conservation period. Nevertheless, the alignment of this partial sequence with others available in public data banks had identity values of 100% with all *rbc*L sequences of *G. turuturu* specimens collected in Japan, Korea, France, UK and USA (Table 1).

Phylogenetic analyses of the *rbc*L sequences (Figure 2) demonstrated that the Taranto and Venice specimens formed a clade with other *Grateloupia turuturu* specimens collected in New York and Rhode Island (USA). Moreover, this clade was had a sister relationship with other *G. turuturu* vouchers sampled in Japan, Korea, France, and UK.

The *cox*2-3 tree (Figure 3) demonstrated that the Taranto and Venice specimens formed a clade together with *G. turuturu* vouchers sampled from New Zealand and USA and reported in GenBank as *G. turuturu* and *G. doryphora,* respectively. This clade was joined to other two groups constituted by other *G. turuturu* specimens collected in France, Japan, The Netherlands and UK.

Description of the Italian specimens

Upright blades, usually in clumps, are attached to the substratum by means of a purplish red to brownish red encrusting base. The plantlets first protruding from encrusting bases have a terete habit, and then they may be dichotomously branched when just 0.5–1.0 mm high (Figure 4). Young blades, up to 4 cm high, are lanceolate, with a terete stipe, ca. 350 μm in diameter and 1–3 mm high, with slightly undulate margins and no proliferations (Figure 5). As plants grow, blades may divide dichotomously one or more times. Mature blades soft and gelatinous, and pinkish to purplish red, up to 80 cm long and 18 cm broad, with marked morphological variability (lanceolate or foliose; cordate or reniform; dichotomously or irregularly divided in one plane). The margins are usually very undulate, smooth or with lateral proliferations. Mature blades may also have more or less round perforations with entire margins from which many proliferations may also issue (Figure 5). Some thalli may contain spherical to irregular gas bubbles of variable dimensions, sometimes involving the whole thallus (Figure 6). Senescent blades take on a greenish colour.

The base is usually irregular in shape, especially on uneven substrata, such as thin ropes and nets, around which it can form a sheath (Figure 7). In radial section, the encrusting base is of the *Hildenbrandia* type with a basal layer of tightly adherent, radiating filaments, with each cell 7–12 μm long and 2–7 μm in diameter, producing an erect, laterally coherent filament of small cubical cells 6–7 μm in diameter (Figure 8). Floridean starch granules ca. 1 μm in diameter are visible in the most basal cells of erect filaments. Primary and secondary pit-connections are present. The crusts, 70–250 μm and 10–30 cells thick, are covered by a mucilage coat (10–12 μm thick) that imparts translucence. In surface view, the cortex is composed of polygonal cells 3 μm in diameter.

In transverse section, the stipe has a compact cellular cortex 3–4 cells thick, and a compact filamentous medulla (Figure 9). Outer cortical cells are elliptical, 3–5 μm in diameter by 4–8 μm in length. Medullary filaments are anticlinally and periclinally arranged, with cells ca. 5–7 μm in diameter by about 50–60 μm in length. In transverse section, mature blades, 150–200 μm thick, have an outer mucilage coat, a compact cellular cortex and a loose medulla of anticlinally and periclinally arranged filaments. Outer and inner cortices are 3–4 and 2–3 cells thick, respectively (Figure 10). Outer cortical cells, 2–3 μm in diameter by 3–4 μm in length, are ovoid to cylindrical (Figure 11). Inner cortical cells are rounded, 6–8 μm in diameter, to spur-like, ca. 12 μm in length (Figure 12). Medullary cells are star-shaped to filamentous, 2–7 μm in diameter by 25–60 μm in length. In young blades, medullary filaments are frequently anticlinally arranged (Figure 13). In surface view, the arrangement of polygonal cortical cells, 5–7.5 μm in diameter, may resemble a mosaic pattern (Figure 14).

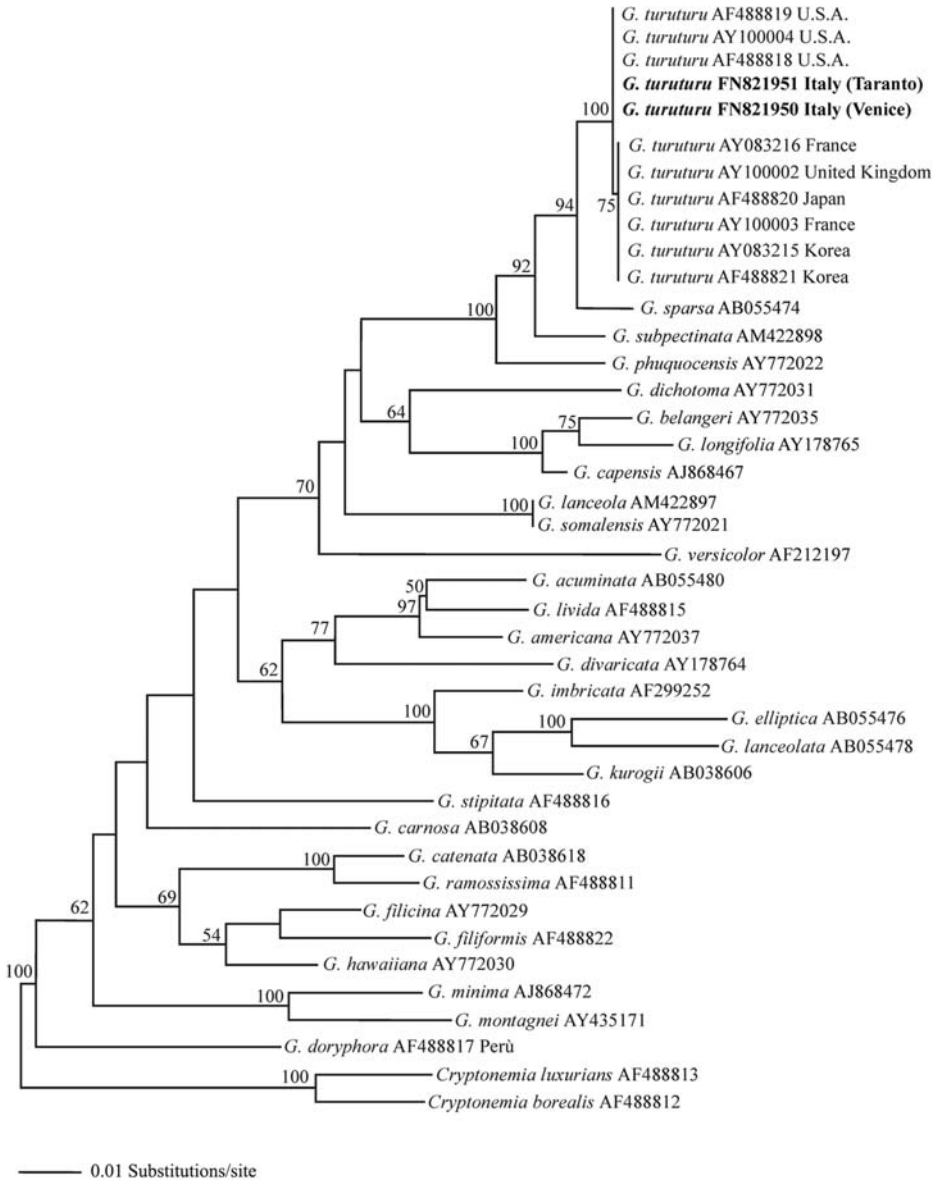


Figure 2 *Grateloupia turuturu*: placement within a phylogenetic tree based on the *rbc*L gene sequences reconstructed using the maximum-likelihood analysis of evolutionary distances determined by the GTR+I+G model (1000 replications).
The numbers near the nodes indicate bootstrap values. Sequences determined in this work are indicated in bold. Bar represents 0.01 nucleotide substitutions per site.

Gametophytes are monoecious and reproductive structures form over the entire thallus except the basal part. Mature cystocarps are spherical, not protruding, 130–250 μm broad, including the pericarp. The ostiole is either not protruding or slightly depressed (Figures 15, 16). Spermatangia, 2–3 μm in diameter by 3–4 μm in length, are cut-off from the outer cortical cells (Figure 17). In surface view they form discontinuous sori on outer cortical cells. Tetrasporangia are oblong, cruciately divided, 30–40 (-50) μm long and 10–20 (-25) μm wide, and scattered over the entire thallus except for the basal portion, arising from the third to fifth cortical cell layers inward from the surface without any modification of the cortex (Figure 18).

Recruitment and growth

The Mar Piccolo of Taranto Thalli of foliose *Grateloupia* were present in a eutrophic zone in the intertidal and in the upper sublittoral, to a depth of 50 cm at most. They were settled both on natural (stones and mussel valves) and artificial (including small ropes, plastic tubular nets used for mussel culture and fishing nets) hard substrata.

Grateloupia populations had a conspicuously clumped spatial pattern among patches of *Dictyota dichotoma* (Hudson) J.V. Lamouroux and *Corallina officinalis* Linnaeus, which were the other dominant algal species in the zone. Many actinians were also present. Blades of *Grateloupia*

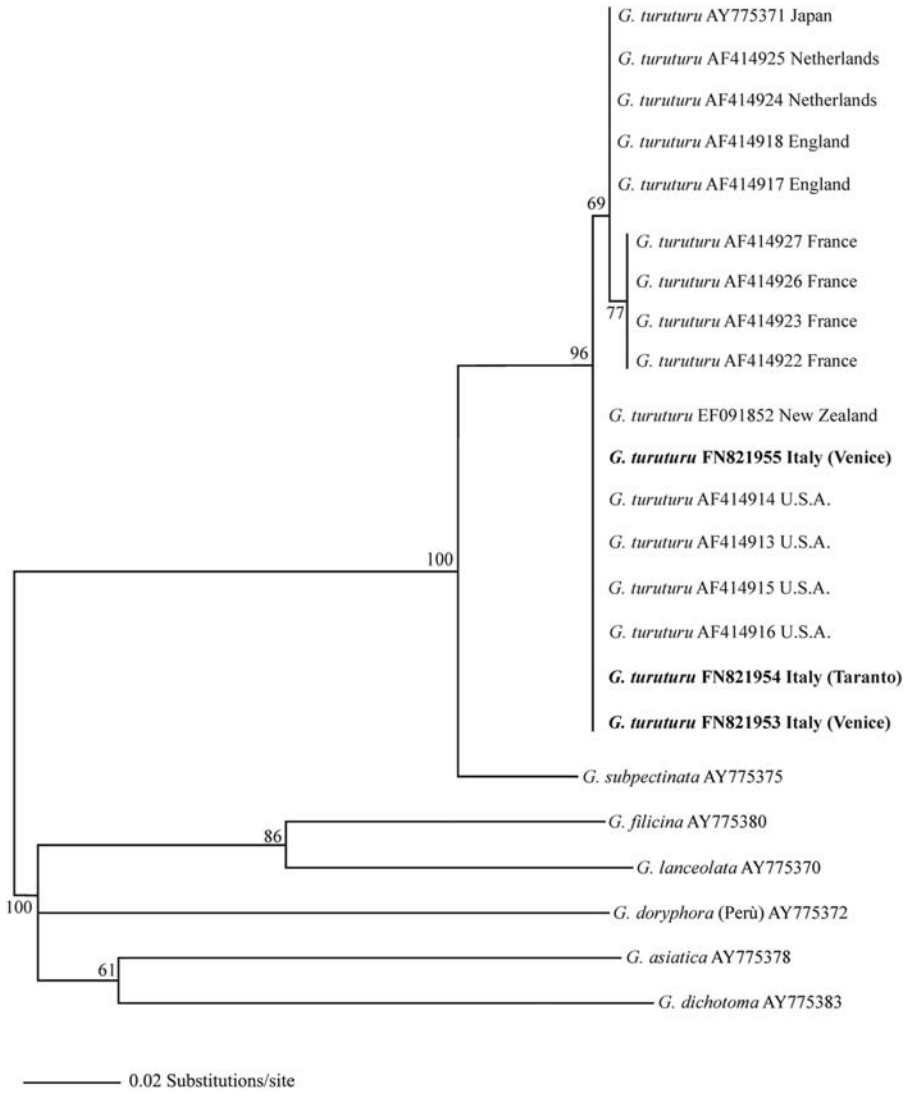


Figure 3 *Grateloupia turuturu*: placement within a phylogenetic tree based on the *cox*2-3 spacer region sequences reconstructed using the maximum-likelihood analysis of evolutionary distances determined by the GTR+I+G model (1000 replications).
The numbers near the nodes indicate bootstrap values. Sequences determined in this work are indicated in bold. Bar represents 0.02 nucleotide substitutions per site.

were poorly epiphytized by members of the Ceramiaceae, while encrusting coralline algae sometimes settled on crusts.

Upright thalli were present from October to June. In this period, uprights sprouted from encrusting thalli every month, although in October and June, only very few thalli up to 1 cm (October) and 2 cm (June) high were observed. In July, August and September, only encrusting thalli were found. Larger blades, up to 80 cm high, were found in April at -50 cm depth. The tetrasporophytes were found in February and March and the gametophytes from March to June.

The Lagoon of Venice In the Lagoon of Venice, thalli of foliose *Grateloupia* colonized both the mesotrophic seaward side artificial substrata and the eutrophic-hypertrophic lagoon shores and docks. The highest number of thalli was recorded on the quays of the Venice historical centre where nutrient concentrations were high (Pavoni et al. 1990). On the stone canal banks, dams and wharfs where boats for public service dock, the alga formed relatively dense populations associated with many filamentous members of the Ulvaceae. Blades were present throughout the year, with two peaks of abundance in spring and autumn. The first seasonal peak of development, with blades up to 80 cm long and 18 cm broad, occurred from March to June, before a summer decline of populations when the seawater temperature reached 25–30°C. However, blades did not disappear completely since some individuals, 10–30 cm long, were still present. The second peak of development started in late August or September when water temperature decreased, and continued through to December when the seawater temperature declined to 10°C. In autumn, *Grateloupia* populations were dominant due to the absence of *Undaria pinnatifida* (Harvey) Suringar and *Sargassum muticum* (Yendo) Fensholt, which had seasonal developments in spring (Sfriso et al. 2009).

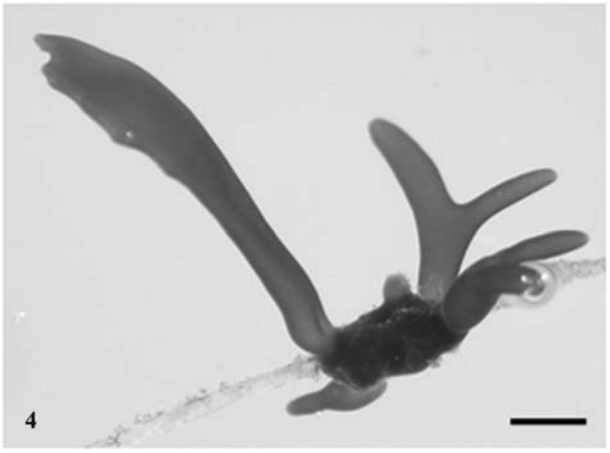


Figure 4 *Grateloupia turuturu*: (Mar Piccolo) cylindrical uprights sprouting from an encrusting base.
Scale bar: 5 mm.

Grateloupia thalli were also present in winter, especially when water temperature was ca. 5–6°C, as in the historical centre of Venice or along the sea coastline and near the lagoon mouths. At lower temperatures and in the inner areas of the lagoon where seawater can freeze, blades disappeared or were very rare, as happened during winter 2009–2010, when we found only encrusting bases.

Discussion

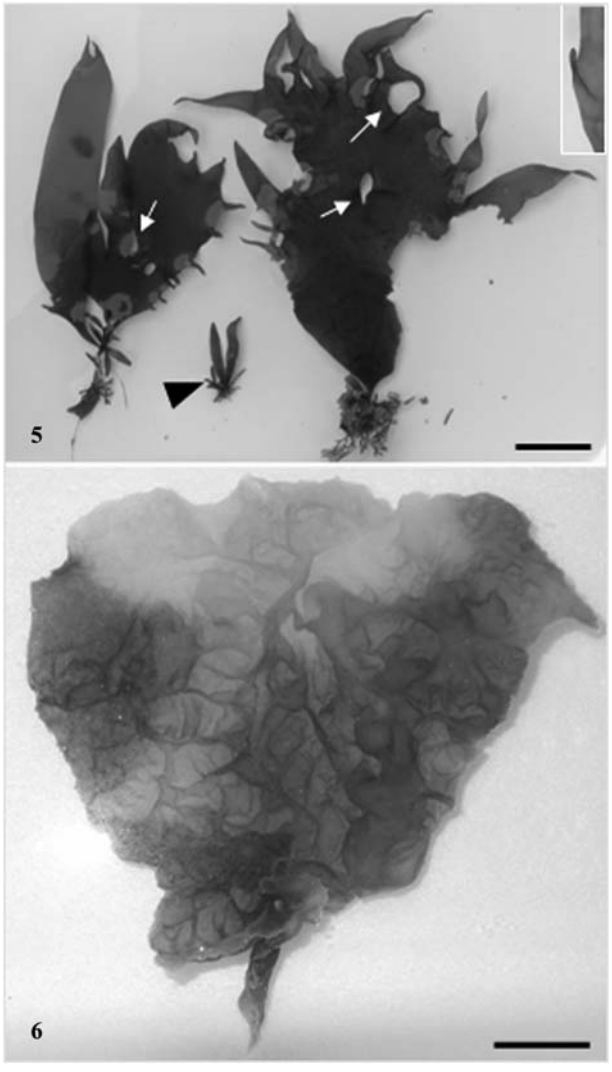
Identity of specimens

Molecular studies and careful morphological observations carried out on specimens collected in France (Thau Lagoon) and North America have shown that the introduced species, previously reported as *Grateloupia doryphora*, belongs to *G. turuturu* or *G. lanceolata* (Verlaque 2001, Gavio and Fredericq 2002, Verlaque et al. 2005). In Italy, molecular and morphological analyses confirmed that foliose *Grateloupia* specimens found in the Mar Piccolo of Taranto and the Lagoon of Venice, including those assigned to *G. doryphora* by Tolomio (1993), also belong to the Japanese *G. turuturu*. Among morphological characteristics, the thickness of blades, abrupt transition between cortex and medulla, anticlinal arrangement of medullary filaments in young blades, and spur-like innermost cortical cells allow easy identification.

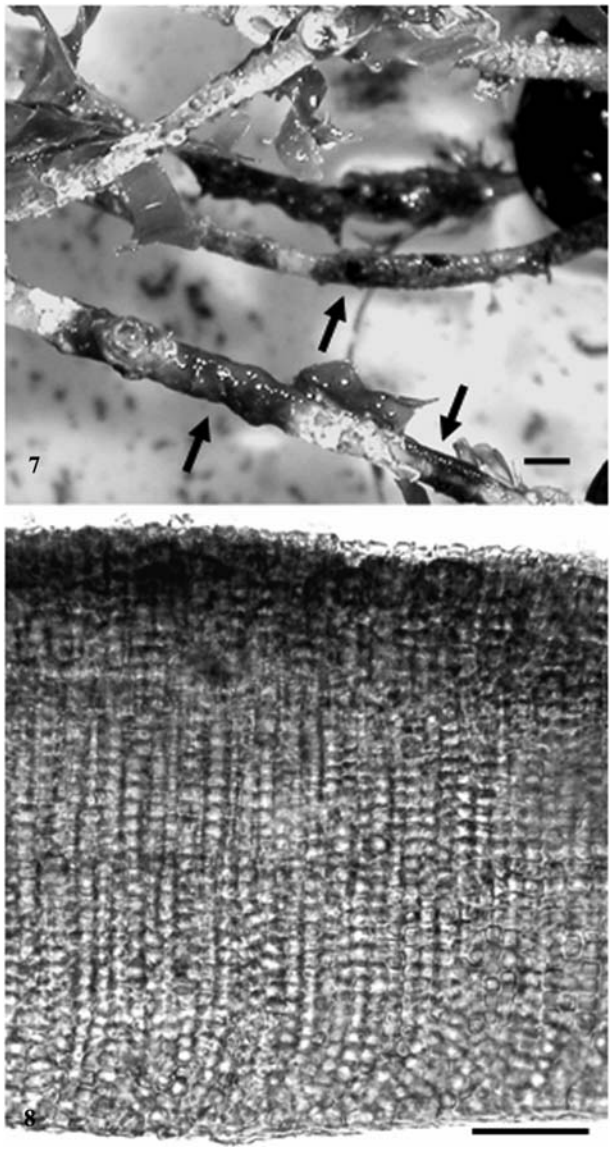
Seasonal cycle of development

Grateloupia turuturu thalli in both the Lagoon of Venice and the Mar Piccolo of Taranto are markedly variable in gross morphology, even within the same population. Such extensive plasticity has already been reported for specimens from other localities throughout the world (Ben Maiz et al. 1986 as *G. doryphora*, Cabioch et al. 1997 as *G. doryphora*, Villalard-Bohnsack and Harlin 1997 as *G. doryphora*, Bárbara and Cremades 2004, D'Archino et al. 2007).

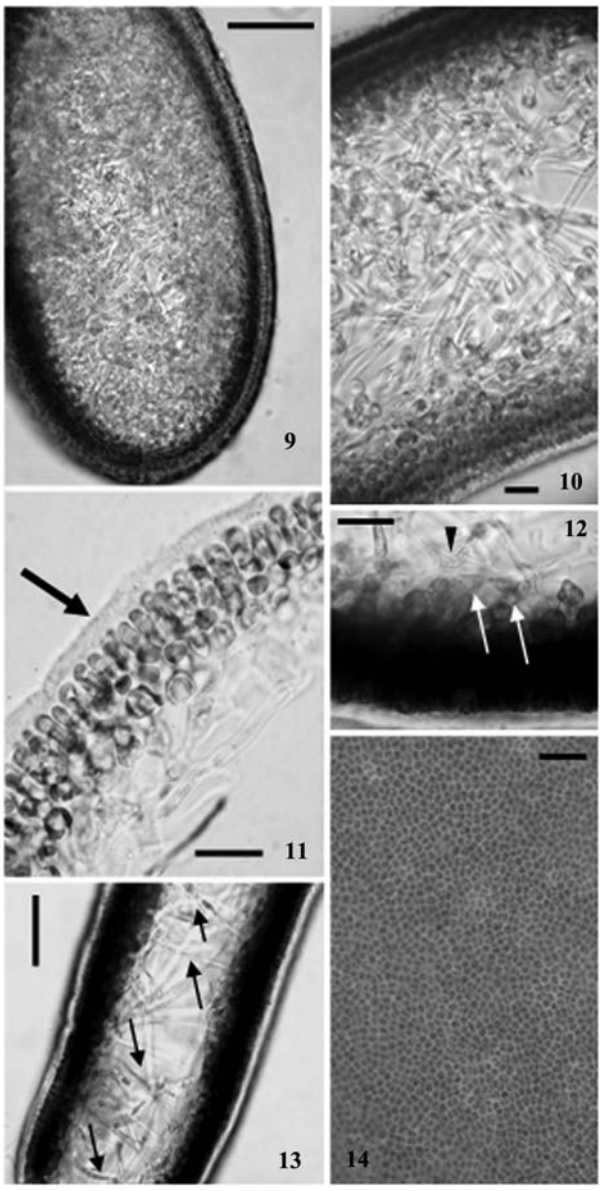
In France, encrusting bases were first described in 1997 (Cabioch et al. 1997). In Rhode Island (USA), these bases are derived from the development of carpospores (Harlin and Villalard-Bohnsack 2001). Similarly, carpospores and tetraspores of *Grateloupia acuminata* Holmes were observed developing into encrusting bases from which uprights arose (Iima et al. 1995). An encrusting base was also observed in *G. minima* P. Crouan *et* H. Crouan (Cabioch and Giraud 1982 as *G. filicina* var. *minima*, De Clerck et al. 2005) and in *G. asiatica* Kawaguchi *et* Wang [Migita (1988) and Chiang (1993) as *G. filicina* (Wulfen) C. Agardh]. Therefore, an encrusting base, which seems to be a frequent feature in the genus *Grateloupia*, might behave as a resting structure for perennial species. In 1982, Cabioch and Giraud have already hypothesised that the *Hildenbrandia* type structure of the



Figures 5–6 *Grateloupia turuturu*.
(5) Specimens with lanceolate (arrow head) and irregularly foliose habit with perforations (arrows) and proliferations (inset image) (Lagoon of Venice). Scale bar: 4 cm. (6) Thallus containing gas bubbles (Mar Piccolo). Scale bar: 2 cm.



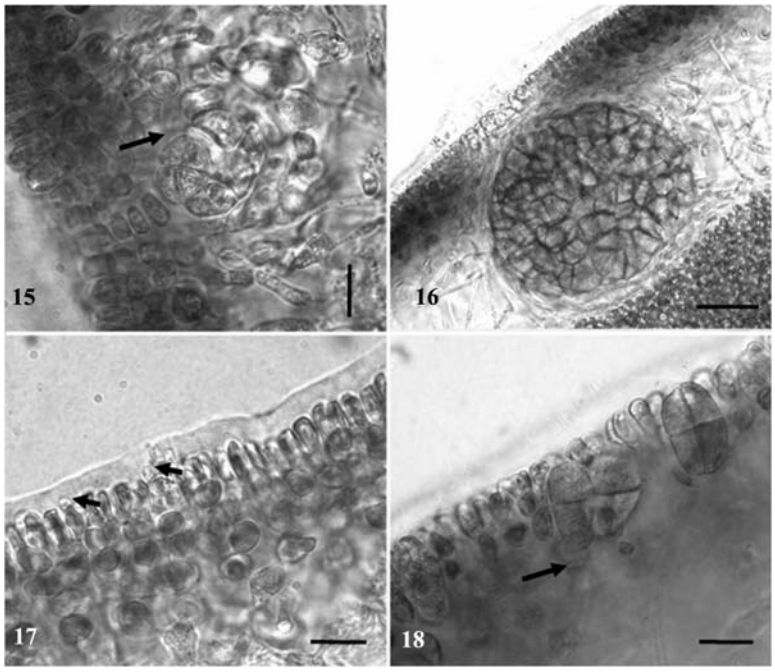
Figures 7–8 *Grateloupia turuturu* (Mar Piccolo).
(7) Encrusting bases (arrows) wrapped around a net used in mussel farming. Scale bar: 1 mm. (8) Radial vertical section of a thick base. Scale bar: 50 μm.



Figures 9–14 *Grateloupia turuturu*.
(9) Transverse section of the stipe of a young blade (Mar Piccolo). Scale bar: 10 μm. (10) Longitudinal section of a mature blade showing abrupt transition between medulla and cortex (Lagoon of Venice). Scale bar: 25 μm. (11) Transverse section of a mature blade showing shape and arrangement of outer cortical cells and the thick mucilage coat (arrow) (Lagoon of Venice). Scale bar: 15 μm. (12) Transverse section of a mature blade showing cortical innermost spur-like cells (arrows) and stellate medullary cells (arrowhead) (Mar Piccolo). Scale bar: 25 μm. (13) Longitudinal section of a young blade showing the anticlinal arrangement of medullary filaments (arrows) (Mar Piccolo). Scale bar: 50 μm. (14) Surface view of cortical cells showing a mosaic arrangement (Mar Piccolo). Scale bar: 25 μm.

encrusting base of *G. filicina* var. *minima* might be a resting form able to overcome adverse environmental conditions. This hypothesis is consistent with the differences in seasonal dynamics among *G. turuturu* populations in different localities of the world.

On Brittany coasts, *G. turuturu* populations have seasonal dynamics similar to that observed in the Lagoon of Venice; in both populations, fertile blades, though rare, were still collected in September (Cabioch et al. 1997). Along the northern coasts of the USA (i.e., Narragansett Bay), *G. turuturu* populations have a clear seasonal pattern. In winter and late spring (from March to June), the percent cover of blades is reduced down to zero in some localities (the coldest) and increases in late summer–autumn (September–October). Since *G. turuturu* survived winter adverse conditions as encrusting bases, these were considered as an overwintering form and the species was classified as pseudoperennial (Harlin and Villalard-Bohnsack 2001).



Figures 15–18 *Grateloupia turuturu*.
(15) Transverse section through a young cystocarp (arrow) (Lagoon of Venice). Scale bar: 10 μm. (16) Transverse section through a mature cystocarp (Lagoon of Venice). Scale bar: 35 μm. (17) Transverse section of cortex with spermatangia (arrows) (Mar Piccolo). Scale bar: 15 μm. (18) Transverse section of cortex with cruciately divided tetrasporangia. Arrow: synapse between the inner cortical cell and the tetrasporangium (Mar Piccolo). Scale bar: 20 μm.

In the Mar Piccolo of Taranto, only encrusting bases of *Grateloupia turuturu* are present throughout summer. Therefore, in this locality in the central Mediterranean Sea, basal crusts are oversummering structures and *G. turuturu* behaves *sensu* Feldmann (1951, 1966), or deciduiphyke in the classification of Chapman and Chapman (1976). In contrast, Venice plants of *G. turuturu* may behave either as hemicryptophytes or phanerophytes *sensu* Feldmann (1951, 1966) [''deciduiphyke'' and ''mesophyke'' *sensu* Chapman and Chapman (1976), respectively] depending on environmental conditions, especially temperature. Indeed, in the Lagoon of Venice, blades are generally present year-round with two seasonal peak of development (spring and autumn), except in the areas where the seawater temperature is lower than 5°C, where only crusts remain.

Therefore, encrusting bases allow *Grateloupia turuturu* to pass the unfavourable season when temperatures are either too high or too low to allow persistence of the blades, especially in intertidal and upper sublittoral zones, where this species usually lives. This particular feature illustrates the plasticity of development in the species.

In the light of these considerations, the differences in thickness observed among encrusting bases certainly reflect the ages of individuals, thinner crusts being those immediately derived from spore development.

Ecological considerations

In both the Mar Piccolo of Taranto and the Lagoon of Venice, *Grateloupia turuturu* specimens occur in eutrophic waters characterized by a wide range of both salinity and temperature, as previously reported for other localities into which it has been introduced (Riouall et al. 1985 as *G. doryphora*, Simon et al. 1999 and Simon et al. 2001 as *G. doryphora*). In both localities, specimens settled in the intertidal and upper sublittoral environments.

Vectors of introduction

Sixteen of 38 alien seaweed species (i.e., 42%) reported for the Apulian coasts are present in the Mar Piccolo of Taranto, often as first reports for the Mediterranean Sea (Gravili et al. 2010); they have commonly been observed for the first time close to the dock or just settled on the dock where there are fish shops and mollusc import-export enterprises (Cecere et al. 2000, Cecere and Petrocelli 2009).

Twenty alien macroalgal species have been introduced into the Lagoon of Venice since 1983 (Sfriso et al. 2009). Most were first recorded near the fish markets of Chioggia and Venice, where fresh molluscs are imported (Curiel and Marzocchi 2010).

In both coastal lagoons, alien macroalgae have probably been introduced as microscopic life stages or resting forms settled either on shellfish or on packing material (nets, boxes). Alien seaweeds can also be used themselves as packing material, as shown in Taranto by the discovery, in summer 2009, of long floating fragments of *Ascophyllum nodosum* (Linnaeus) Le Jolis (Ochrophyta, Fucales) near a mussel culture farm (Petrocelli and Cecere 2010).

Probability of invasion

Grateloupia turuturu proved to be an invasive species along the coasts of the NW and NE Atlantic (Villalard-Bohnsack and Harlin 1997, Harlin and Villalard-Bohnsack 2001, Simon et al. 2001, Bárbara and Cremades 2004). In the Lagoon of Venice, *Sargassum muticum*, *Undaria pinnatifida*, *Scytosiphon dotyi* M.J. Wynne, *Polysiphonia morrowii* Harvey, *Antithamnion hubbsii* Dawson and *Ulva fasciata* Delile are seasonally invasive, whereas others such as *Heterosiphonia japonica* Yendo, *Cladosiphon zosterae* (J. Agardh) Kylin have spread only locally. *Grateloupia turuturu* is very frequent but it never reaches abundant biomasses. Therefore, its development seems to have minor impact on native species. This species does not show either increasing or shrinking trends but has been stable for some years since introduction. Though it has been successfully established for the past 25 years and has developed reproductive populations, it has not become invasive.

In the Mar Piccolo of Taranto, although its population is reproductive and has spread slightly along the dock where it was first found, *Grateloupia turuturu* does not yet have the features of an invasive species (i.e., ecologically and/or economically harmful species). However, *G. turuturu* has been present for only three years. The spreading and the phenology of its population will be monitored in future in order to ascertain whether the species will remain established and persistent (Valentine et al. 2007) or, on the contrary, will disappear as in the cases of *U. pinnatifida* and *C. fragile* (Gravili et al. 2010).

Acknowledgements

The authors acknowledge G. Portacci for his help during sampling in the Mar Piccolo of Taranto, the support of the National Research Project V.E.C.T.O.R. (VulnErabilità delle Coste e degli ecosistemi marini italiani ai cambiamenti climaTici e loro ruolO nei cicli del caRbonio mediterraneo; web site: http://vector.conismamibi.it.) and Dr. C. Tolomio who kindly gave us his specimen of *Grateloupia* of the Lagoon of Venice.

References

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol. 215*: 403–410.

Bárbara, I. and J. Cremades. 2004. *Grateloupia lanceola* versus *Grateloupia turuturu* (Gigartinales, Rhodophyta) en la Península Iberíca. *Anales del Jardin Botánico de Madrid 61*: 103–118.

Ben Maiz, N., Ch.F. Boudouresque and M. Gerbal. 1986. Flore algale de l'étang de Thau: *Grateloupia doryphora* (Montagne) Howe et *G. filicina* (Wulfen) C. Agardh. *Thalassographica 9*: 39–49.

Cabioch, J. and G. Giraud. 1982. La structure hildenbrandioïde, stratégie adaptative chez les Floridées. *Phycologia 21*: 307–315.

Cabioch, J., A. Castric-Fey, M.T. L'Hardy-Halos and A. Rio. 1997. *Grateloupia doryphora* et *Grateloupia filicina* var. *luxurians* (Rhodophyta, Halymeniaceae) sur le côtes de Bretagne (France). *Cryptogamie, Algol. 18*: 117–137.

Cecere, E. and A. Petrocelli. 2007. Prima segnalazione di *Grateloupia turuturu* nel Mar Piccolo di Taranto. *In*: (C. Micheli, ed.) *Abstract book Riunione Annuale del Gruppo per l'Algologia*. ENEA Casaccia, Roma. p. 43.

Cecere, E. and A. Petrocelli. 2009. The Mar Piccolo of Taranto. *In*: (E. Cecere, A. Petrocelli, G. Izzo and A. Sfriso, eds) *Flora and vegetation of the Italian transitional water systems*. CORILA, Multigraf Spinea, Venezia. pp. 195–227.

Cecere, E., A. Petrocelli and O.D. Saracino. 2000. *Undaria pinnatifida* (Fucophyceae, Laminariales) spread in the central Mediterranean: its occurrence in the Mar Piccolo of Taranto (Ionian Sea, southern Italy). *Cryptogamie, Algol. 21*: 305–309.

Chapman, V.J. and D.J. Chapman. 1976. Life forms in the algae. *Bot. Mar. 19*: 65–74.

Chiang, Y.M. 1993. The developmental sequence of the marine red alga *Grateloupia filicina* in culture. *Korean J. Phycol. 8*: 231–237.

Curiel, D. and M. Marzocchi. 2010. Stato delle conoscenze nella laguna di Venezia di due alien species: *Undaria pinnatifida* e *Sargassum muticum*. *Lavori Soc. Ven. Sc. Nat. 35*: 93–105.

D'Archino, R., W.A. Nelson and G.C. Zuccarello. 2007. Invasive marine red alga introduced to New Zealand waters: first record of *Grateloupia turuturu* (Halymeniaceae, Rhodophyta). *NZ J. Mar. Freshw. Res. 41*: 35–42.

De Clerck, O., B. Gavio, S. Fredericq, I. Bárbara and E. Coppejans. 2005. Systematics of *Grateloupia filicina* (Halymeniaceae, Rhodophyta), based on *rbc*L sequence analyses and morphological evidence, including the reinstatement of *G. minima* and the description of *G. capensis* spec. nov. *J. Phycol. 41*: 391–410.

De Masi, F. and G.M. Gargiulo. 1982. *Grateloupia doryphora* (Mont.) Howe (Rhodophyta, Cryptonemiales) en Méditerranée. *Allionia 25*: 105–108.

Elliot, M. 2003. Biological pollutants and biological pollution – an increasing cause for concern. *Mar. Poll. Bull. 46*: 275–280.

Feldmann, J. 1951. Ecology of marine algae. *In*: (G.M. Smith, ed.) *Manual of phycology – an introduction to the algae and their biology*. Chronica Botanica Company, Waltham, MA. pp. 313–334.

Feldmann, J. 1966. Les types biologiques des cryptogames non-vasculaires. *B. Soc. Bot. Fr.-Mém. 1965*: 45–60.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evolution 39*: 783–791.

Freshwater, D.W. and J. Rueness. 1994. Phylogenetic relationships of some European *Gelidium* (Gelidiales, Rhodophyta) species, based on *rbc*L nucleotide sequence analysis. *Phycologia 33*: 187–194.

Gavio, B. and S. Fredericq. 2002. *Grateloupia turuturu* (Halymeniaceae, Rhodophyta) is the correct name of the non-native species in the Atlantic known as *Grateloupia doryphora*. *Eur. J. Phycol. 37*: 349–359.

Gravili, C., G. Belmonte, E. Cecere, F. Denitto, A. Giangrande, P. Guidetti, C. Longo, F. Mastrototaro, S. Moscatello, A. Petrocelli, S. Piraino, A. Terlizzi and F. Boero. 2010. Non-indigenous species (NIS) along the Apulian coast, Italy. *Chem. Ecol. 26*: 121–142.

Guindon, S. and O. Gascuel. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol. 52*: 696–704.

Harlin, M.M. and M. Villalard-Bohnsack. 2001. Seasonal dynamics and recruitment strategies of the invasive seaweed *Grateloupia doryphora* (Halymeniaceae, Rhodophyta) in Narragansett Bay and Rhode Island Sound, Rhode Island, USA. *Phycologia 40*: 468–474.

Iima, M., T. Kinoshita, S. Kawaguchi and S. Migita. 1995. Cultivation of *Grateloupia acuminata* (Halymeniaceae, Rhodophyta) by regeneration from cut fragments of basal crusts and upright thalli. *J. Appl. Phycol. 7*: 583–588.

Lanave, C., G. Preparata, C. Saccone and G. Serio. 1984. A new method for calculating evolutionary substitution rates. *J. Mol. Evol*. *20*: 86–93.

Marston, M. and M. Villalard-Bohnsack. 2002. Genetic variability and potential sources of *Grateloupia doryphora* (Halymeniaceae, Rhodophyta), an invasive species in Rhode Island waters (USA). *J. Phycol. 38*: 649–658.

Mathieson, A.C., C.J. Dawes, J. Pederson, R.A. Gladych and J.T. Carlton. 2008. The Asian red seaweed *Grateloupia turuturu* (Rhodophyta) invades the Gulf of Maine. *Biol. Invasions 10*: 985–988.

Mc Lusky, D.S. and M. Elliot. 2007. Transitional waters: a new approach, semantics or just muddying the waters? *Estuar. Coast. Shelf Sci. 71*: 359–363.

Migita, S. 1988. Cultivation of *Grateloupia filicina* (Rhodophyta, Cryptonemiales) by regeneration of crusts. *Nippon Suisan Gakk. 54*: 1923–1927 (in Japanese).

Minchin, D. 2007. Aquaculture and transport in a changing environment: overlap and links in the spread of alien biota. *Mar. Poll. Bull. 55*: 302–313.

Olenin, S., D. Minchin and D. Daunys. 2007. Assessment of biopollution in aquatic ecosystems. *Mar. Poll. Bull. 55*: 379–394.

Pavoni, B., A. Sfriso, R. Donazzolo and A.A. Orio. 1990. Influence of waste waters from the city of Venice and the hinterland on the eutrophication of the Lagoon. *Sci. Tot. Environ. 96*: 235–252.

Petrocelli, A. and E. Cecere. 2010. Biodiversity and mollusk transfer: need of observance of the laws to avoid alien seaweeds introduction. *Biol. Mar. Medit. 17*: 175–176.

Riouall, R., M.D. Guiry and L. Codomier. 1985. Introduction d'une espèce foliacée de *Grateloupia* dans la flore marine de l'étang de Thau (Hérault, France). *Cryptogamie, Algol. 6*: 1–98.

Sfriso, A., C. Facca and P.F. Ghetti. 2006. Utilizzo delle macroalghe e di variabili ecologiche per la valutazione della qualità ambientale degli ambienti marini di transizione. *Biol. Mar. Medit. 13*: 434–445.

Sfriso, A., D. Curiel and A. Rismondo. 2009. The Lagoon of Venice. *In*: (E. Cecere, A. Petrocelli, G. Izzo and A. Sfriso, eds) *Flora and vegetation of the Italian transitional water systems*. CORILA, Multigraf Spinea, Venezia. pp. 195–227.

Simon, C., E. Ar Gall, G. Levavasseur and E. Deslandes. 1999. Effects of short-term variations of salinity and temperature on the photosynthetic response of the red alga *Grateloupia doryphora* from Brittany (France). *Bot. Mar. 42*: 437–440.

Simon, C., E. Ar Gall and E. Deslandes. 2001. Expansion of the red alga *Grateloupia doryphora* along the coasts of Brittany (France). *Hydrobiologia 443*: 23–29.

Thompson, J.D., D.G. Higgins and T.J. Gibson. 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res. 22*: 4673–4680.

Tolomio, C. 1993. Prima segnalazione di *Grateloupia doryphora* (Mont.) Howe (Rhodophyceae) nella Laguna di Venezia. *Lav. Soc. Ven. Sc. Nat. 18*: 215–220.

Valentine, J.P., R.H. Magierowski and C.R. Johnson. 2007. Mechanisms of invasion: establishment, spread and persistence of introduced seaweed populations. *Bot. Mar. 50*: 351–360.

Verlaque, M. 2001. Check-list of the macroalgae of Thau lagoon (Hérault, France): a hot spot of marine species introduction in Europe. *Oceanol. Acta 24*: 29–49.

Verlaque, M., P.M. Brannock, T. Komatsu, M. Villalard-Bohnsack and M. Marston. 2005. The genus *Grateloupia* C. Agardh (Halymeniaceae, Rhodophyta) in the Thau Lagoon (France, Mediterranean): a case study of marine plurispecific introductions. *Phycologia 44*: 477–496.

Verlaque, M., C.F. Boudouresque and F. Mineur. 2007. Oyster transfers as a vector for marine species introductions: a realistic approach based on the macrophytes. *CIESM Workshop Monographs, Monaco 32*: 39–48.

Villalard-Bohnsack, M. and M. Harlin. 1997. The appearance of *Grateloupia doryphora* (Halymeniaceae, Rhodophyta) on the northeast coast of North America. *Phycologia 36*: 324–328.

Wilkes, R.J., M. Morabito and G.M. Gargiulo. 2006. Taxonomic consideration of a foliose *Grateloupia* species from the Strait of Messina. *J. Appl. Phycol. 18*: 663–669.

Zuccarello, G.C., G. Burger, J.A. West and R.J. King. 1999. A mitochondrial marker for red algal intraspecific relationships. *Mol. Ecol. 8*: 1443–1447.

Received 2 July, 2010; accepted 16 September, 2010

Biological Invasions

Spreading of the invasive macroalga Gracilaria vermiculophylla (Gracilariales, Rhodophyta) in the North Adriatic Sea (Mediterranean Sea, Italy)

--Manuscript Draft--

Manuscript Number:	
Full Title:	Spreading of the invasive macroalga Gracilaria vermiculophylla (Gracilariales, Rhodophyta) in the North Adriatic Sea (Mediterranean Sea, Italy)
Article Type:	Research paper
Keywords:	Gracilaria vermiculophylla; invasive alien seaweeds; Mediterranean Sea; Po Delta; Venice Lagoon
Corresponding Author:	Isabella Moro, Ph.D. University of Padova Padova, ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Padova
Corresponding Author's Secondary Institution:	
First Author:	Katia Sciuto, Ph.D.
First Author Secondary Information:	
Order of Authors:	Katia Sciuto, Ph.D.
	Marion Adelheid Wolf, Ph.D.
	Adriano Sfriso, Prof.
	Carlo Andreoli, Prof.
	Silvia Maistro, Ph.D.
	Isabella Moro, Ph.D.
Order of Authors Secondary Information:	
Abstract:	The invasive alien species Gracilaria vermiculophylla (Ohmi) Papenfuss, after the first record noticed by molecular analyses in the Po Delta lagoons (Adriatic Sea) in May 2008, was also found in the Venice Lagoon (Veneto) in March 2009 and in 'Sacca di Goro' and 'Pialassa della Baiona' (Emilia-Romagna) in May 2009. This species shows high growth rates, forming abundant floating thalli with biomasses up to 15 kg fwt m-2 that cover completely bottoms, and overcomes the growth of the local species, including laminar Ulvaceae. G. vermiculophylla was recorded in the northern coasts of the Atlantic Sea only in the 2000s and it is rapidly colonizing many European States including the Italian regions of the North Adriatic Sea. The most probable introduction vector for Europe has been oyster farms that imported these bivalves from the Indo-Pacific area. The present study supplies further information on the spreading of this alien species in the North Adriatic transitional systems and on the most probable introduction and diffusion vectors.
Suggested Reviewers:	M. Antonia Ribera Siguan riberasiguan@ub.edu
	Marina Morabito morabitom@unime.it
	Amelia Gómez Garreta ameliagomez@ub.edu

Katia Sciuto[1§], Marion A. Wolf [1§], Adriano Sfriso[2], Carlo Andreoli[1], Silvia Maistro[1], Isabella Moro[1*]

Spreading of the invasive macroalga *Gracilaria vermiculophylla* (Gracilariales, Rhodophyta) in the North Adriatic Sea (Mediterranean Sea, Italy)

1) Department of Biology, University of Padova Via Ugo Bassi 58b, I-35131 Padova, Italy

2) Department of Environmental Sciences, Informatics and Statistics, University of Venice, Calle Larga 2137, 30123-Venice, Italy

[§]These authors contributed equally to this work

*Corresponding author: e-mail isabella.moro@unipd.it

Abstract The invasive alien species *Gracilaria vermiculophylla* (Ohmi) Papenfuss, after the first record noticed by molecular analyses in the Po Delta lagoons (Adriatic Sea) in May 2008, was also found in the Venice Lagoon (Veneto) in March 2009 and in 'Sacca di Goro' and 'Pialassa della Baiona' (Emilia-Romagna) in May 2009. This species shows high growth rates, forming abundant floating thalli with biomasses up to 15 kg fwt m^{-2} that cover completely bottoms, and overcomes the growth of the local species, including laminar Ulvaceae.

G. vermiculophylla was recorded in the northern coasts of the Atlantic Sea only in the 2000s and it is rapidly colonizing many European States including the Italian regions of the North Adriatic Sea. The most probable introduction vector for Europe has been oyster farms that imported these bivalves from the Indo-Pacific area.

The present study supplies further information on the spreading of this alien species in the North Adriatic transitional systems and on the most probable introduction and diffusion vectors.

Keywords : *Gracilaria vermiculophylla*, invasive alien seaweeds, Mediterranean Sea, Po Delta, Venice Lagoon.

Running title: *G. vermiculophylla* spreading in the North Adriatic Sea

Introduction

The Mediterranean Sea is one of the marine aquatic environments most affected by biological invasions of allochthonous species (Zenetos et al. 2010) mainly coming from the Indo-Pacific area (Boudouresque and Verlaque 2002; Cormaci et al. 2004; Occhipinti-Ambrogi 2002; Verlaque 1994). Most of these alien species have been introduced via the Straits of Suez or the Straits of Gibraltar. Moreover, such invasions were supported by the increase of touristic and commercial shipping traffic with extra-Mediterranean countries, the import of fish products and aquaculture activities (Sfriso and Curiel 2007). In particular, touristic and commercial shipping traffic concern the Venice Lagoon with an increasing trend. In 2006, over 5800 ships reached the ports of Venice and Chioggia (http://it.wikipedia.org/wiki/Porto_di_Venezia) and most of them unballasted in the port areas. Moreover, in the lagoons of the Northern Adriatic Sea ca. 6000 ha are used for clam-farming with a production estimated in ca.55,000 tonnes in 2007 (Turolla et al. 2008). In the same basins there are several decontamination plants, important fishing harbours and fish wholesale markets such as: Chioggia and Venice in the Venice Lagoon and Pila and Goro in the Po Delta. In 2003 the markets of Chioggia and Venice had a business volume of ca. 42 and 55 million Euros, respectively, which

represented ca. the 61% of the whole North Adriatic activity. Most of the sold products came from extra-Mediterranean countries. If appropriate precautions are not taken, the touristic-commercial traffic, the transfer of the products at different age-stages and the seed recruitment will greatly favour the alien species introduction and spreading. Due to such activities, every year new species are recorded, especially in the Venice Lagoon, the most studied Mediterranean environment (Mizzan 1995; Occhipinti-Ambrogi 2000), and most of these are macroalgae (Curiel et al., 1996a, b, 1998, 2001, 2002, 2003, 2004, 2005; Bellemo et al. 1999, 2001; Sfriso 1987, 2006, 2007, 2009; Sfriso and La Rocca 2005; Sfriso and Curiel 2007; Tolomio 1993).

Climate changes, triggering water temperature increase, favour the spreading and colonization of alien species, i.e. non-indigenous species (NIS), with a rhythm never observed before. The check-list of NIS in the Mediterranean Sea was updated to December 2010 (Zenetos et al. 2010). On the whole, a list of 955 taxa was produced and out of these 125 are seaweed taxa. In the Adriatic Sea alien macrophytes are 49, ca. 39% of the total (Zenetos et al. 2010), and 33 are present in the Lagoon of Venice.

The species considered invasive or potentially invasive are six: i.e. *Codium fragile* subsp. *fragile* (Suringar) Hariot, *Gracilaria vermiculophylla* (Ohmi) Papenfuss, *Grateloupia turuturu* Yamada, *Heterosiphonia japonica* Yendo, *Sargassum muticum* (Yendo) Fensholt and *Undaria pinnatifida* (Harvey) Suringar.

S. muticum and *U. pinnatifida* are the most abundant and diffuse species that colonize the hard substrata of the historical centre of Venice, Chioggia and many islands with people concern. However they are cold attached algae, which are not able to grow in a floating form and, in summer, disappear with the temperature increase.

Codium, *Grateloupia* and *Heterosiphonia* are also attached taxa, present in many areas of the Lagoon, but with a negligible biomass.

Gracilaria vermiculophylla, a species native from Japan, is the most recent introduction. Literature shows that it usually grows mainly from spring to autumn in the floating form, behaving invasive and reaching high biomasses. It is a potentially dangerous species that, in eutrophic and turbid environments, can grow very rapidly replacing the other species including Ulvaceae taxa. In the Mediterranean Sea it was recorded for the first time in May 2008 in some lagoons of the Po Delta (Sfriso et al. 2010), but at the time it was not very abundant and diffuse. No records in others lagoons were reported.

This paper reports the introduction of this species also in other Adriatic transitional systems. In particular, *G. vermiculophylla* was recorded in the Venice Lagoon (Veneto) and in 'Sacca di Goro' and 'Pialassa della Baiona' (Emilia-Romagna). Here detailed distribution maps are produced.

For a correct identification of the specimens molecular analyses were also carried out. The sequences of the plastid *rbc*L gene of the specimens collected in Emilia-Romagna and in the Venice Lagoon were compared with those available in GenBank and the ones of thalli previously sampled in the Po Delta (Sfriso et al. 2010). Moreover, to understand the possible provenance area of this species, the more variable mitochondrial *cox*2-3 intergenic spacer region, useful for population studies, was also analyzed.

Materials and Methods

Sampling

Samples were collected both in Emilia-Romagna transitional systems ('Pialassa della Baiona' and 'Sacca di Goro') in May-October 2009 and in the Venice Lagoon in May-October 2010 and 2011 (Fig. 1), during some campaigns carried out to evaluate the ecological state of these environments by the study of macrophytes (macroalgae and

angiosperms according to the Water Framework Directive (2000/60/EC)). Other samples were collected monthly during one year (March 2009-February 2010) in some areas of the Lagoon of Venice during a survey to study the submerged flora and vegetation in confined areas.

Macroalgal biomass was obtained according to the procedure by Sfriso et al. (1991) set up to determine the pleustophytic biomasses of soft substrates in transitional systems. Macroalgae were collected in a sampling area of 15x15 m² by using an aluminium box of 0.5 m² (base: 71 x 71 cm, height: 70 cm). The inside biomass collected by a rake was drained, fresh weighed and the final results was the mean of 3-6 replicates. The accuracy ranged from 5 to 10% depending on the biomass amount and increasing with the biomass itself.

Some samples were preserved in 4% formaldehyde up to laboratory identification by means of a stereo zoom microscope E-1654ZT45 (Euromex microscopes, Holland) and a light microscope-353Ph (Optika microscopes, Italy). Other samples were also freeze dried for the molecular analyses.

Study areas (Fig. 1)

All the spreading areas of *G. vermiculophylla*, with exception of the Marano-Grado Lagoon (Falace et al., 2009), are located in the north-western transitional systems of the Italian coasts: Venice Lagoon, Po Delta and Pialassa della Baiona.

The Venice Lagoon (45°34'-45°12'N; 12°09'-12°36'E) with a total surface of ca. 549 km² is the largest lagoon of the Mediterranean Sea. The mean depth is ca 1m and ca. 60% of the water is exchanged with the sea at any tidal cycle (12 hrs). It is subdivided in many sub-basins and in high renewal and confined areas characterized by very different morphological and physico-chemical characteristics that allow the growth

of a polymorphic vegetation and the presence of more than 300 macrophytes (Sfriso et al., 2009), out of these 33 are alien species (Zenetos et al., 2010).

The Po Delta lagoons (45°08'-44°48'N; 12°16'-12°34'E) are a mosaic of small basins that cover ca. 100 km². This value doubles by considering fishing ponds. Some of these are very shallow and exhibit a mean depth <1 m (Caleri, Barbamarco, Canarin, Marinetta, Vallona,), others are 2-3 m deep (Goro, Scardovari). The water exchange is high but waters are eutrophic and very turbid. The vegetation is characterized by the dominance of Ulvaceae, Gracilariaceae and Solieriaceae, whereas other taxa are scarce.

Pialassa della Baiona (44°31'-44°28'N; 12°14'-12°16'E) is a semi-closed basin of ca. 10 km² and 0.6-1.4 m deep located near the port of Ravenna. Nutrient amounts are high and waters are clear. The vegetation is dominated by Ulvaceae and Gracilariaceae and the number of taxa is poor.

Physico-chemical parameters were measured according to Facca and Sfriso (2007).

Molecular analyses

In order to precisely identify the collected specimens the DNA barcoding approach was adopted, using the first part of the *rbc*L gene (about 700 bp) and the mitochondrial *cox*2-3 intergenic spacer region as DNA barcodes.

DNA extraction was carried out using the Genomic DNA purification kit (Fermentas©). The *rbc*L gene fragment was amplified using the primer pair F7 and R753 (Freshwater and Rueness 1994; Gavio and Fredericq 2002). The *cox*2-3 intergenic spacer region was obtained as reported in Zuccarello et al. (1999).

Positive PCR products were cleaned by using the ExoSAP-ITTM kit (Amersham Biosciences) or, in case of multiple bands, purified with the DNA Gel extraction kit (Millipore©). The sequencings were performed at the BMR Genomics Sequencing Service (Padova University) on automated ABI DNA sequencers, with the same primer

pairs used in the amplification reactions. Final consensus sequences were assembled using the SeqMan II program from the Lasergene software package (DNAStar©, Madison, WI) and then manually refined by eye.

Identity of new sequences was checked by using the BLAST program (Altschul et al. 1990), available at the USA National Center for Biotechnology Information (NCBI) web server (http://www.ncbi.nlm.nih.gov), and the sequence alignments were obtained by the ClustalW computer program (Thompson et al. 1994).

The *rbc*L and *cox*2-3 spacer barcode fragments of the *G. vermiculophylla* isolates collected in Emilia-Romagna and in the Venice Lagoon were deposited in DDBJ/GenBankTM/EBI Data Bank with the following accession numbers: HE652864, HE652865 (*rbc*L) and HE653918, HE653919 (*cox*2-3 spacer).

To better infer the provenance of our isolates, a dataset with almost complete sequences of the *rbc*L gene (>1200 bp) was constructed and used for a phylogenetic analysis. It included the *rbc*L gene sequence previously obtained from the Po Delta isolate (Sfriso et al. 2010) and other published sequences of *G. vermiculophylla* sampled from different geographic areas. The phylogenetic analysis was performed according to the Neighbor-Joining method (Saitou and Nei 1987) with the program MEGA5 (Tamura et al. 2011) and the evolutionary distances, given in the units of the number of base substitutions per site, were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). The analysis involved 31 nucleotide sequences for a total of 1278 aligned positions. A bootstrap consensus tree (Felsenstein 1985), inferred from 1000 replicates, was taken to represent the evolutionary relationships among the isolates.

Results

Morphology, habitat, and biomass estimation

Gracilaria vermiculophylla thallus filaments, brownish to a greyish wine-red in colour, are from cylindrical to feebly compressed, wrinkled and very long: 0.5-1.5 m (Fig. 2). In the old parts they are thick and long, ploughed by longitudinal grooves. Thalli present 1-2 percurrent axes of 1.5-3 mm in diameter, often larger in the central part, which arise from a small basal disk. Branching is scarce in reproductive thalli and very abundant and highly irregular in vegetative samples. *G. vermiculophylla* thalli occur in the loose-lying form on mud or fine sand, or more rarely they are attached to mollusc shells or stones (Fig. 3).

In cross section, except for filament ends, thalli are hollow (Fig. 4) and this gives them a consistency extremely elastic allowing to wave when unattached.

The habitats colonized by this species are shallow, strongly eutrophicated and turbid environments, where salinity can change according to the outflows of rivers. During samplings in Emilia-Romagna, for example, the Po river outflow in the Delta lagoons was regular and salinity ranged between 14.9 and 29.3 psu, but, during the river flood, salinity decreased up to values lower than 10 psu. Water transparency measured by the Secchi disk ranged between 0.5 to 2.2 m, whereas the oxygen saturation fluctuated from 75 to 334%. High variations showed also nutrient concentrations. In particular, the reactive phosphorus (RP) showed a peak of 20.1 µM, a value so high that was never recorded in other lagoons of the northern Adriatic Sea such as Venice or Grado-Marano. *G. vermiculophylla* completely covered the bottoms, forming abundant pleustophytic thalli with biomasses up to 15 kg fwt m^{-2}.

Molecular results

Identification of the isolates from Emilia-Romagna and the Venice Lagoon as *Gracilaria vermiculophylla* was obtained through molecular analyses using the plastid *rbc*L gene, being useful for examining species relationships in *Gracilaria*.

The partial plastid gene sequences got in this study, compared with data available in GenBank, showed 100% identity with the sequence of *G. vermiculophylla* from Po Delta (FN400862) and 14 sequences of *G. vermiculophylla* collected in South Korea and Japan.

The phylogenetic reconstruction (Fig. 5a), based on the almost complete *rbc*L gene sequence, detected 6 haplotypes, differing from 1 to 4 base pairs (Fig. 5b). Their geographical distribution is shown in Fig. 6.

Most of the isolates divided into two clades, corresponding to the haplotypes A and B. The first clade (haplotype A) included Japan and South Korea isolates, together with the North Adriatic representative (Italy). The samples with haplotype B were from South Korea and Russia. The rest of haplotypes were represented by one isolate each. Of these, one was from France (North Atlantic), two from Japan (Kyushu and Chiba), and the most divergent one from North Carolina, USA (Atlantic Ocean).

The mitochondrial *cox*2-3 intergenic spacer region, used to infer population relationships, was also analyzed. The obtained sequences, 359 bp long, were 100% identical with the 367 bp sequence FN400863, previously obtained for the Po Delta samples (North Adriatic). Thus, this last, including the *cox*2-3 spacer (136 bp) and the flanking regions of *cox*2 gene (180 bp) and *cox*3 gene (51 bp), was used in comparisons with other published *G. vermiculophylla cox*2-3 sequences. The North Adriatic mitochondrial spacer showed a percentage of 100% similarity with those of 10 entities of *G. vermiculophylla* sampled in France, one specimen from Portugal, and one from Netherlands. Shorter sequences of the same DNA region, belonging to other specimens attributed to *G. vermiculophylla* and collected in Japan (362 bp), South Korea (356 bp), Spain (351 bp), and Sweden (345 bp), also resulted identical to that of the Italian isolates. A 0.54% nucleotide divergence (2 bp), instead, was observed with 3 sequences

(DQ173304, DQ173303, DQ173302) belonging to specimens of *G. vermiculophylla* from different areas of Hog Island Bay in Virginia, USA (Atlantic Ocean).

Discussion

Gracilaria vermiculophylla was introduced in the European coasts at least since 1996 (Rueness 2005) and behaves as an invasive species that rapidly spreads along the Atlantic, North Sea, and Baltic Sea coasts, being not limited by low salinity waters. In fact, *G. vermiculophylla* tolerates salinities lower than 10 psu and its diffusion is increased by the frequent thallus fragmentation, normally caused by grazing (Thomsen et al. 2007).

The species usually forms loose-lying thalli on mud or fine sand in shallow protected bottoms of bays and estuaries, more rarely it is attached to mollusc shells or stones. It is commonly found in seagrass beds of *Zostera marina* Linnaeus or *Nanozostera noltii* (Honermann) Tomlinson and Posluzny, but it can also form tangled mats together with other species such as members of the Ulvaceae. The species usually grows in association with many macrofaunal organisms (e.g. calcareous or membranaceous tube-building worms, mussels, gastropods), creating a compact substratum that favours the growth of all the associate organisms and supplies new substratum for the attachment of further thalli.

The most probable introduction vector for Europe has been oyster farms that import these bivalves from the Indo-Pacific area, in particular the Japan coasts, where *G. vermiculophylla* is abundant (Rueness 2005), as hypothesized for other red algae by Maggs and Stegenga (1999).

In Italy *G. vermiculophylla* was recorded for the first time in the North Adriatic Sea, (Po Delta Lagoons) (Sfriso et al. 2010). Being not affected by touristic and commercial traffics, the most probable introduction vector of this red alga in the Po Delta Lagoons

could be clam farming and, in particular, the importation of the Manila clam *Ruditapes philippinarum* Adams & Reeve (Sfriso and Curiel 2007).

Based on the molecular data obtained in this study for both the *rbc*L gene and the *cox*2-3 spacer region, we can hypothesize that the North Adriatic *G. vermiculophylla* isolates have been introduced by the Japan or the Korean populations. More in detail, the 100% identity of the North Adriatic *cox*2-3 sequences with the South Korean and Japan ones strongly suggests the introduction from these localities, being the mitochondrial marker useful in demographic studies of red algae (Zuccarello et al. 1999). The results obtained with the *rbc*L marker support this hypothesis one more time. In particular, the North Adriatic haplotype corresponds with the most spread one (haplotype A) in the South-East Pacific area.

The nucleotide differences observed among the haplotypes lead also to suppose that the USA *G.vermiculophylla* population was introduced and settled before the European ones. This is confirmed by the genetic divergence (0.54%) in the *cox*2-3 spacer region between the population from Virginia, USA, and the native Indo-Pacific one.

About the European population, the French North Atlantic *G. vermiculophylla* probably settled before the Italian one, based on the differences between the *rbc*L haplotypes. Unfortunately no more *rbc*L sequences of *G. vermiculophylla* from other European regions were available for comparison.

The absence of differences in the mitochondrial marker between the French and all the other European isolates, including the Adriatic ones, suggests that for this species the *cox*2-3 spacer has a lower rate of evolution than the plastid *rbc*L gene.

Besides the already suggested vector of introduction, in the North Adriatic Sea the spreading of *G. vermiculophylla* can be due to other transport mechanisms, such as small boats, anchors, nets, and other fishing tools.

The high growth rates and the ability to tolerate also low salinities, make *G. vermiculophylla* able to live in environments with strong eutrophication and turbidy, as well as wide salinity changes, like the North Adriatic transitional systems, where the diffusion of many other indigenous macroalgal and seagrass species is limited.

For this reason, in some areas *G. vermiculophylla* has overcame the growth of the local species, including the laminar Ulvaceae.

According to the survey by Freshwater et al. (2006) on the spreading of *G. vermiculophylla* in North Carolina, USA, this species is to considered highly invasive, meeting 6 of the 10 criteria described by Chapman and Carlton (1991, 1994) for the identification of invasive taxa. Taken into account our results and those considerations, the spreading of *G. vermiculophylla* in the North Adriatic Sea can seriously threaten the local biodiversity of this area.

Acknowledgments

We thank the "Agenzia Regionale per la Prevenzione e Protezione Ambientale del Veneto" (ARPAV) which supported the sampling of the Po Delta lagoons and Dr. Bruno Sebastiano and Dr. Daniele Bon for the sampling facilities.

This work was supported by grants provided by Progetto di Ateneo of the University of Padova.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Molec Biol 215:403-410. doi:10.1016/S0022-2836(05)80360-2

Bellemo G, Curiel D, Marzocchi M, Iuri M, Pavan M, Vecchiato N, Scattolin M (1999) Aggiornamento di nuove specie algali per la laguna di Venezia. Lavori Soc Ven Sc Nat 24:55-66

Bellemo G, Curiel D, La Rocca B, Scattolin M, Marzocchi M (2001) First Report of a filamentous species of *Desmarestia* (Desmarestiaceae, Fucophyceae) in the Lagoon of Venice (Italy, Mediterranean Sea). Bot Mar 44:541-545 doi:10.1515/BOT.2001.060

Boudouresque CF, Verlaque M (2002) Biological pollution in the Mediterranean sea: invasion versus introduced macrophytes. Mar Poll Bull 44:32-38. doi:10.1016/S0025-326X(01)00150-3

Chapman JW, Carlton JT (1991) A test of criteria for introduced species: the global invasion by the isopod *Synidotea laevidorsalis* (Miers, 1881). J Crustacean Biol 11:386-400

Chapman JW, Carlton JT (1994) Predicted discoveries of the introduced isopod *Synidotea laevidorsalis* (Miers, 1881). J Crustacean Biol 14:700-714

Cormaci M, Furnari G, Giaccone G, Serio D (2004) Alien macrophytes in the Mediterranean sea: A review. In: Pandalai SG (ed) "Recent Research Developmtents in Environment Biology", pp. 153-202

Curiel D, Bellemo G, Marzocchi M (1996a) New records of marine algae in the lagoon of Venice. Giorn Bot Ital 130:352

Curiel D, Marzocchi M, Bellemo G (1996b) First report of fertile *Antithamnion pectinatum* (Ceramiales, Rhodophyceae) in the North Adriatic Sea (Lagoon of Venice, Italy). Bot Mar 39:19-22. doi:10.1515/botm.1996.39.1-6.19

Curiel D, Bellemo G, Marzocchi M, Scattolin M, Parisi G (1998) Distribution of introduced Japanese macroalgae *Undaria pinnatifida*, *Sargassum muticum* (Phaeophyta) and *Antithamnion pectinatum* (Rhodophyta) in the lagoon of Venice. Hydrobiologia 385:17-22. doi: 10.1023/A:1003437105147

Curiel D, Bellemo G, La Rocca B, Scattolin M, Marzocchi M (2001) Note su specie algali nuove per la laguna di Venezia. Lavori Soc Ven Sc Nat 26:101-102

Curiel D, Bellemo G, La Rocca B, Scattolin M, Marzocchi M (2002) First report of *Polysiphonia morrowii* Harvey (Ceramiales, Rhodophyta) in the Mediterranean Sea. Bot Mar 45:66-70. doi:10.1515/BOT.2002.008

Curiel D, Scattolin M, Marzocchi M (2003) Modificazioni dei popolamenti fitobentonici di substrato duro nella laguna di Venezia in seguito all'introduzione di *Undaria pinnatifida* (Harvey) Suringar. Lavori Soc Ven Sc Nat 28:25-31

Curiel D, Checchin E, Dri C, Miotti C, Bellemo G, Rismondo A, Scattolin M, Marzocchi M (2004) Brevi note su alcuni ritrovamenti algali per la laguna di Venezia. Lavori Soc Ven Sc Nat 29:59-61

Curiel D, Bellemo G, Checchin E, Dri C. Miotti C, Marzocchi M (2005) Segnalazione di nuove macroalghe per la laguna di Venezia. Lavori Soc Ven Sc Nat 30:41-44

Facca C, Sfriso A (2007) Epipelic diatom spatial and temporal distribution and relationship with the main environmental parameters in coastal waters. Estuar Coast Shelf Sci 75:35-49. doi:10.1016/j.bbr.2011.03.031

Falace A, Curiel D, Sfriso A (2009) Study of the Macrophyte Assemblages and Application of Phytobenthic Indices to Assess the Ecological Status of the Marano and Grado Lagoon (Italy). Mar Ecol 30:480-494. doi:10.1111/j.1439-0485.2009.00300.x

Felsenstein J (1985) Confidence limits on phylogenies: an approach using bootstrap. Evolution 39:783-791

Freshwater DW, Rueness J (1994) Phylogenetic relationships of some European *Gelidium* (Gelidiales, Rhodophyta) species, based on *rbc*L nucleotide sequence analysis. Phycologia 33:187-194. doi: 10.2216/i0031-8884-33-3-187.1

Freshwater DW, Montgomery F, Green JK, Hamner RM, Williams M, Whitfield PE (2006) Distribution and identification of an invasive *Gracilaria* species that is

hampering commercial fishing operations in southeastern North Carolina, USA. Biol Invasions 8: 631-637. doi: 10.1007/s10530-005-1809-5

Gavio B, Fredericq S (2002) *Grateloupia turuturu* (Halymeniaceae, Rhodophyta) is the correct name of the non-native species in the Atlantic known as *Grateloupia doryphora*. Eur J Phycol 37:349–359. doi: 10.1017/S0967026202003839

Guindon S, Gascuel O (2003) A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696-704. doi: 10.1080/10635150390235520

Maggs CA, Stegenga H (1999) Red algal exotics on North Sea coasts. Helgoländer Meeresuntersuchungen 52:243-258

Mizzan L (1995) Note on presence and diffusion of *Dyspanopeus sayi* (Smith, 1869) (Crustacea, Decapoda, Xanthidae) in the Venetian lagoon. Boll Mus Civ Storia Nat Venezia 44:121-129

Occhipinti-Ambrogi A (2000) Biotic invasion in a Mediterranean lagoon. Biol Invasions 2:165-176. doi: 10.1023/A:1010052809567

Occhipinti-Ambrogi A (2002) Current Status of Aquatic Introductions in Italy. In: Leppäkoski E, Gollasch S, Olenin S (eds) Invasive aquatic species of Europe - distribution, impact and management. Kluwer Academic Publishers, Dordrecht, Boston, London, pp 311-324

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14:817-818. doi:10.1093/bioinformatics/14.9.817

Posada D, Buckley TR (2004) Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. Syst Biol 53:793-808. doi:10.1080/10635150490522304

Rueness J (2005) Life history and molecular sequences of *Gracilaria vermiculophylla* (Gracilariales, Rhodophyta), a new introduction to European waters. Phycologia 44:120-128. doi: 10.2216/0031-8884(2005)44

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425

Sfriso A (1987) Flora and vertical distribution of macroalgae in the lagoon of Venice: a comparison with previous studies. Giorn Bot Ital 121:69-85

Sfriso A, Raccanelli S, Pavoni B, Marcomini A (1991) Sampling strategies for measuring macroalgal biomass in the shallow waters of the Venice lagoon. Environ Technol 12:263-269. doi: 10.1080/09593339109385004

Sfriso A (2006) Rinvenimento di nuove macroalghe nei bassofondali e nei litorali marini della laguna di Venezia. Lavori Soc Ven Sc Nat 31:17-24

Sfriso A (2007) Descrizione di macroalghe nuove per la laguna di Venezia. Lavori Soc Ven Sc Nat 32:25-32

Sfriso A (2009) Segnalazione di macroalghe nuove per la laguna di Venezia. Lavori Soc Ven Sc Nat 34:65-71

Sfriso A, La Rocca B 2005 Aggiornamento di taxa macroalgali nei litorali e nei bassofondali della laguna di Venezia. Lavori Soc Ven Sc Nat 30:45-58

Sfriso A, Curiel D (2007) Check-list of marine seaweeds recorded in the last 20 years in Venice lagoon and a comparison with the previous records. Bot Mar 50:22-58. doi:10.1515/BOT.2007.004

Sfriso A, Maistro S, Andreoli C, Moro I (2010) First record of *Gracilaria vermiculophylla* (Gracilariales, Rhodophyta) in the Po Delta lagoons, Mediterranean Sea (Italy). J Phycol 46:1024-1027. doi: 10.1111/j.1529-8817.2010.00893.x

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 101:11030-11035. doi: 10.1073/pnas.0404206101

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol 28: 2731-2739. doi:10.1093/molbev/msr121

Thompson JD, Higgins DG, Gibson TJ (1994) Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680. doi: 10.1093/nar/22.22.4673

Thomsen MS, Gurgel CFD, Fredericq S, McGlathery KJ (2005) *Gracilaria vermiculophylla* (Rhodophyta, Gracilariales) in Hog Island Bay, Virginia: a cryptic alien and invasive macroalga and taxonomic correction. J Phycol 42:139-141. doi: 10.1111/j.1529-8817.2005.00160.x

Thomsen MS, Staehr PA, Nyberg CD, Schwaerter S, Krause-Jensen D, Silliman BR (2007) *Gracilaria vermiculophylla* (Ohmi) Papenfuss, 1967 (Rhodophyta, Gracilariaceae) in northern Europe, with emphasis on Danish conditions, and what to expect in the future. Aquat Invasion 2:83-94. doi: 10.3391/ai.2007.2.2.1

Tolomio C (1993) Prima segnalazione di *Grateloupia doryphora* (Mont.) Howe (Rhodophyceae) nella laguna di Venezia. Lavori Soc Ven Sci Nat 18:215-220

Turolla E, Rossetti E, Pellizzato N, Zentilin A (2008) La venericoltura in Italia a 25 anni dal suo esordio. Il Pesce 3:31-40

Verlaque M (1994) Inventaire des plantes introduites en Mediterranée; origines et répercussions sur L'environnnement et les activities humaines. Oceanol Acta 17:1-23

Zenetos A (2010) Trend in aliens species in the Mediterranean. An answer to Galil, 2009 «Taking stock: inventory of alien species in the Mediterranean Sea». Biol Invasions12:3379-3381. doi:10.1007/s10530-009-9679-x

Zuccarello GC, Burger G, West JA, King RJ (1999) A mitochondrial marker for red algal intraspecific relationship. Mol Ecol 8:1443-1447. doi:10.1046/j.1365-294x.1999.00710.x

FIGURE CAPTIONS

Fig. 1 Map showing sampling sites. **a** Venice Lagoon **b,c** Emilia-Romagna transitional systems ('Pialassa della Baiona' and Delta del Po Lagoons)

Fig. 2 Greyish wine-red in colour thalli of *Gracilaria vermiculophylla* collected in the Venice Lagoon

Fig. 3 Thalli of *G. vermiculophylla* attached to rocks

Fig. 4 Cross section of the median region of a thallus filament

Fig. 5 Molecular analyses of the *G. vermiculophylla rbc*L haplotypes. **a** Unrooted phylogenetic consensus tree obtained with the Neighbor-Joining method. Haplotypes are highlighted by different colours. The Italian representative is evidenced with a blue ellipse. Bar = 0.0001 nucleotide substitutions per site.**b** Compressed alignment, showing position in sequence and nucleotide changes in each haplotype

Fig. 6 Geographical distribution of *G. vermiculophylla rbc*L haplotypes, with inset enlargements of the French and Italian coasts and the Russian, Korean, and Japan interested areas. Haplotypes for each locality are indicated by the corresponding letters inside white circles

Fig.1
Click here to download Figure: Fig. 1.pdf

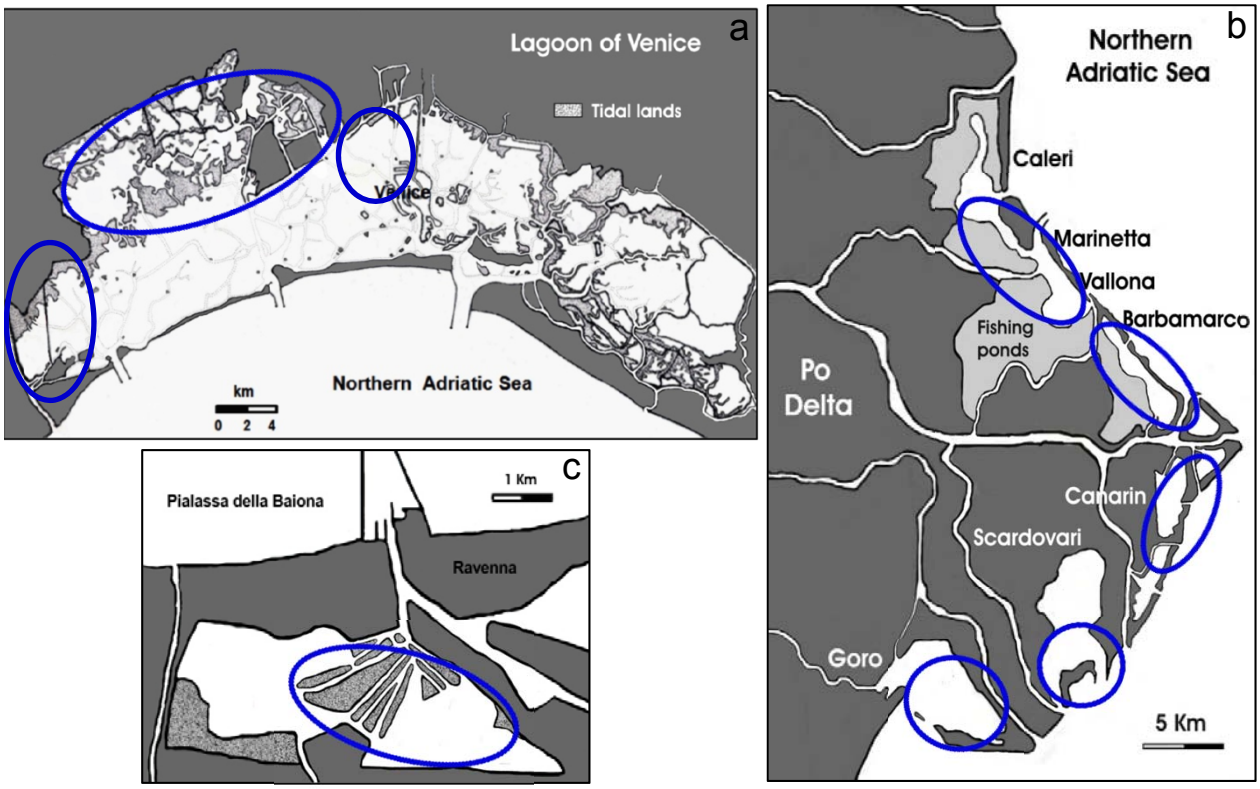
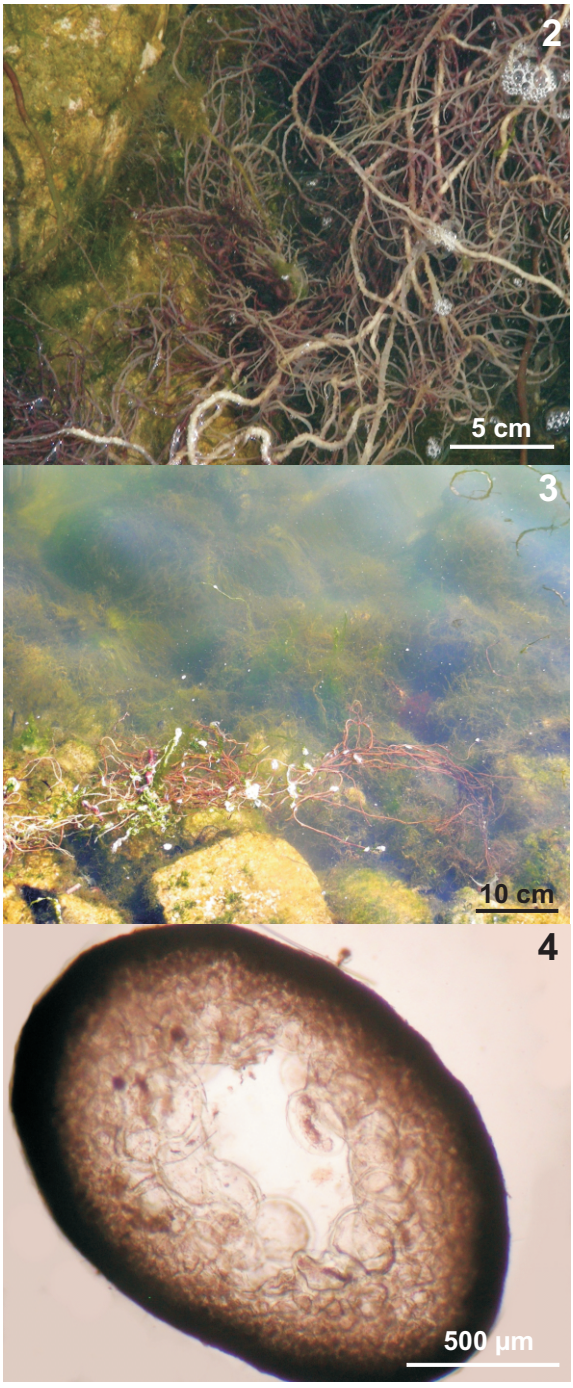


Fig. 1

Figs 2-4
Click here to download Figure: Figs 2-4.pdf



Figs 2-4

Fig. 5
Click here to download Figure: Fig. 5.pdf

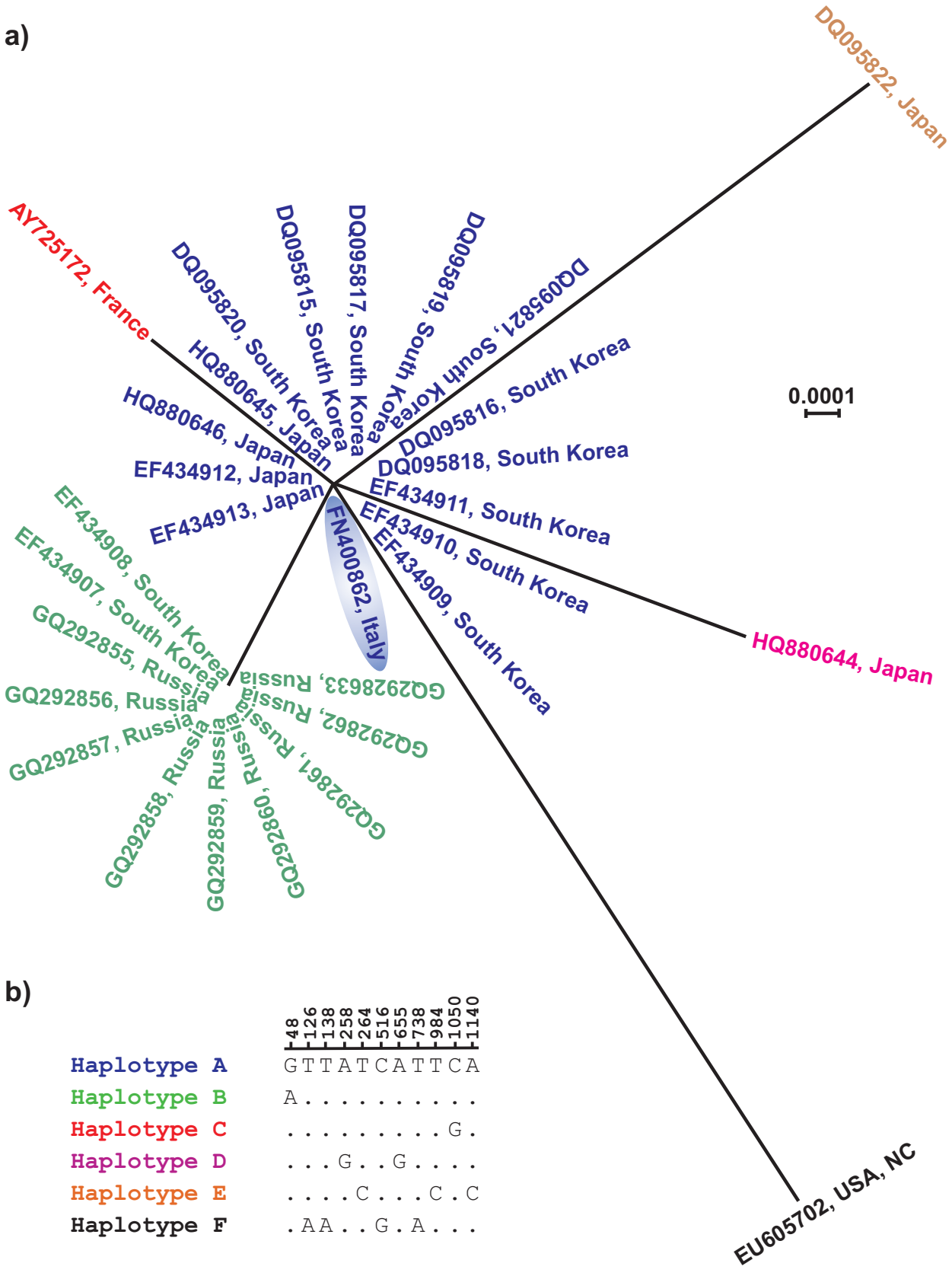


Fig. 5

Fig. 6
Click here to download Figure: Fig. 6.pdf

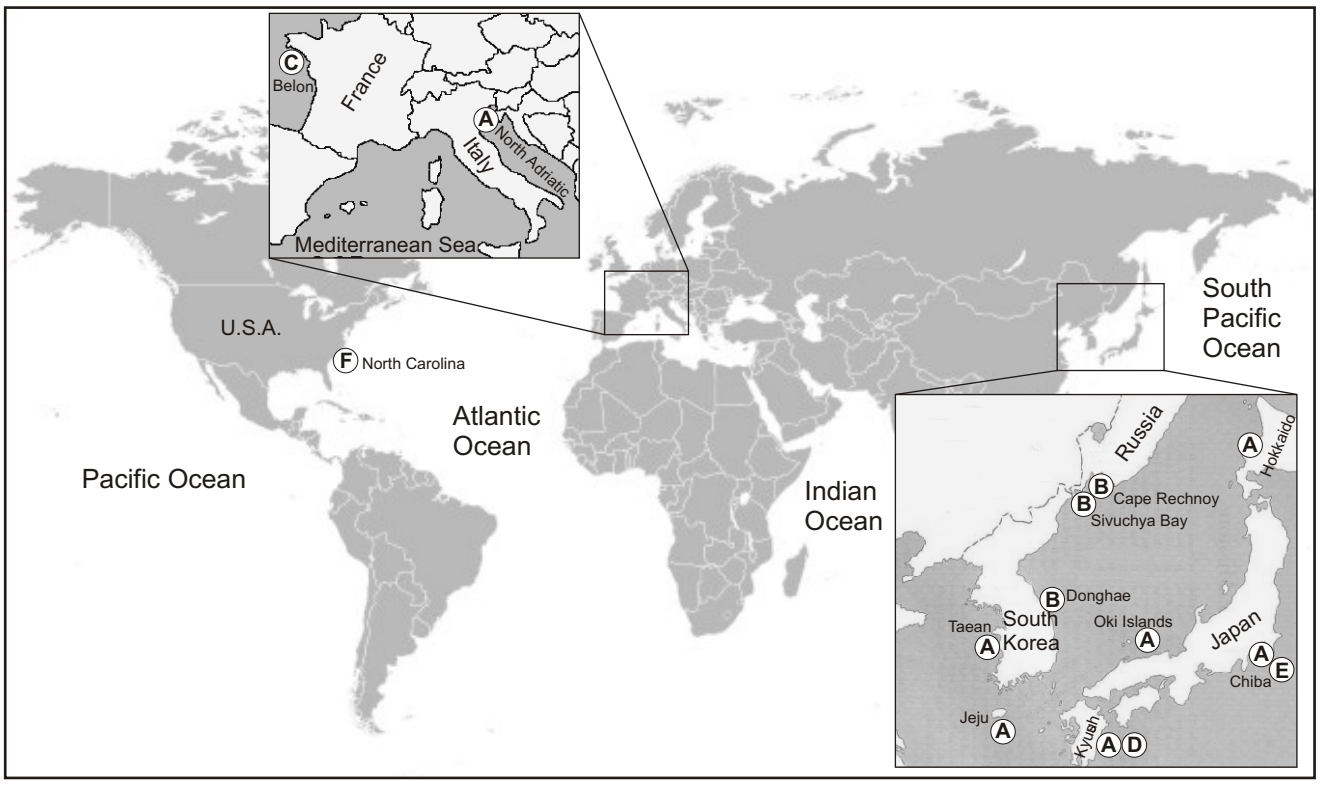


Fig. 6

Journal of PHYCOLOGY
An International Journal of Algal Research

ULVA (CHLOROPHYTA, ULVALES) BIODIVERSITY IN THE NORTH ADRIATIC SEA (MEDITERRANEAN, ITALY): CRYPTIC SPECIES AND NEW INTRODUCTIONS

Journal:	*Journal of Phycology*
Manuscript ID:	JPY-11-228-ART
Manuscript Type:	Regular Article
Date Submitted by the Author:	04-Oct-2011
Complete List of Authors:	Wolf, Marion; University of Padova, Dept. of Biology Sciuto, Katia; University of Padova, Dept. of Biology Andreoli, Carlo; University of Padova, Dept. of Biology Moro, Isabella; University of Padova, Dept. of Biology
Keywords:	alien species, cryptic diversity, Mediterranean Sea, phylogeny, rbcL, tufA, Ulva
Category:	Phylogenetics and Taxonomy

TITLE

ULVA (CHLOROPHYTA, ULVALES) BIODIVERSITY IN THE NORTH ADRIATIC SEA (MEDITERRANEAN, ITALY): CRYPTIC SPECIES AND NEW INTRODUCTIONS

Marion Adelheid Wolf*, Katia Sciuto*, Carlo Andreoli, Isabella Moro[2]

Department of Biology, University of Padova, via U. Bassi 58/B, 35131 Padova, Italy

* these authors contributed equally to this work

[2] corresponding author: isabella.moro@unipd.it, phone: +39 049 8276255, Fax: +39 049 8276260

RUNNING TITLE

Ulva species in the North Adriatic Sea

ABSTRACT

The genus *Ulva* Linnaeus (Ulvophyceae, Ulvales) is cosmopolitan in its distribution, with species occurring in all aquatic habitats from freshwater through brackish to fully saline environments. Members of this genus show a very simple morphology and a certain degree of phenotypic plasticity, heavily influenced by environmental conditions, making difficult the delineation of species by morphological features alone.

In spite of this, most of the studies dealing with *Ulva* biodiversity in Italian waters have been based only on morphological characters. For this reason we have recently started surveys on *Ulva* biodiversity in the North Adriatic Sea (Mediterranean, Italy) basing on DNA barcoding.

As maritime traffic is considered one of the major causes of species introduction, for example through ballast waters, hull fouling, and ship sea chests, we have focused on three places for sampling: Venice Lagoon, Chioggia inlet, and Lido of Venice. The first two are greatly affected by naval traffics, while the last one is much less influenced by this phenomenon.

The molecular analyses, carried out using the *rbc*L-3P region and *tuf*A gene as molecular markers, revealed the presence of six different species, often with overlapping morphologies: *U. californica* Wille, *U. flexuosa* Wulfen, *U. rigida* C. Agardh, *U. compressa* Linnaeus, *U. pertusa* Kjellman, and one probable new taxon. Some of the recognized species have not been reported up to now in the investigated areas.

KEY INDEX WORDS

alien species, cryptic diversity, Mediterranean Sea, phylogeny, *rbc*L, *tuf*A, *Ulva*

INTRODUCTION

Ulva Linnaeus (Ulvophyceae, Ulvales), one of the first described algal taxa, is an ubiquitous genus of macroalgae, with species occurring in all aquatic habitats from freshwater through estuarine to marine environments (Guiry and Guiry 2011). *Ulva* thalli are either distromatic and foliose (blades) or monostromatic and tubular. Thallus of blade species can be broadly expanded, irregularly lobed, cuneate, linear, lanceolate, oblanceolate, or deeply divided into linear lacinae. Some members of this group show regular perforations, such as *U. reticulata*, or marginal dentations, such as *U. rigida* and *U. taeniata*. The tubular forms were previously regarded as belonging to a distinct taxon, *Enteromorpha* Link, until recent phylogenetic studies have demonstrated that these two genera are not separate (Hayden et al. 2003). As presently circumscribed, 127 species are recognized under the genus *Ulva* (Guiry and Guiry 2011).

Besides their importance as components of biodiversity and indicators of eutrophic environments, several members (particularly the tubular enteromorphoid taxa) are notorious biofoulers of ships' hulls and ballast waters and, for this reason, they result among the most commonly transported and widely introduced macroalgal species (Nelson et al. 2007).

Being frequently sites of aquaculture activities, lagoons are particularly subject to species introductions, either accidental or voluntary, with all possible negative effects from cell to community level (McLusky and Elliot 2007). In particular, the increasing of naval traffic with extra-Mediterranean countries, for both the import of fish products and aquaculture activities, introduces new macroalgal species in the Venice Lagoon every year (Sfriso and Curiel 2007). Since 1983, twenty alien macroalgae have been introduced into this environment (Sfriso et al. 2009) and more are reported by recent studies (Cecere et al. 2011; Wolf et al. 2011a).

These considerations highlight the importance of cataloguing the macroalgal biodiversity in such areas to preserve its conservation and utilization.

Identification of *Ulva* spp. has challenged taxonomists and field botanists since the time of Linnaeus. Members of this genus show a very simple morphology and a certain degree of phenotypic plasticity, heavily influenced by environmental conditions, making difficult the delineation of species only by morphological features (Loughnane et al. 2008). In fact, morphology can vary with age of the thallus, time of the year, salinity, and life style (e.g. whether the thallus is attached or floating) (e.g. Malta et al. 1999). Moreover the existence of cryptic species in this group further complicates *Ulva* identification merely on a morphological basis and, for this reason, up to recent times Mediterranean communities have been largely underestimated due to the co-occurrence of genetically distinct but morphologically overlapping species or species complexes (Boudouresque and Verlaque 2002; Bickford et al. 2007).

With the spread of the DNA techniques, more and more authors have shown how the use of molecular markers can shed light on the systematics of problematic taxa when clear morphological diagnostic criteria are lacking (Saunders 2009; Destombe et al. 2010). DNA barcoding, a method which identifies organisms through comparisons of short standardized DNA sequences, as agreed upon by the Consortium for the Barcode of Life (CBOL; http://barcoding.si.edu/), is a revolutionary system for identification and discovery of all of the world's eukaryotic organisms (Hebert et al. 2003a; 2003b).

About seaweeds, several markers of different DNA regions (nuclear, plastid, and mitochondrial) have been used to study phylogenetic relationships at the interspecific and intraspecific level. Among these, the plastid large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) is one of the most commonly employed genetic regions (Freshwater et al. 1994); the mitochondrial *cox*1 gene, coding for the subunit I of cytochrome oxidase enzyme, was proposed as barcode marker for red algae (Saunders 2005), and the nuclear internal transcribed spacer of the ribosomal cistron (ITS) is extensively used for investigations of phylogeny, molecular ecology, and evolution of marine green macroalgae (Hayden and Waaland 2004). Other interesting loci are the plastid

elongation factor *tuf*A, used to discriminate among green algal species (Fama et al. 2002; Zuccarello et al. 2009; Händeler et al. 2010); the plastid *rbc*L-*rbc*S spacer region, proven to be a good marker for species discrimination being essentially invariant within a species, but extremely variable at the interspecific level (Cho et al. 2003; Skage et al. 2005, Wolf et al. 2011b); and the more variable mitochondrial *cox*2-*cox*3 spacer, useful for population studies (Zuccarello et al. 1999).

Here we report the first results of a survey on *Ulva* biodiversity in the North Adriatic Sea (Mediterranean, Italy). To start we focused on three sampling sites: Venice Lagoon, Chioggia inlet, and Lido of Venice. The first two areas are greatly affected by naval traffics, while the last one is much less influenced by this phenomenon. The phylogenetic analyses, carried out using the *rbc*L-3P region and the *tuf*A gene as molecular markers, revealed the presence of six different species with overlapping morphologies: *U. californica* Wille, *U. flexuosa* Wulfen, *U. rigida* C. Agardh, *U. compressa* Linnaeus, *U. pertusa* Kjellman, and one probable new taxon. The molecular approach used in this study allowed the discrimination among morphologically similar species and the identification of some new alien and/or invasive entities, never reported before in these areas.

MATERIALS AND METHODS

Field collections and sample preparation

Samplings were carried out at the end of January and during February 2011 in the Lagoon of Venice (S. Marta: 45° 26' 17.11"N, 12° 18' 54.65"; Piazzale Roma: 45° 26' 14.26"N, 12° 19' 12.84E), Chioggia inlet (Fondamenta Merlin: 45° 13' 25.55"N, 12° 16' 47.19"E; Ponte dell'Unione: 45° 12' 56.83"N, 12° 17' 3.10"E; Hydrobiological Station: 45° 13' 23.00"N, 12° 17' 0.24"E) and in the Lido of Venice (Cà Bianca: 45° 23' 28.38"N, 12° 21' 25.75"E) (Fig. 1). The narrow intertidal zone and shallow subtidal zones (to 1 m depth) were sampled.

Specimens for the analyses were assigned letters corresponding to the different sampling sites (Table 1). Each sample was pressed on herbarium paper as a voucher, while a subsample was divided, with part of it preserved in 4% formalin/seawater for morphological observations and some dried in silica gel and kept at -80°C for genomic DNA extraction. Voucher specimens are available at the laboratory of Dr. Moro, University of Padova (Italy).

Sections for microscopic observations were made by hand using a razor blade under a stereo zoom microscope. Photographs were taken with a digital camera attached to a light microscope (Leica DMR5000).

DNA extraction, amplification, and sequencing

Genomic DNA was isolated from 5 mm^2 of dried algal tissue, ground in liquid nitrogen following procedures outlined in Saunders (1993) with modifications (Saunders 2008). Extracted DNA was stored at -80°C and used for amplification of the 3' region of the plastid *rbc*L gene and the elongation factor *tuf*A gene. PCR amplification was carried out using the Ex Taq™ DNA Polymerase (Takara, Shiga, Japan) following the manufacturer's instructions with a final volume of 20µl per reaction. For the *rbc*L-3P region, the primer pair used was G*rbcL*Fi (Saunders and Kucera 2010) and 1385R (Manhart 1994). To amplify *tuf*A gene primers *tuf*GF4 (Saunders and Kucera 2010) and *tuf*AR (Fama et al. 2002) were employed.

The PCR amplification profile for each marker followed those listed in Saunders and Kucera (2010). PCR products were purified using Exo-Sap-IT kit (USB, Cleveland, Ohio, USA).

Sequencing for all *rbc*L-3P and *tuf*A PCR products was carried out using the PE Applied Biosystems Big Dye (version 3.1) kit (Foster City, CA, USA), employing the same primers as for the PCR. Sequence trace files were generated on an Applied Biosystems 3130 XL automated sequencer (PE Applied Biosystems; Foster City, CA, USA). Forward and reverse sequence reads were edited to produce contigs in Sequencher™ 4.8 (Gene Codes Corporation, Ann Arbor,

149 Michigan, USA), and a multiple sequence alignment was constructed for each gene region using
150 MacClade version 4.08 (Maddison and Maddison 2005).
151 The nucleotide sequences obtained during this study were deposited in the DDBJ/GenBankTM/EBI
152 Data Bank under the accession numbers reported in bold on the trees.

153 *Phylogenetic analyses*

154 Separate datasets were prepared for the *rbc*L-3P region and the *tuf*A gene, using the new sequences
155 plus other *Ulva* sequences in the DDBJ/GenBankTM/EBI Data Bank. Multiple alignments for
156 phylogenetic analyses were generated with the ClustalW computer program (Thompson et al.
157 1994).
158 Maximum Likelihood (ML) analyses were carried out with PHYML 3.065 (Guindon and Gascuel
159 2003). Non-parametric bootstrap re-sampling (Felsenstein 1985) was performed to test the
160 robustness of the tree topologies (1000 replicates). All Bayesian Inference (BI) analyses were
161 carried out with MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) and included two
162 separate concurrent MCMC runs, each composed of four chains, three heated and one cold. Each
163 Markov chain ran for 1,500,000 generations, in the case of the *rbc*L-3P gene region, and 10,000,000
164 generations, in the case of the *tuf*A gene, sampling trees every 100 generations. At the end of each
165 run we considered the sampling of the posterior distribution to be adequate if the average standard
166 deviation of split frequencies was < 0.01. The first 3,750 trees, for the *rbc*L-3P region, and 25,000
167 trees, for the *tuf*A gene, were discarded as burn-in, as determined by stationarity of lnL assessed
168 using Tracer v. 1.5 (Rambaut and Drummond 2007). Consensus topologies and posterior
169 probability values were then calculated from the remaining trees. The nexus files for the BI analyses
170 were generated with the Mesquite 2.71 software package (Maddison and Maddison 2009).

For ML and BI analyses, the models that best fit our data were found using the program jMODELTEST under the Akaike Information Criterion (AIC) (Posada and Crandall 1998; Posada and Buckley 2004).

Tree topologies were visualized with the NJplot computer program (Perrière and Gouy 1996).

RESULTS AND DISCUSSION

In this paper we report the results of a preliminary survey on *Ulva* biodiversity in the North Adriatic Sea, based on molecular analyses performed using the *rbc*L-3P region and the *tuf*A gene as molecular markers. The phylogenetic analyses carried out according to the two different methods gave comparable results, thus, for each molecular marker, we depicted only the ML topologies, reporting both the ML bootstrap values and the posterior probabilities at the nodes.

The *rbc*L-3P region dataset included 75 sequences, 41 of which were retrieved from DDBJ/GenBankTM/EBI Data Bank. The sequence of *Ulvaria oscura* was chosen as outgroup. The alignment consisted of a total of 713 positions, out of which 73 were parsimony-informative. The TrN+I+G model, as selected by jMODELTEST, was used in both the ML and BI analyses.

The *tuf*A gene dataset included 49 sequences, of which only 16 were available for comparison in the public databases. Again, *Ulvaria obscura* was selected as outgroup. The resulting alignment consisted of a total of 774 positions, of which 106 were parsimony-informative. The GTR+I+G model, as indicated by jMODELTEST, was used for the ML and BI phylogenetic analyses.

In the *rbc*L-3P phylogenetic reconstruction (Fig. 2) *Ulva* Adriatic samples formed five distinct clades (A, B, C, D, E) with other *Ulva* species whose sequences were available in the public databases. The nucleotide divergence values between the Adriatic specimen sequences and those which grouped with them in the *rbc*L-3P phylogenetic tree were calculated and are reported in Table 2.

The *tuf*A phylogenetic reconstruction (Fig. 3) confirmed the above cited clades, but in this case the phylogenetic relationships resulted better resolved, also if less sequences from public databases could be included in the analyses. This is in agreement with other authors who state that the *tuf*A gene has a higher resolution power in respect to other molecular markers, including the *rbc*L gene (Saunders and Kucera 2010).

More in detail, both in the *rbc*L-3P and *tuf*A obtained trees, samples #VE3, #VE9, and #VE10 clustered with specimens of *U. californica* Wille (Clade A) (#VE11 *rbc*L sequence failed to amplify). The nucleotide divergence percentage value between the Adriatic specimen and *Ulva californica tuf*A sequences was 0.39%, a value inside the intraspecific divergence range (0%-0.39%) reported in previous studies on the genus *Ulva* using the same marker (Saunders and Kucera 2010). Strengthened by the diverrgence percentage found for the *rbc*L-3P region sequences (Table 2), this result confirms the belonging of our samples to the species *U. californica*.

U. californica has never recorded before in the Mediterranean according to the current check-lists. This species is native of California (type locality: La Jolla, Collins et al. 1899: no. 611) and was recently recorded in Ireland (Loughnane et al. 2008) and Britain (Hayden and Waaland 2004, Brodie et al. 2007), probably introduced to European coasts by naval traffic (as hull-fouling components).

Blades of *Ulva californica* Adriatic specimens were mostly linear to oblanceolate and much crisped (Fig. 4A). Cells in surface view were irregularly polygonal with rounded corners and irregular arrangement, 6-15 µm in diameter (Fig. 5A). The chloroplast presented one or two pyrenoids (Fig. 5A).

Clade A resulted sister to clade B, represented by sample #VE7 and a specimen of *U. flexuosa* Wulfen from Canada in both the trees. The *tuf*A nucleotide divergence between our sample and *U. flexuosa* (0.13%) indicates the conspecificity of these two entities.

In the case of *rbc*L tree, also other two specimens deposited as *Ulva* sp. LGKK2008f and *Enteromorpha* sp. WTU344779 were included in clade B. Hayden and collaborators (2003) and Kraft and collaborators (2010), described these last two isolates as *U. prolifera* O.F. Müller and *U. stipitata* var. *linzoides* L.G.Kraft, Kraft & R.F.Waller respectively. The belonging of *Ulva* sp. LGKK2008f to the species *U. prolifera* seems very improbable, because another *Ulva prolifera* sample (Genbank accession number HQ603634) placed outside clade B. Moreover the divergence values between the Adriatic specimen and each of the others included in clade B (0%-0.28%) lead to reject the hypothesis that they could belong to different species. In fact, if the value 0.28% is slightly higher respect to the intraspecific divergence range (0%-0.27%) reported for the 3'-742 bp region of *Ulva rbc*L gene (Saunders and Kucera 2010), at the same time it is much outside of the interspecific range calculated for the same region of *Ulva* (0.95%-4.76%) (Saunders and Kucera 2010).

One more time, this underlines the difficulty in morphological identification of *Ulva* species and highlights also another current problem with *Ulva* systematics, that is the number of superfluous and synonymised species entities recorded: more than 550 historical names for 127 currently recognized species (Guiry and Guiry 2011). The use of distinct names for a same species sampled from different regions is a great hindrance for the development of a more universal, rather than more restrictive, taxonomy of algae.

U. flexuosa #VE7 specimen exhibited a tube-like filamentous light green thallus (Fig. 4B). Cells in surface view were arranged not in a distinct order in most of the areas. The single cells were angular, often squarish to elongate, 5-10 µm wide and 10-20 µm long. The chloroplast was parietal, almost filling cell, and with one or two pyrenoids (Fig. 5B).

Both according to the *tuf*A (0% nucleotide divergence) and *rbc*L-3P analyses, #LI4, #LI5, #LI6, #LI7, and #LI9, collected from Lido of Venice, result belonging to the species *U. rigida* C. Agardh (Clade C). Commonly named Sea Lettuce, this species, originally described from Cadiz (Spain), has

an ubiquitous distribution. There is an open question about the coexistence in the Venice Lagoon of this species and another laminar *Ulva*, named *U. laetevirens* Areshoug. The confusion about this topic is due to their very similar morphology. According to some authors the two species grow and coexist in the same environments and on the same substrata and the only diagnostic character to distinguish the two entities is the cell shape of rhizoidal and basal regions (Sfriso 2010). On the contrary, our results show the presence of only one species, identified as *U. rigida*. Moreover, in the *rbc*L-3P tree the clade formed by the Adriatic specimens and *U. rigida* includes also a *U. laetevirens* sample. This and the percentage of nucleotide divergence (0.14%) suggest the potential conspecificity of these two species, as proposed by previous studies (Loughnane et al. 2008; Kraft et al. 2010).

Morphological characters for Adriatic specimens of this group corresponded to published information used to delineate this species. The formation of denticulations at the edge of the thallus was observed in most of the specimens (Fig. 4C). Thalli were generally stiff in texture when fresh, sometimes perforated (Fig. 4D), and varied in colour from light to dark green. All specimens examined had more than two pyrenoids per cell (Fig. 5C) and cells were rounded in shape. In the middle region, the cell length varied between 10 and 22 µm and the cell width between 8 and 15 µm.

Two well-resolved clades were that formed by samples #LI1, #LI3 (#LI10 *tuf*A sequence failed to amplify) and *U. compressa* Linnaeus (clade D) and that constituted by the most of the Adriatic samples (22 isolates) and *U. pertusa* Kjellman (clade E). While *U. compressa* is endemic, *U. pertusa* has never been recorded in the Adriatic Sea up to now.

U. pertusa (common name: Lacy Sea Lettuce) is a Japanese species already widely introduced in Europe. In fact, it is present in France (Thau Lagoon, Atlantic coasts) (Verlaque 2001), Netherlands (Stegenga et al.2007, Gittenberger et al. 2010), Spain (Galicia, Atlantic Coast) (López et al. 2007), and Italy (Naples) (Flagella et al. 2010). This species was often misidentified as *U. rigida* (López et

al. 2007) due to its cryptic morphology. Indeed, it was the most abundant species collected during this survey, with specimens largely present in all sampling sites. Like many components of this genus, *U. pertusa* tolerates wide ranges of salinity, temperature, and water quality and grows rapidly in nutrient-rich habitats. The formation of extensive *Ulva* mats is an indicator of eutrophication in coastal marine systems (Kim et al. 2004). According to these findings, in the Lido of Venice, the less polluted of the collection sites considered (Solidoro et al., 2010; Facca et al. 2011; ARPAV, http://www.arpa.veneto.it), we found only a *U. pertusa* specimen (#LI8) in contrast with the high abundance of this species in the other areas. For these reasons it could be a probable new invasive species, in agreement with recent studies (Aguilar-Rosas et al. 2008).

Adriatic *Ulva compressa* had tubular thalli with the upper portion of main axis compressed and tapering to base. Thallus was compressed, narrowed at base and broadening distally, often with hollow margins (Fig. 4F). Cells in surface view were irregularly arranged, polygonal, usually with rounded corners, 5-10 µm in diameter. The chloroplast was laminate with one pyrenoid (Fig. 5E).

The macroscopic morphology of Adriatic *Ulva pertusa* thalli looked very similar to those of *U. rigida*, as blades were distromatic, foliose, from light to dark green in colour and often extensively perforated (Figs 4G, H). In surface view chloroplasts were confined to one side of the cell and most cells had one pyrenoid (Fig. 5F).

A probably undescribed species was discovered in the marine site of Lido of Venice. The thallus of the only specimen found (#LI2) had thin filaments departing from the base (lacinae), light green in colour (Fig. 4E). Cells in surface view were irregularly arranged, polygonal, usually with rounded corners, 6-10 µm in diameter. The chloroplast had one big or two small pyrenoids in central position (Fig. 5D).

Sample #LI2 placed far from other recognized *Ulva* species in both the trees. In the phylogenetic reconstruction based on the *rbc*L-3P region it resulted sister taxon to *Ulva* sp. B15b from the Yellow Sea (China), but the nucleotide divergence between their *rbc*L-3P sequences (0.70%)

suggests that they could be different species. Further samplings and analyses are required to determine if this specimen could represent a new entity.

CONCLUSIONS

Algal biodiversity can be impacted upon negatively (overall reduction and/or shift in composition) by factors such as global warming, increased environmental stress arising from fisheries and aquaculture activities, and accidental introductions of invasive species.

The number of these introduced species and their impact on the Mediterranean communities have been largely underestimated due to the difficulties incurring in species identification. Misidentifications are common in the case of cryptic species (i.e. species with morphologies identical or similar, though genetically different) or for environmental influences on morphological characters. Precise species recognition is needed in order to identify invasive organisms, implement biological controls, and preserve and manage the local marine resources (Bickford et al. 2007). DNA barcoding removes the reliance on morphological feature in order to discriminate species and for this reason it is a good way to have an objective survey of the entities present in the different regions (Wattier and Maggs 2001; Gabrielson et al. 2003; Andreakis et al. 2004, 2007).

This survey on *Ulva* biodiversity shows the presence of two new alien species in the North Adriatic Sea: *U. californica*, never recorded before in the Mediterranean, and the cryptic invasive species *U. pertusa*, reported for the first time in the Adriatic area. In addition we found a potential new *Ulva* species, which needs further investigations to be confirmed.

Moreover our data support the probable conspecificity between *U. rigida* and *U. laetevirens*, recently suggested by other authors. This case and that of *U. flexuosa* underline the problem of superfluous and synonymised species in *Ulva* systematics, leading to a very high number of entities listed under this taxon, and therefore the necessity of a more universal taxonomy of algae.

Finally, once again the DNA barcoding method revealed essential to solve taxonomic problems of identification in a complex macroalgal genus with hidden diversity of species, for which the morphological criteria outlined up to now look ambiguous and not diagnostic.

ACKNOWLEDGEMENTS

We thank Dr. G.W. Saunders for allowing us to carry out the amplification and sequencing reactions in his laboratory.

This work was supported by grants provided by Progetto di Ateneo of the University of Padova.

REFERENCES

Aguilar-Rosas, R., Aguilar.Rosas L. E. & Shimada, S. 2008. First Record of *Ulva pertusa* Kjellman (Ulvales, Chlorophyta) in the Pacific Coast of Mexico. *Algae* 23: 201-7.

Andreakis, N., Procaccini, G. & Kooistra, W. H. C. F. 2004. *Asparagopsis taxiformis* and *Asparagopsis armata* (Bonnemaisoniales, Rhodophyta): genetic and morphological identification of Mediterranean populations. *Eur. J. Phycol.* 39: 273-83.

Andreakis, N., Procaccini, G., Maggs, C. A. & Kooistra, W. H. C. F. 2007. Phylogeography of the invasive seaweed *Asparagopsis* (Bonnemaisoniales, Rhodophyta) reveals cryptic diversity. *Mol. Ecol.* 16: 2285-9.

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K. L., Meier, R., Winker, K., Ingram, K. K. & Das, I. 2007. Cryptic species as a window on diversity and conservation. *Trends Ecol. Evol.* 22: 148-55.

Boudouresque, C. F. & Verlaque, M. 2002. Assessing scale and impact of ship-transported alien macrophytes in the Mediterranean Sea. *In* Briand, F. [Ed.] *Alien marine organisms introduced by ships in the Mediterranean and Black seas*. CIESM 2002. Workshop Monographs 20, Monaco, pp. 53-61.

343 Brodie, J., Maggs, C. A. & John, D. M. 2007. *Green seaweeds of Britain and Ireland*. British
344 Phycological Society, London, 242 pp.
345 Cecere, E., Moro, I., Wolf, M. A., Petrocelli, A., Verlaque, M. & Sfriso, A. 2011. The introduced
346 seaweed *Grateloupia turuturu* (Rhodophyta, Halymeniales) in two Mediterranean transitional
347 water systems. *Bot. Mar.* 54: 23-33.
348 Cho, T. O., Fredericq, S. & Boo, S. M. 2003. *Ceramium inkyuii* sp. nov. (Ceramiaceae,
349 Rhodophyta) from Korea; a new species based on morphological and molecular evidence. *J.*
350 *Phycol.* 39: 237-47.
351 Collins, F. S., Holden, I. & Setchell, W. A. 1899. *Phycotheca boreali-americana. A collection of*
352 *dried specimens of the algae of North America*. Vol. Fasc. XII-XIII. Malden, Massachusetts, pp.
353 551-650.
354 Destombe, C., Valero, M. & Guillemin, M. L. 2010. Delineation of two sibling red algal species,
355 *Gracilaria gracilis* and *Gracilaria dura* (Gracilariales, Rhodophyta), using multiple DNA markers:
356 resurrection of the species *G. dura* previously described in the northern Atlantic 200 years ago. *J.*
357 *Phycol.* 46: 720-7.
358 Facca, C., Pellegrino, N., Ceoldo, S., Tibaldo, M. & Sfriso, A. 2011. Trophic Conditions in the
359 Waters of the Venice Lagoon (Northern Adriatic Sea, Italy). *The Open Oceanography Journal* 5: 1-
360 13.
361 Fama, P., Wysor, B., Kooistra, W. & Zuccarello, G. C. 2002. Molecular phylogeny of the genus
362 *Caulerpa* (Caulerpales, Chlorophyta) inferred from chloroplast *tuf*A gene. *J. Phycol.* 38: 1040-50.
363 Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evolution* 39:
364 783-791.
365 Flagella, M.M., Andreakis, N., Hiraoka, M., Verlaque, M. & Buia, M.C. 2010. Identification of
366 cryptic *Ulva* species (Chlorophyta, Ulvales) transported by ballast water. *J. Biol. Res.-Thessalon.*
367 13: 47-57.

Freshwater, D.W., Fredericq, S., Butler, B.S., Hommersand, M.H. & Chase, M.W. 1994. A gene phylogeny of the red algae (Rhodophyta) based on plastid *rbc*L. *Proc. Natl. Acad. Sci.* USA 91: 7281-7285.

Gabrielson, T. M., Brochmann, C. & Rueness, J. 2003. Phylogeny and interfertility of North Atlantic populations of "*Ceramium strictum*" (Ceramiales, Rhodophyta): how many species? *Eur. J. Phycol.* 38: 1-13.

Gittenberger, A., Rensing, M., Stegenga, H. & Hoeksema, B. 2010. Native and non-native species of hard substrata in the Dutch Wadden Sea. *Nederlandse faunistische Mededelingen* 33: 21-75.

Guindon, S. & Gascuel, O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol*. 52: 696-704.

Guiry, M. D. & Guiry, M. G. 2011. *AlgaeBase Version 4.2.* World-wide Electronic Publication, National University of Ireland, Galway. Available at: http://www.aglaebase.org (last accessed 15 September 2011).

Händeler, K., Wägele, H., Wahrmund, U., Rüdinger, M. & Knoop, V. 2010. Slugs'last meals: molecular identification of sequestered chloroplast from different algal origins in Sacoglossa (Opisthobranchia, Gastropoda). *Mol. Ecol. Resour.* 10: 968-78.

Hayden, H. S., Blomster, J., Maggs, C. A., Silva, P. C., Stanhope, M. J. & Waaland, J. R. 2003. Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. *Eur. J. Phycol.* 38: 277-94.

Hayden, H. S. & Waaland, J. R. 2004. A molecular systematic study of *Ulva* (Ulvaceae, Ulvales) from the northeast Pacific. *Phycologia* 43: 364-82.

Hebert, P. D. N., Cywinska, A., Ball, S. L. & Deeward, J. R. 2003a. Biological identifications through DNA barcodes. *P. Roy. Soc. B-Biol. Sci*. 270: 313-21.

Hebert, P. D. N., Ratnasingham, S. & Deeward, J. R. 2003b. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *P. Roy. Soc. B-Biol. Sci*. 270: 96-9.

Kim, K. Y., Choi, T. S., Kim, J. H., Han, T., Shin, H. W. & Garbary, D. J. 2004. Physiological ecology and seasonality of *Ulva pertusa* on a temperate rocky shore. *Phycologia* 43: 483-92.

Kraft, L. G. K., Kraft, G. T. & Waller, R. F. 2010. Investigations into southern Australian *Ulva* (Ulvophyceae, Chlorophyta) taxonomy and molecular phylogeny indicate both cosmopolitanism and endemic cryptic species. *J. Phycol.* 46: 1257-77.

López, S. B., Fernàndez, I. B., Lozano, R. B. & Ugarte, J. C. 2007. Is the cryptic alien seaweed *Ulva pertusa* (Ulvales, Chlorophyta) widely distributed along European Atlantic coasts? *Bot. Mar.* 50: 267-74.

Loughnane, C. J., McIvor, L. M., Rindi, F., Stengel, D. B. & Guiry, M. D. 2008. Morphology, *rbc*L phylogeny and distribution of distromatic *Ulva* (Ulvophyceae, Chlorophyta) in Ireland and southern Britain. *Phycologia* 47: 416-29.

Maddison, W. D. & Maddison, D. R. 2005. *MacClade v4.08*. Sinauer Associates, Sunderland, Massachusetts.

Maddison, W.P. & Maddison, D.R. 2009. *Mesquite: a modular system for evolutionary analysis. Version 2.71.* Available at: http://mesquiteproject.org (last accessed 7 September 2009).

Malta, E.-J., Draisma, S. G. A. & Kamermans, P. 1999. Free floating *Ulva* in the southwest Netherlands: species or morphotypes? A morphological, molecular and ecological comparison. *Eur. J. Phycol.* 34: 443-54.

Manhart, J. R. 1994. Phylogenetic Analysis of Green Plant *rbc*L Sequences. *Mol. Phyl. Evol.* 3: 114-27.

McLusky, D. S. & Elliot, M. 2007. Transitional waters: a new approach, semantics or just muddying the waters? *Estuar. Coast. Shelf Sci.* 71: 359-63.

Nelson, W., Heesch, S., Broom, J., Neill, K., Farr, T. & Dalen, T. 2007. Using molecular tools to tackle troublesome genera: Ulvaceae in New Zealand – diversity and introduced species revealed.

Proceedings of the 22nd Annual Conference of the Australasian Society of Phycology and Aquatic Botany, 26–29 November 2007. Deakin University, Warrnambool, Victoria, Australia, p. 22.

Perrière, G. & Gouy, M. 1996. WWW-Query: An on-line retrieval system for biological sequence banks. *Biochimie* 78: 364-369.

Posada, D. & Crandall, K.A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817-8.

Posada, D. & Buckley, T.R. 2004. Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. *Syst. Biol.* 53: 793-808.

Rambaut, A. & Drummond, A.J. 2007. *Tracer v1.5*. Available at: http://beast.bio.ed.ac.uk/Tracer (last accessed 4 August 2009).

Ronquist, F. & Huelsenbeck, J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572-4.

Saunders, G. W. 1993. Gel purification of red algal genomic DNA - an inexpensive and rapid method for the isolation of polymerase chain reaction-friendly DNA. *J. Phycol.* 29: 251-4.

Saunders, G. W. 2005. Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. *Philos. Trans. SerB* 360: 1879-88.

Saunders, G. W. 2008. A DNA barcode examination of the red algal family Dumontiaceae in Canadian waters reveals substantial cryptic species diversity. 1. The foliose *Dilsea-Neodilsea* complex and *Weeksia. Botany* 86: 773-89.

Saunders, G. W. 2009. Routine DNA barcoding of Canadian Gracilariales (Rhodophyta) reveals the invasive species *Gracilaria vermiculophylla* in British Columbia. *Mol. Ecol. Resour.* 9: 140-50.

Saunders, G. W. & Kucera H. 2010. An evaluation of *rbc*L, *tuf*A, UPA, LSU and ITS as DNA barcode markers for the marine green macroalgae. *Cryptog. Algol.* 31: 487-528.

Sfriso, A. & Curiel, D. 2007. Check-list of marine seaweeds recorded in the last 20 years in Venice lagoon and a comparison with the previous. *Bot. Mar*. 50: 22-58.

Sfriso, A., Curiel, D. & Rismondo, A. 2009. The Lagoon of Venice. *In* Cecere E., Petrocelli A., Izzo G. & Sfriso A. [Eds.] *Flora and vegetation of the Italian transitional water systems*. Corila, Multigraf Spinea, Venezia, pp. 195-227.

Sfriso, A. 2010. Coexistence of *Ulva rigida* and *Ulva laetevirens* (Ulvales, Chlorophyta) in Venice Lagoon and other Italian transitional and marine environments. *Bot. Mar.* 53: 9-18.

Skage, M., Gabrielsen, T. M. & Rueness, J. 2005. A molecular approach to investigate the phylogenetic basis of three widely used species groups in the red algal genus *Ceramium* (Ceramiales, Rhodophyta). *Phycologia* 44: 353-60.

Solidoro, C., Bandelj, V., Aubry Bernardi, F., Camatti, E., Ciavatta, S., Cossarini G., Facca, C., Franzoi, P., Libralato, S., Melaku Canu, D., Pastres, R., Pranovi, F., Raicevich, S., Socal, G., Sfriso, A., Sigovini, M., Tagliapietra, D. & Torricelli, P. 2010. Response of Venice Lagoon ecosystem to natural and anthropogenic pressures over the last 50 years. *In* Kennish M.H.K. & Paerl H. [Eds.] *Coastal Lagoons: Critical Habitats of Environmental Change*. CRC Press, Boca Raton, FL, pp. 483-512.

Stegenga, H., Karremans, M. & Simons, J. 2007. Zeewieren van de voormalige oesterputten bij Yerseke. *Gorteria* 32: 125-143.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-80.

Verlaque, M. 2001. Checklist of the macroalgae of Thau Lagoon (Hérault, France), a hot spot of marine species introduction in Europe. *Oceanologica Acta* 24: 29-49.

Wattier, R. & Maggs, C. A. 2001. Intraspecific variation in seaweeds: The application of new tools and approaches. *Adv. Bot. Res.* 35: 171-212.

Wolf, M. A., Sfriso, A., Andreoli, C. & Moro, I. 2011a. The presence of exotic *Hypnea flexicaulis* (Rhodophyta) in the Mediterranean Sea as indicated by morphology, *rbc*L and *cox*1 analyses. *Aquat. Bot*. 95: 55-8.

Wolf, M. A., Sciuto, K., Maggs, C. A., Andreoli, C. & Moro, I. 2011b. *Ceramium* Roth (Ceramiales, Rhodophyta) from the Adriatic Sea (Venice lagoon, Italy): comparative studies of Mediterranean and Atlantic taxa provide new taxonomic insights into a complex genus. *Taxon* (in press).

Zuccarello, G. C., Burger, G., West, J. A. & King, R. J. 1999. A mitochondrial marker for red algal intraspecific relationships. *Mol. Ecol.* 8: 1443-47.

Zuccarello, G., Price, N., Verbruggen, H. & Leliaert, F. 2009. Analysis of plastid multigene dataset and the phylogenetic position of the marine macrolaga *Caulerpa filiformis* (Chlorophyta). *J. Phycol.* 45: 1206-12.

LEGENDS

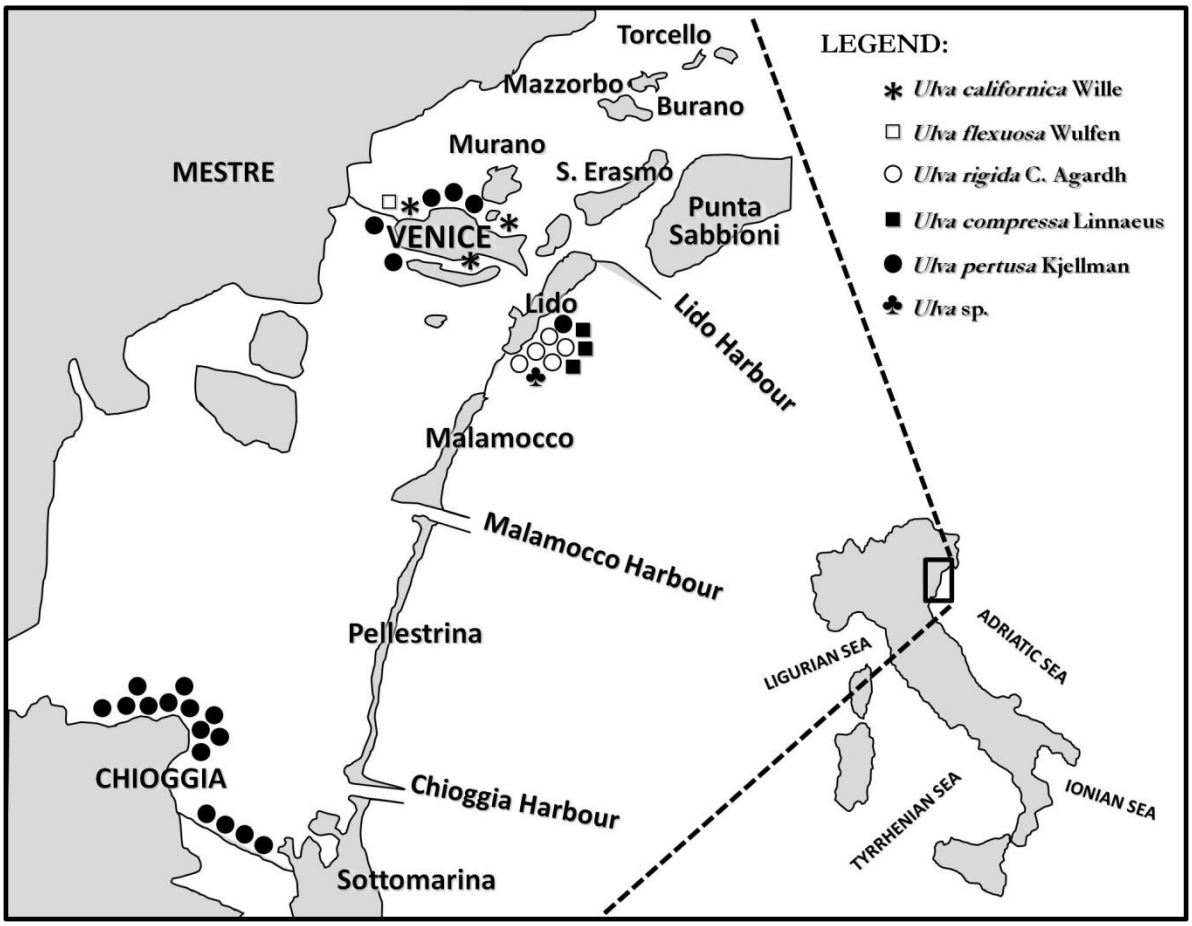
Fig. 1. Map showing the sampling sites of the Venice Lagoon (Italy). Each symbol represents a given *Ulva* specimen in the considered areas. Map and symbols are not in scale.

Fig. 2. Maximum-likelihood (ML) tree inferred from *rbc*L sequences calculated using the TrN+I+G evolutionary model. Numbers near each node refer to bootstrap values (1000 replications) and Bayesian posterior probabilities (PP), denoted in bold. Nodes with <50% bootstrap support and <0.90 Bayesian support are not labeled. As the Adriatic *U. pertusa* sequences were identical and given the high number of samples for this taxon, only a sequence for each collection site was submitted.

Fig. 3. Maximum-likelihood (ML) tree of *tuf*A sequences calculated using the evolution model GTR + I +G. ML bootstrap values (1000 replications) and Bayesian posterior probabilities (PP) (in bold) are shown for each clade. Nodes with <50% bootstrap support and <0.90 Bayesian support are not labeled. As the Adriatic *U. pertusa* sequences were identical and given the high number of samples for this taxon, only a sequence for each collection site was submitted.

Fig. 4. Habit of *Ulva* spp. Bars = 2 cm. (A) *Ulva californica* Wille, specimen #VE9. (B) *Ulva flexuosa* Wulfen, specimen #VE7. (C, D) *Ulva rigida* C. Agardh, specimens #LI5 and #LI7 respectively. (E) *Ulva* sp., specimen #LI2. (F) *Ulva compressa* Linnaeus, specimen #LI3. (G, H) *Ulva pertusa* Kjellman, specimens #LI8 and #PU2 respectively.

Fig. 5. Surface view through middle region of the thalli of *Ulva* spp. Bars = 20 µm. Arrows indicate the presence of pyrenoids in the cells. (A) *Ulva californica* Wille, specimen #VE9. (B) *Ulva flexuosa* Wulfen, specimen #VE7. (C) *Ulva rigida* C. Agardh, specimen #LI5. (D) *Ulva* sp., specimen #LI2. (E) *Ulva compressa* Linnaeus, specimen #LI3. (F) *Ulva pertusa* Kjellman, specimen #LI8.


Torcello
Mazzorbo
Burano
Murano
MESTRE
S. Erasmo
Punta
Sabbioni
VENICE
Lido
Lido Harbour
Malamocco
Malamocco Harbour
Pellestrina
CHIOGGIA
Chioggia Harbour
Sottomarina
LEGEND:
Ulva californica Wille
Ulva flexuosa Wulfen
Ulva rigida C. Agardh
Ulva compressa Linnaeus
Ulva pertusa Kjellman
Ulva sp.
LIGURIAN SEA
ADRIATIC SEA
TYRRHENIAN SEA
IONIAN SEA

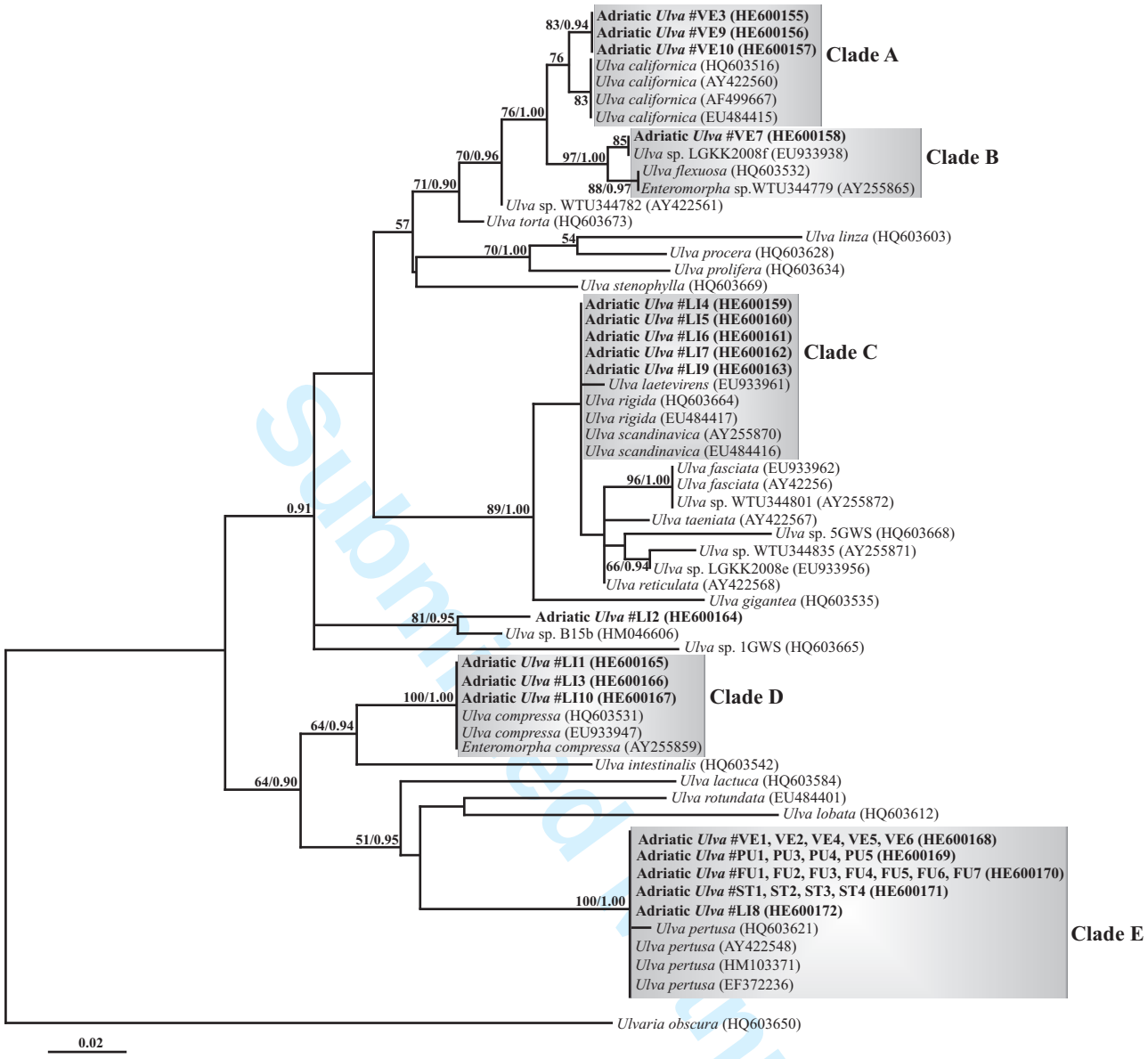


Fig. 2

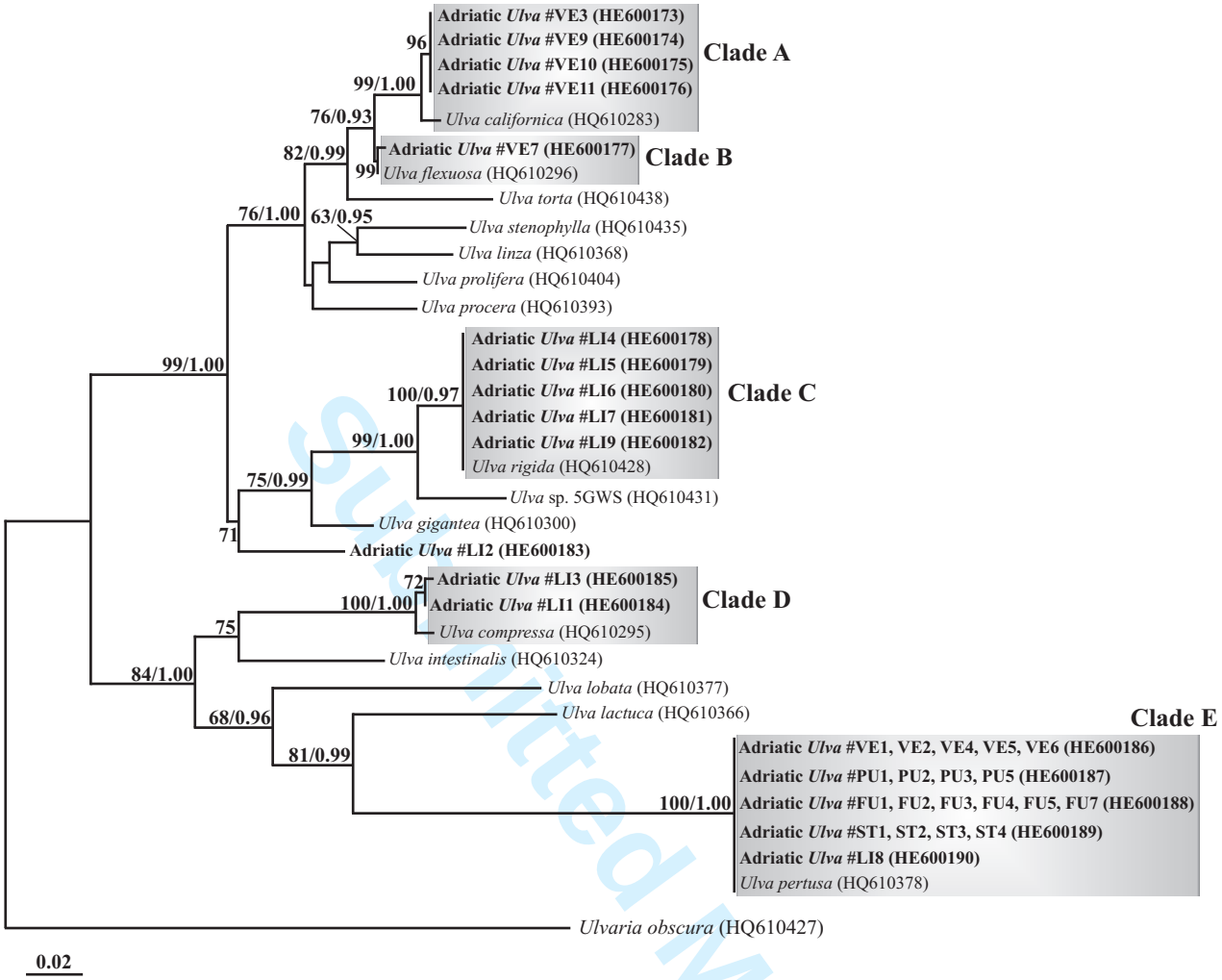


Fig. 3

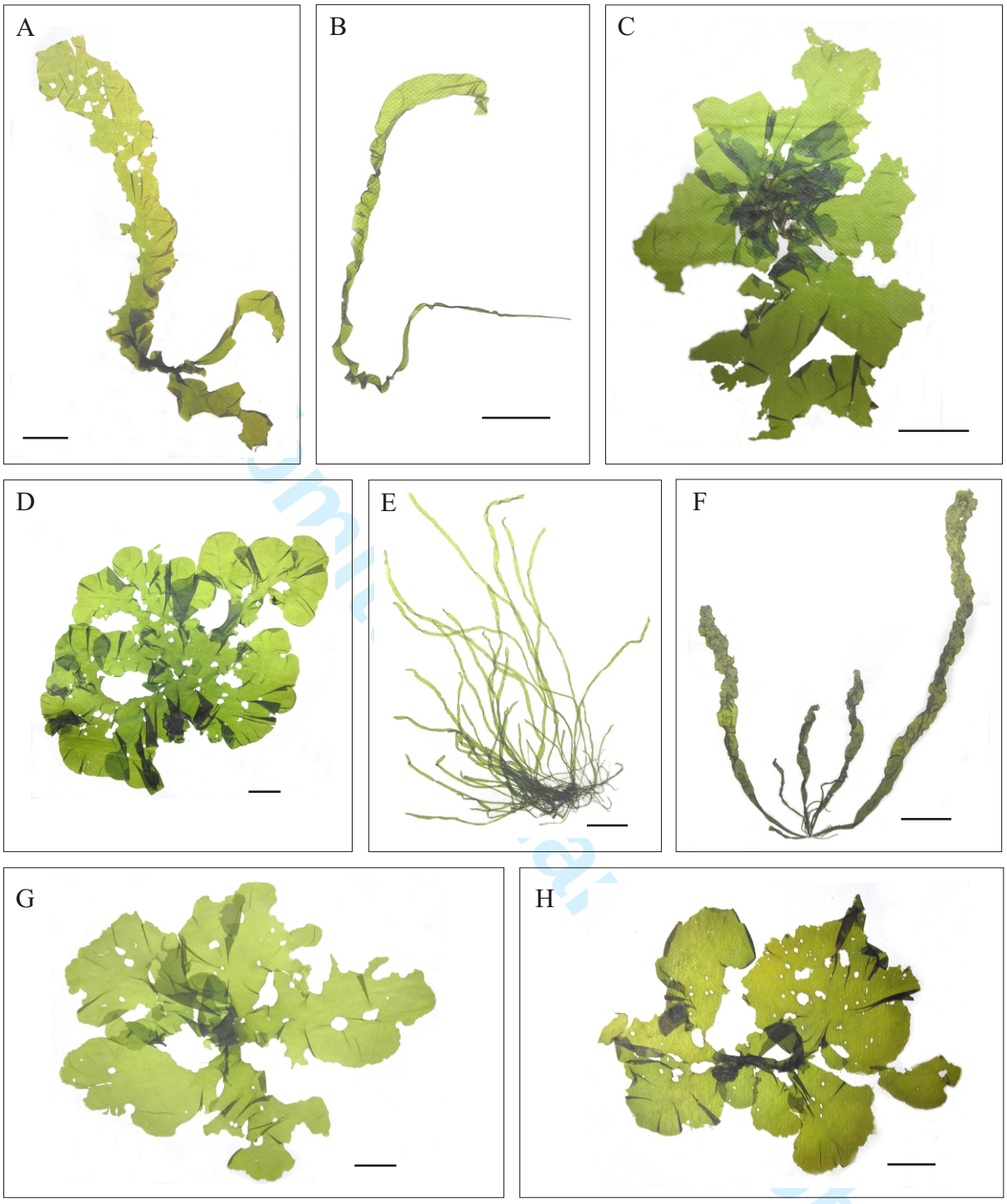


Fig. 4

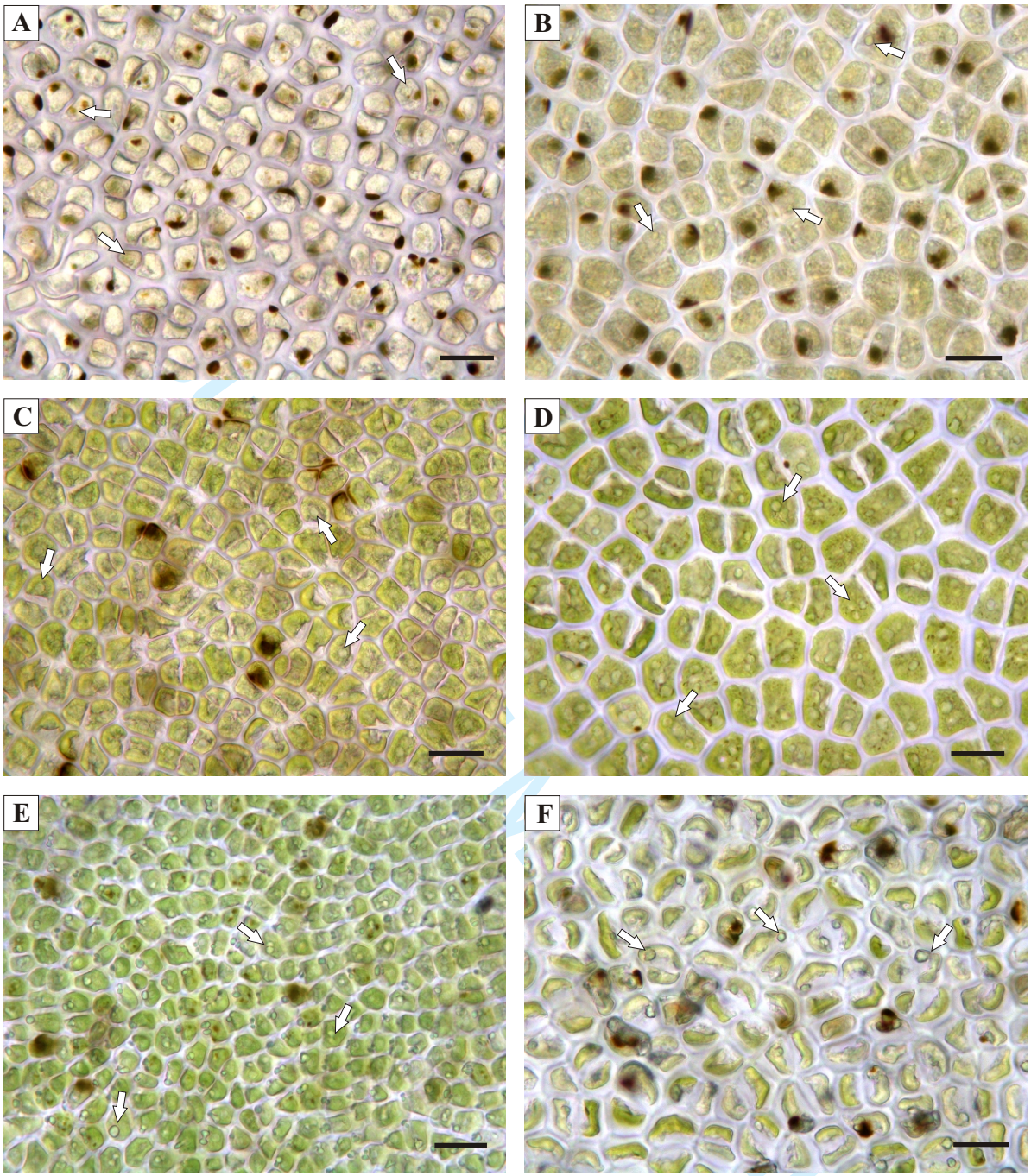


Fig. 5

Table 1. Collection data of the Adriatic *Ulva* samples.

Sample ID	Collection Date	Area	Site	Latitude	Longitude
FU1-FU7	24/01/2011	Chioggia inlet	Fondamenta Merlin	45° 13' 25.55"N	12° 16' 47.19"E
LI1-LI10	19/02/2011	Lido of Venice	Ca' Bianca	45° 23' 28.38"N	12° 21' 25.75"E
PU1-PU5	24/01/2011	Chioggia inlet	Ponte dell'Unione	45° 12' 56.83"N	12° 17' 3.10"E
ST1-ST4	24/01/2011	Chioggia inlet	Hydrobiological Station	45° 13' 23.00"N	12° 17' 0.24"E
VE1-VE8	10/02/2011	Venice Lagoon	S. Marta	45° 26' 17.11"N	12° 18' 54.65"E
VE9-VE11	10/02/2011	Venice Lagoon	Piazzale Roma	45° 26' 14.26"N	12° 19' 12.84"E

Table 2. Divergence percentages of *rbc*L-3P region sequences of the Adriatic samples and other *Ulva* specimens available in GenBank, that grouped in the *rbc*L-3P phylogenetic tree.
* synonym of *U. rigida* in Algaebase.

SPECIES	COLLECTION SITES	NUCLEOTIDE DIVERGENCES
Adriatic specimens of CLADE A VS.		
Ulva californica Wille HQ603516	Canada, British Columbia	0.28%
Ulva californica Wille AY422560	USA, California	0.28%
Ulva californica Wille AF499667	USA, Colture Collection	0.28%
Ulva californica Wille EU484415	Ireland	0.28%
Adriatic specimens of CLADE B VS.		
Ulva flexuosa Wulfen HQ603532	Canada, British Columbia	0.28%
Enteromorpha sp. AY255865	USA, California	0.28%
Ulva sp. EU933938	South Australia	0%
Adriatic specimens of CLADE C VS.		
Ulva rigida C. Agardh EU484417	Ireland	0%
Ulva rigida C. Agardh HQ603664	Canada, New Brunswick	0%
Ulva scandinavica Bliding AY255870*	England	0%
Ulva scandinavica Bliding EU484416*	Ireland	0%
Ulva laetevirens Areschoug EU933961	South Australia	0.14%
Adriatic specimens of CLADE D VS.		
Ulva sp. HM046606	Yellow Sea	0.70%
Adriatic specimens of CLADE E VS.		
Ulva compressa Linnaeus HQ603531	Canada, New Brunswick	0%
Ulva compressa Linnaeus EU933947	Australia, Victoria	0%
Enteromorpha compressa (Linnaeus) Nees AY255859	British Isles	0%
Adriatic specimens of CLADE F VS.		
Ulva pertusa Kjellman HM103371	Iberian Islands	0%
Ulva pertusa Kjellman EF372236	European Atlantic Coasts	0%
Ulva pertusa Kjellman AY422548	USA, California	0%
Ulva pertusa Kjellman HQ603621	Canada, British Columbia	0.14%

Ceramium Roth (Ceramiales, Rhodophyta) from Venice lagoon (Adriatic Sea, Italy): Comparative studies of Mediterranean and Atlantic taxa

Marion A. Wolf,[1*] Katia Sciuto,[1*] Christine A. Maggs,[2] Maria Beatriz B. de Barros-Barreto,[3] Carlo Andreoli[1] & Isabella Moro[1]

1 *Department of Biology, University of Padova, via U. Bassi 58/B, 35131 Padova, Italy*

2 *School of Biological Sciences, Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, U.K.*

3 *Departamento de Botânica, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Laboratório Integrado de Ficologia, Bloco A, sala 99, sub-solo 2562-6331, Brazil*

* These authors contributed equally to this work.

Author for correspondence: *Isabella Moro, isabella.moro@unipd.it*

Abstract The cosmopolitan genus *Ceramium* (Ceramiaceae, Rhodophyta) is a large and systematically complex group. The taxonomy of this genus remains in a chaotic state due to the high degree of morphological variation. Culture studies, suggesting a strong influence of environment on phenotype, and the use of molecular tools have recently questioned the validity of morphological features used in species recognition. Here we compare three *Ceramium* taxa from Venice lagoon with samples from northwest Europe using the plastid ribulose-1,5-bisphosphate carboxylase/oxygenase gene (*rbcL*) and the *rbcL-rbcS* intergenic spacer combined with morphological observations. A strongly banded species, previously identified as member of a poorly understood and misnamed group, the *Ceramium diaphanum* complex sensu Feldmann-Mazoyer, is probably conspecific with British samples of *Ceramium diaphanum* sensu Harvey, for which no valid name has been identified up to now. We show that *Ceramium polyceras* (Kützing) Zanardini is a valid name for this species. A fully corticated *Ceramium* species morphologically resembling *C. secundatum* differs at the species level from Atlantic *C. secundatum*; a valid name for this entity is *Ceramium derbesii* Solier ex Kützing, described from Mediterranean France. A third species characterized by cortical spines, previously listed as *Ceramium ciliatum* var. *robustum* (J. Agardh) Mazoyer, is shown to be *Ceramium nudiusculum* (Kützing) Rabenhorst, originally described from Venice.

Keywords *Ceramium*; Mediterranean seaweeds; phylogeny; *rbcL*; *rbcL-rbcS* spacer; species delimitation; Venice lagoon

Supplementary Material The alignment files are available in the Supplementary Data section of the online version of this article (http://ingentaconnect.com/content/iapt/tax).

■ INTRODUCTION

The cosmopolitan genus *Ceramium* Roth (Ceramiaceae) is one of the most speciose genera in the Rhodophyta. It typically occurs in eulittoral or shallow subtidal habitats and has a worldwide distribution (Maggs & al., 2002). The genus is characterized by cylindrical or slightly compressed thalli, axial cells bearing regular rings of periaxial cells and becoming incompletely to completely covered by cortical cells, alternate to pseudo-dichotomous branching, and straight to inrolled apices (Dixon, 1960; Maggs & Hommersand, 1993). Traditionally, three morphological groups have been recognized (Skage & al., 2005): (1) species that bear cortical spines; (2) species able to form cortication that completely covers the internodes; and (3) species with ecorticate internodes. However, these groups are not resolved as clades in molecular studies (Skage & al., 2005; Barros-Barreto & al., 2006).

Currently, the systematics of this genus is still rather chaotic despite several recent studies (Maggs & al., 2002; Cho & al., 2003, 2008; Skage & al., 2005; Barros-Barreto & al., 2006). Taxonomic problems are linked to the high degree of variation in morphological characters classically used in species recognition: presence of cortical spines, numbers of periaxial cells, developmental patterns of the corticating filaments, branching pattern, and tetrasporangial features (Maggs & Hommersand, 1993). In addition, culture studies, suggesting a strong influence of the environment on morphology, and the results of molecular analyses have questioned the validity of morphological features for discriminating *Ceramium* species (Maggs & al., 2002). The identification of new *Ceramium* species is often complicated by their small size and epiphytic habit (South & Skelton, 2000). Molecular markers are therefore of increasing importance for screening and assessing *Ceramium* biodiversity (Maggs & al., 2002; Cho & al., 2003, 2008; Gabrielsen & al., 2003; Skage & al., 2005; Barros-Barreto & al., 2006).

Difficulties in delimiting species result in and are affected by nomenclatural problems. Since the establishment of the genus *Ceramium* by Roth (1797), taxon circumscriptions have undergone considerable changes. Currently, 893 species (and infraspecific) names are reported in the international database AlgaeBase (Guiry & Guiry, 2011), of which only 177 have been flagged as currently accepted taxonomically. A particular

problem concerning the nomenclature of the European species (with implications for the rest of the world) is that recent studies combining morphological with molecular investigations have mostly been based on Atlantic coasts (Maggs & al., 2002; Skage & al., 2005; Barros-Barreto & al., 2006), whereas a large number of potentially valid species names in *Ceramium* are based on Mediterranean types. For example, *Ceramium circinatum* Kützing (1842) was described from the Mediterranean Sea under nine different names by Kützing (1842, 1843, 1862) and then reported from the Atlantic under another name (Maggs & Hommersand, 1993: 43). Furthermore, one of the most detailed morphological and ecological studies of the genus *Ceramium* was restricted to the western Mediterranean (Feldmann-Mazoyer, 1941). Therefore it has been difficult to compare Atlantic and Mediterranean *Ceramium* taxa in order to evaluate species status and morphological variation, and, thereby, to determine geographical ranges as well as correct nomenclature. Indeed, despite extensive literature and herbarium searches, Maggs & Hommersand (1993) were unable to establish valid names for several common species of *Ceramium* in the British Isles.

The aim of the present study was to use combined morphological and molecular phylogenetic analyses to compare some common *Ceramium* entities from the Venice lagoon (Adriatic Sea) with morphologically similar species from the Atlantic Ocean. Phylogenetic analyses were based on the *rbcL* gene, very commonly employed in molecular systematics of red algae (Freshwater & al., 1994; Fredericq & al., 1996; Hommersand & al., 1999; Gurgel & Fredericq, 2004), and the more variable intergenic *rbcL-rbcS* spacer.

■ MATERIALS AND METHODS

Field collections and sample preparation. — Sampling was carried out in May and June 2010 (late spring/early summer) at the Alberoni breakwater (45°33′N, 12°33′E) in the lagoon of Venice (Italy). The narrow intertidal zone (ca. 20 cm) and shallow subtidal zones (to 1 m depth) were sampled by snorkeling. Macroscopic *Ceramium* samples representing examples of the three morphological groups (spiny; fully corticated; banded) were collected, transported to the laboratory, and sorted under a stereo zoom microscope (Leica Zoom 2000).

A preliminary identification of each group of species was made using Maggs & Hommersand (1993), and for the spiny species we also used information in Dixon (1960) and Itono (1972). Several specimens of each group were selected. Each specimen was divided, with part of it preserved in 4% Formalin/seawater for morphological observations, some dried in silica gel for genomic DNA extraction, and some pressed as a voucher. Voucher specimens have been deposited at PAD, with the following codes: HA 3150-1, HA 3150-2, HA 3150-3, HA 3150-4, HA 3150-5, HA 3150-6, HA 3150-7, HA 3150-8, HA 3150-9, HA 3150-10, HA 3150-11.

Sections for microscopic observations were made by hand using a razor blade under a stereo zoom microscope (Leica Zoom 2000). Photographs were taken with a digital camera attached to a light microscope (Leica DMR 5000).

DNA extraction, amplification, and sequencing. — Genomic DNA was isolated from 5 to 15 mg of dried material, ground in liquid nitrogen, and extracted using the Genomic DNA purification kit (Fermentas International, Burlington, Ontario, Canada) according to the manufacturer's instructions. Extracted DNA was stored at −20°C and used for amplification of the plastid *rbcL* gene and the intergenic *rbcL-rbcS* spacer.

The primers employed to amplify the *rbcL* gene, F57 and RrbcSstart, are listed in Freshwater & Rueness (1994). For the *rbcL-rbcS* spacer the RrbcSstart primer was used in combination with the forward primer F1320 designed by Freshwater & al. (2006). PCR reactions were performed as follows: an initial denaturation step of 2 min at 94°C, 30 cycles of 94°C for 40 s, 50°C for 40 s, 72°C for 40 s, and a final extension step at 72°C for 5 min. The amplification products were cleaned with ExoSAP-ITTM kit (GE Healthcare, Uppsala, Sweden), following the manufacturer's protocol.

Sequences were determined for both forward and reverse strands at the BMR Genomics Sequencing Center (Padova University, Italy). The final consensus sequences were assembled using the SeqMan II program from the Lasergene software package (DNAStar, Madison, Wisconsin, U.S.A.) and compared with others available online using BLAST v.2.0 software (Altschul & al., 1990). The nucleotide sequences obtained during this study were deposited in the DDBJ/GenBankTM/EBI Data Bank under the accession numbers reported in bold on the trees. Voucher specimen codes and collection data of the other *Ceramium* species whose *rbcL* gene sequences have been produced during this work for comparison are reported in the Appendix.

Phylogenetic analyses. — Separate datasets were prepared for the *rbcL* gene and the *rbcL-rbcS* spacer, using the new sequences plus other *Ceramium* sequences in the DDBJ/GenBankTM/EBI Data Bank. Multiple alignments for phylogenetic analyses were generated with the Clustal W v.2.0 computer program (Thompson & al., 1994) and are given both in PHYLIP and NEXUS formats.

Maximum Parsimony (MP) analyses were performed by means of MEGA v.4.0 software (Tamura & al., 2007). Maximum Likelihood (ML) analyses were carried out with PHYML v.3.065 (Guindon & Gascuel, 2003). Non-parametric bootstrap re-sampling (Felsenstein, 1985) was performed to test the robustness of the tree topologies (1000 replicates). All Bayesian Inference (BI) analyses were carried out with MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003) and included two separate concurrent MCMC runs, each composed of four chains, three heated and one cold. Each Markov chain ran for 3,000,000 generations, in the case of *rbcL* gene, and 1,100,000 generations, in the case of *rbcL-rbcS* spacer, sampling trees every 100 generations. At the end of each run we considered the sampling of the posterior distribution to be adequate if the average standard deviation of split frequencies was <0.01. The first 7500 trees, for the *rbcL* gene, and 2500 trees, for the *rbcL-rbcS* spacer, were discarded as burn-in, as determined by stationarity of lnL assessed using Tracer v.1.5 (Rambaut & Drummond, 2007). Consensus topologies and posterior probability values were then calculated from the remaining trees. The nexus files for

Table 1. Nucleotide divergence percentages between the Venice lagoon taxa and other key species for both *rbcL* and *rbcL-rbcS* markers.

Taxa	*rbcL* [%]	*rbcL-rbcS* [%]
***Ceramium polyceras* (Kützing) Zanardini (clade A)**		
C. polyceras–C. diaphanum sensu Harvey	0.80	
C. polyceras–C. pallidum (Nägeli ex Kützing) Maggs & Hommersand	1.88	2.56
C. polyceras–C. siliquosum (Kützing) Maggs & Hommersand	3.39	1.54
C. polyceras–Ceramium sp. Ischia (Italy)	3.57	
C. polyceras–C. tenuicorne (Kützing) Waern	3.48	2.05
C. polyceras–C. strictum sensu Harvey	4.29	3.59
C. polyceras–C. "*diaphanum*" Atlantic North America	6.61	
C. polyceras–C. diaphanum (Lightfoot) Roth	8.75	
C. polyceras–Ceramium sp. Nova Scotia (Canada)		5.13
***Ceramium derbesii* Solier ex Kützing (clade B)**		
C. derbesii–C. secundatum Lyngbye	2.05	3.08
C. derbesii–C. botryocarpum A.W. Griffiths ex Harvey	2.50	2.56
C. derbesii–C. barbatum (J.E. Smith) Duby from Italy	3.30	
C. derbesii–C. virgatum Roth	4.55	
***Ceramium nudiusculum* (Kützing) Rabenhorst (clade C)**		
C. nudiusculum–C. ciliatum (J. Ellis) Ducluzeau	5.27	8.21
C. nudiusculum–C. circinatum (Kützing) J. Agardh	5.98	

the BI analyses were generated with the Mesquite v.2.71 software package (Maddison & Maddison, 2009).

For ML and BI analyses, the models that best fit our data were found using the program jMODELTEST v.0.1.1 under the Akaike Information Criterion (AIC) (Posada & Crandall, 1998; Posada & Buckley, 2004). Tree topologies were visualized with the NJplot v.2.3 computer program (Perrière & Gouy, 1996). Nucleotide sequence divergence values within the clades obtained were calculated (Table 1).

■ RESULTS

Habitat and morphology of clade A samples (HA 3150-8, HA 3150-9, HA 3150-10, HA 3150-11). — Tetrasporangial and cystocarpic thalli growing on algae (e.g., *Cystoseira* sp.) and mussels (e.g., *Mytilus edulis*); thalli bushy, 2–9 cm high (Fig. 1A), dull purple in colour, bleaching to yellowish with less pigmented older axes, consisting of entangled axes giving rise to dense hemispherical to cylindrical tufts. Axes pseudodichotomously branched and distinctly banded to the naked eye. Main axes (Fig. 1B) incompletely corticated, branching at intervals of 6 to 7 segments with branch angles of 30° to 45° and abundant adventitious branchlets. Apices tightly inrolled and nodes consisting of 6 periaxial cells (Fig. 1C). Tetrasporangia (Fig. 1D) borne in whorls in every node of fertile axes, entirely covered by cortical filaments. Cystocarps (Fig. 1E–F) consisting of globular gonimolobes, up to 180 µm in diameter, surrounded when mature by a whorl of 4 to 6 incurved involucral branchlets. Gonimolobes (Fig. 1G) containing numerous angular carposporangia, 20–35 µm in diameter.

Habitat and morphology of clade B samples (HA 3150-1, HA 3150-2, HA 3150-3).— Thalli (Fig. 2A) epilithic on bedrock, often associated with *Polysiphonia* species; 4–10 cm high, brownish-red in colour, consisting of irregular-shaped tufts of one to several erect axes attached by a dense mass of multicellular rhizoidal filaments. Numerous, extremely irregular, lateral branches developing from main axes. Axes (Fig. 2B) up to 500 µm in diameter, sometimes swollen with internodal constrictions when older, typically fully corticated, rarely incompletely corticated with apices forcipate to tightly inrolled. Branching interval of 13–23 segments; adventitious branches abundant in fertile female thalli (Fig. 2C). Nodes consisting of 7 periaxial cells. Tetrasporangia not found. Cystocarps either lateral (Fig. 2D), with fertile female axes commonly deflected about 45° away from them, or terminal, due to deciduous fertile axes. Gonimoblasts (Fig. 2E) globose, sessile, consisting of a single central gonimolobe of about 400 µm in diameter surrounded, when mature, by 5 to 6 short involucral branchlets, with continuous cortication. Carposporangia angular, 30–35 µm in diameter.

Habitat and morphology of clade C samples (HA 3150-4, HA 3150-5, HA 3150-6, HA 3150-7). — Thalli (Fig. 3A) epilithic on bedrock and epiphytic on various algae (e.g., *Ulva* sp.); 3–7 cm high, red-brown in colour, consisting of interwoven prostate axes giving rise to groups of erect pseudodichotomously branched axes (Fig. 3B) corticated only at the nodes. Main axes up to 700 µm in diameter, forcipate with strongly inrolled apices (Fig. 3C), and sparse to numerous adventitious branches on older axes. Nodes consisting of 6 periaxial cells bearing cortical filaments. Spines (Fig. 3C) present on nodal bands of younger axes, normally singly but occasionally paired, typically in an adaxial or abaxial row, 2–4 cells long, mostly

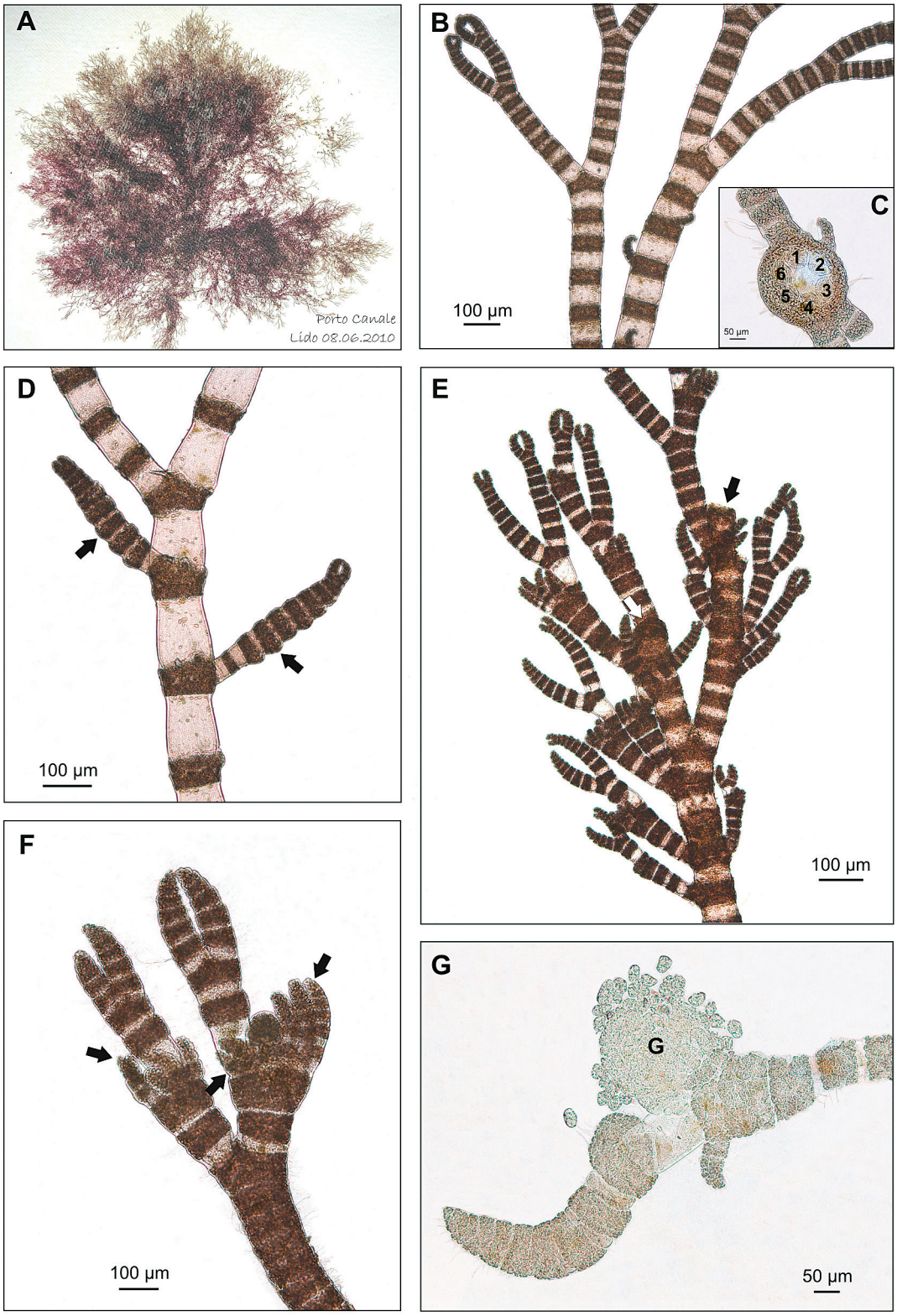


Fig. 1. *Ceramium polyceras* (previously identified as *Ceramium diaphanum* sensu Harvey). **A,** Habit of thallus; **B,** incompletely corticated axes, branching at intervals of 6 segments with branch angles of 30°; **C,** node consisting of 6 periaxial cells (numbered); **D,** fertile axes bearing tetrasporangia in whorls in every node (arrows); **E–F,** female plant with fertile axes bearing cystocarps surrounded by 4 to 6 involucral branchlets (arrows); **G,** gonimolobe (G) containing numerous angular carposporangia.

4-celled, almost straight, acropetally directed, 100–125 µm long × 26–36 µm wide. Rhizoids (Fig. 3D) formed by cortical cells in whorls around the nodes, simple, multicellular, 16–32 µm wide and terminating in multicellular discoid pads. Tetrasporangia (Fig. 3E) ellipsoid (65–70 × 40–56 µm), borne on younger axes in whorls, entirely covered by cortical cells. Cystocarps (Fig. 3F) consisting of globular gonimolobes, about 400 µm in diameter, enclosed in a membrane and surrounded by a whorl of 6 simple, inrolled, involucral branchlets. Carposporangia angular, 20–40 µm in diameter.

Molecular analyses. — Phylogenetic analyses using different methods yielded comparable results, except for the positions

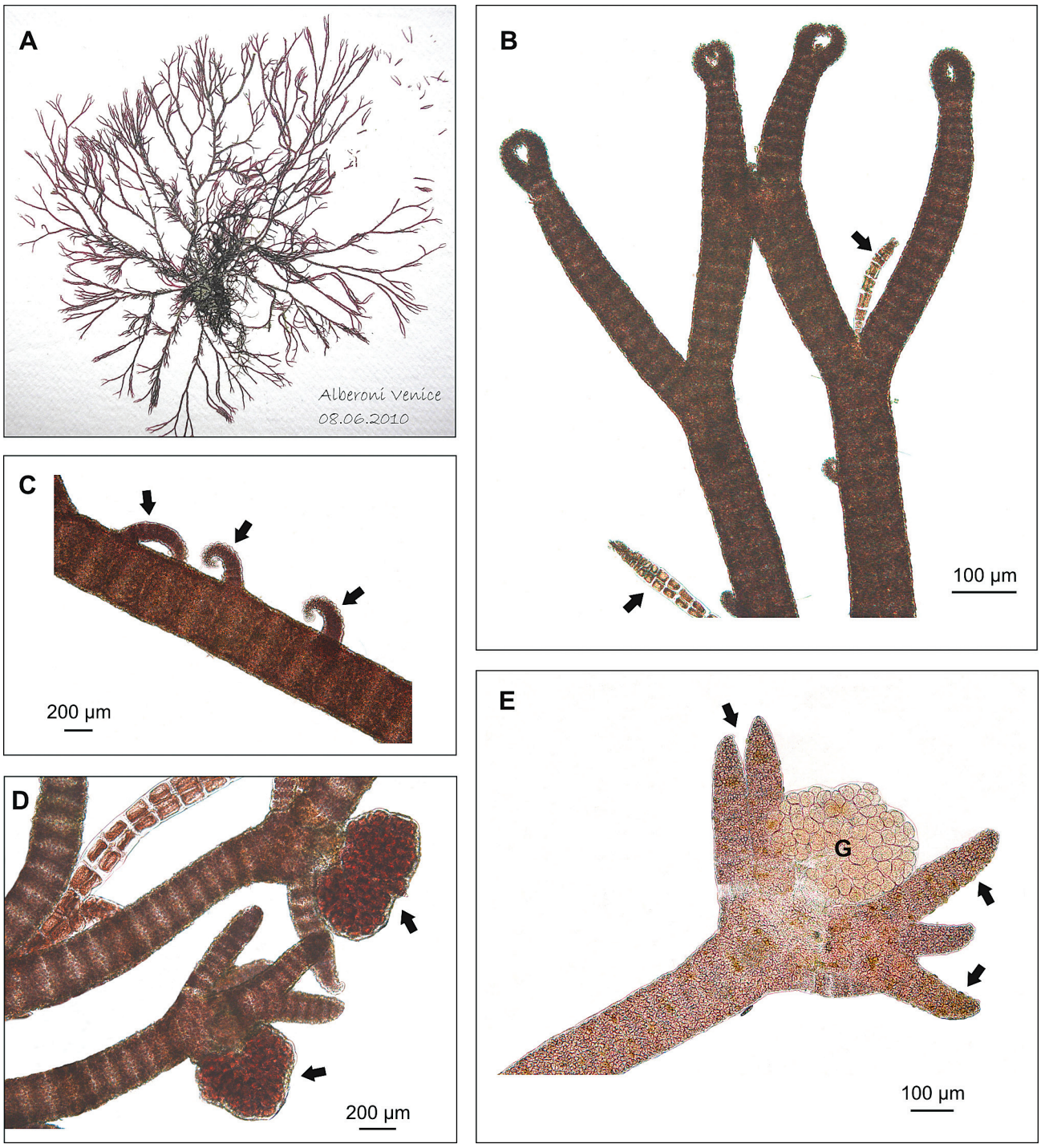


Fig. 2. *Ceramium derbesii* (previously identified as *C. secundatum* or *C. rubrum* var. *barbatum*). **A,** Habit of the thallus; **B,** axes, typically fully corticated, sometimes swollen or nodular, with epiphytic *Polysiphonia* sp. (arrows); **C,** adventitious branchlets (arrows) in female thalli; **D,** lateral cystocarps (arrows) deflected about 45° from female axes; **E,** gonimoblast consisting of a single central gonimolobe (G) containing numerous angular carposporangia surrounded by 5 involucral branchlets (arrows).

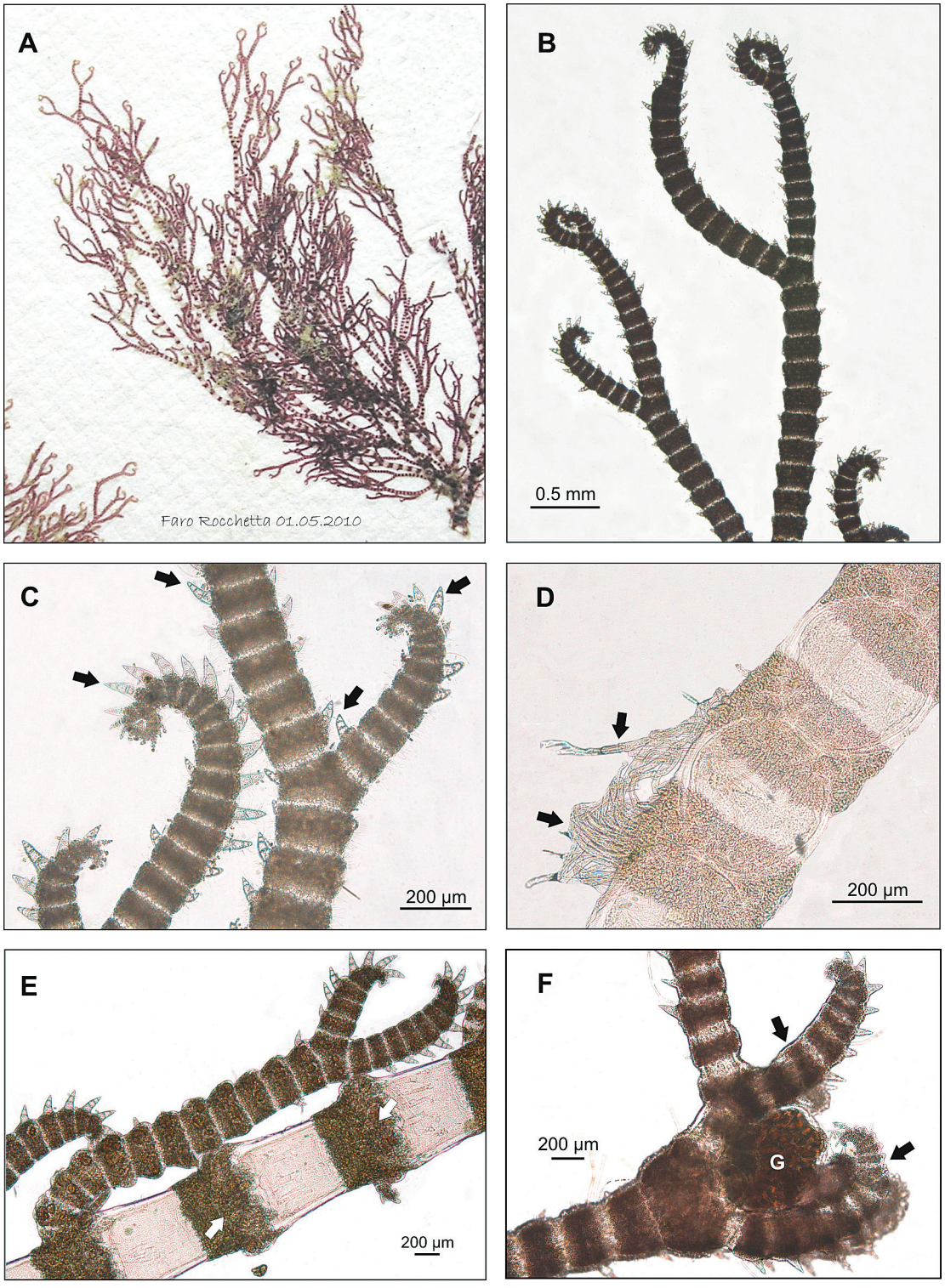


Fig. 3. *Ceramium nudiusculum* (previously identified as *Ceramium ciliatum* var. *robustum*). **A,** Habit of thallus; **B,** axes, pseudodichotomously branched and corticated only at the nodes; **C,** young axes with strongly inrolled apices bearing 4-celled spines (arrows); **D,** rhizoids formed by cortical cells, simple, multicellular (arrows); **E,** younger axes bearing tetrasporangia in cortical whorls (arrows); **F,** gonimolobe (G) containing numerous carposporangia surrounded by a whorl of 6 involucral branchlets (arrows).

of some internal nodes. As the same clades were detected with all the methods, we show only the ML topologies, reporting the ML and MP bootstrap values and the posterior probabilities at the congruent nodes.

Our complete *rbcL* dataset included all the sequences from this survey plus other 51 *Ceramium* sequences, and the outgroup sequence of *Spyridia filamentosa* (Wulfen) Harvey. The alignment consisted of a total of 1099 positions, out of which 306 were parsimony-informative. The GTR+I+G model, as selected by jMODELTEST, was used in both the ML and BI analyses.

The *rbcL* phylogenetic tree (Fig. 4) showed the four clade A samples clustering with strong bootstrap supports and posterior probability. This clade was resolved as the sister taxon to *C. diaphanum* sensu Harvey (1848: pl. 193) from Cornwall (U.K.), in a well-resolved clade which also included *C. siliquosum* (Kützing) Maggs & Hommersand, *C. pallidum* (Nägeli ex Kützing) Maggs & Hommersand, *C. tenuicorne* (Kützing) Waern, *C. strictum* sensu Harvey, and a *Ceramium* sp. sample from Ischia (Italy). This cultured isolate (CAM 1130) had been morphologically identified as "*C. diaphanum* sensu lato". Two '*C. diaphanum*' specimens from Atlantic North America and Canada were basal to this clade, which did not include authentic *C. diaphanum* (Lightfoot) Roth. The two samples HA 3150-1 and HA 3150-3 constituted together the strongly supported clade B and were included in a group with other Atlantic *C. secundatum* samples, *C. botryocarpum*, and '*C. barbatum*' Italy. *Ceramium virgatum* was sister to this clade. The spiny sample HA 3150-4 was placed near *C. ciliatum* from Ireland and *C. circinatum* from England, U.K., though this grouping was very weakly supported. Partial *rbcL* sequences of two additional spiny specimens (FR775781 of sample HA 3150-5 and FR775782 of sample HA 3150-7) were identical to that of HA 3150-4.

For the *rbcL-rbcS* spacer region sequences were obtained from 11 samples, representing three distinct genotypes. Sequences of clades A and B were 195 bp long, with the *rbcL-rbcS* spacer consisting of 93 bp and the flanking regions of 85 bp (*rbcL*) and 17 bp (*rbcS*). Sequences of clade C were 193 bp long, of which the *rbcL-rbcS* spacer was 91 bp. Only 16 *Ceramium rbcL-rbcS* spacer sequences were available in public databases for use in this study (Fig. 5). Again, *Spyridia filamentosa* was selected as outgroup. The resulting alignment consisted of a total of 213 positions, of which 43 were parsimony-informative.

In the ML and BI phylogenetic reconstructions using the TIM3+G model, as indicated by jMODELTEST, the sequences of our Venice lagoon specimens formed three well-resolved clades, labelled A, B, and C (Fig. 5). The four samples of clade A were part of a larger well-supported clade including *C. tenuicorne* from Finland, *C. siliquosum*, *C. pallidum*, *C. strictum* sensu Harvey from Ireland, with a *Ceramium* sp. specimen from Antigonish, Nova Scotia, Canada basal to this clade. Divergences between our sequences and other members of the clade were: 1.54% from *C. siliquosum*, 2.05% from *C. tenuicorne*, 2.56% from *C. pallidum*, 3.59% from *C. strictum* sensu Harvey, and 5.13% from *Ceramium* sp. (AY254300). The three clade B samples were related to *C. botryocarpum* from Northern Ireland and *C. secundatum* from the Faroe Islands, though with weak support. The *rbcL-rbcS* spacer region nucleotide divergence was 2.56% from *C. botryocarpum* and 3.08% from *C. secundatum*. The four clade C samples clustered with strong support, while their relationship with *Ceramium ciliatum* from Banyuls, Mediterranean France, was not supported. The *rbcL-rbcS* spacer region of clade C samples and this Mediterranean *C. ciliatum* specimen differed by 16 bp, corresponding to 8.21% nucleotide divergence.

■ DISCUSSION

Taxonomy and nomenclature. — The genus *Ceramium* Roth (Ceramiaceae, Rhodophyta) is one of the most systematically complex groups of the Rhodophyta due to the high degree of morphological variability, which has led to the increasing importance of molecular approaches in resolving taxonomic issues. Despite the trend towards molecular systematics, to date knowledge of this genus in the Mediterranean Sea has generally been confined to morphological observations. Therefore, in this survey we combined morphological observations with molecular phylogenetic analyses to identify different *Ceramium* samples from Venice lagoon (Italy). The Venice specimens could each be attributed to one of three phylogenetic species, representing the three morphological groups of *Ceramium*, i.e., spiny, fully corticated and partially corticated (Skage & al., 2005). The Adriatic samples were identifiable as being within the morphological range of one or more Atlantic species, but each of the three species was shown to be phylogenetically distinct from its Atlantic counterpart. The implications for the taxonomy and nomenclature of these species are discussed in detail below.

***Ceramium polyceras* (Kützing) Zanardini (clade A). —** Our partially corticated samples were initially identified as a member of the *Ceramium diaphanum* complex sensu Feldmann-Mazoyer (1941). In particular, our Adriatic banded species resembles *Ceramium diaphanum* var. *zostericola* f. *acrocarpum* (Kützing) Feldmann-Mazoyer (1941: 318), which is based on *Hormoceras acrocarpum* Kützing (1863: pl. 1, figs. a, b) from the Adriatic Sea. However Kützing's illustrations show that cystocarps of *Hormoceras acrocarpum* are always terminal and not surrounded by involucral branchlets, whereas in our samples cystocarps are involucrate. Several other Adriatic specimens were described as new members of the genus *Hormoceras* by Kützing (1842): *Hormoceras polyceras* (p. 752), *Hormoceras nodosum* (p. 733), and *Hormoceras gracillimum* (p. 753). The last two species are currently regarded as taxonomic synonyms of *Ceramium diaphanum* (Lightfoot) Roth, a morphologically distinctive species with conspicuous gland cells, protruding nodes, and strongly inrolled apices (Maggs & Hommersand, 1993). Our samples resemble the description by Kützing (1842) of *Hormoceras polyceras* (illustrated by Kützing 1862: pl. 66) because the cystocarps consist of globular gonimolobes surrounded generally by five involucral branchlets. Moreover, *H. polyceras* was originally described from material collected both at Spalato (Adriatic Sea) and in the Venice lagoon. *Hormoceras polyceras* was transferred to *Ceramium* by Zanardini (1847). We therefore propose that the correct name for the

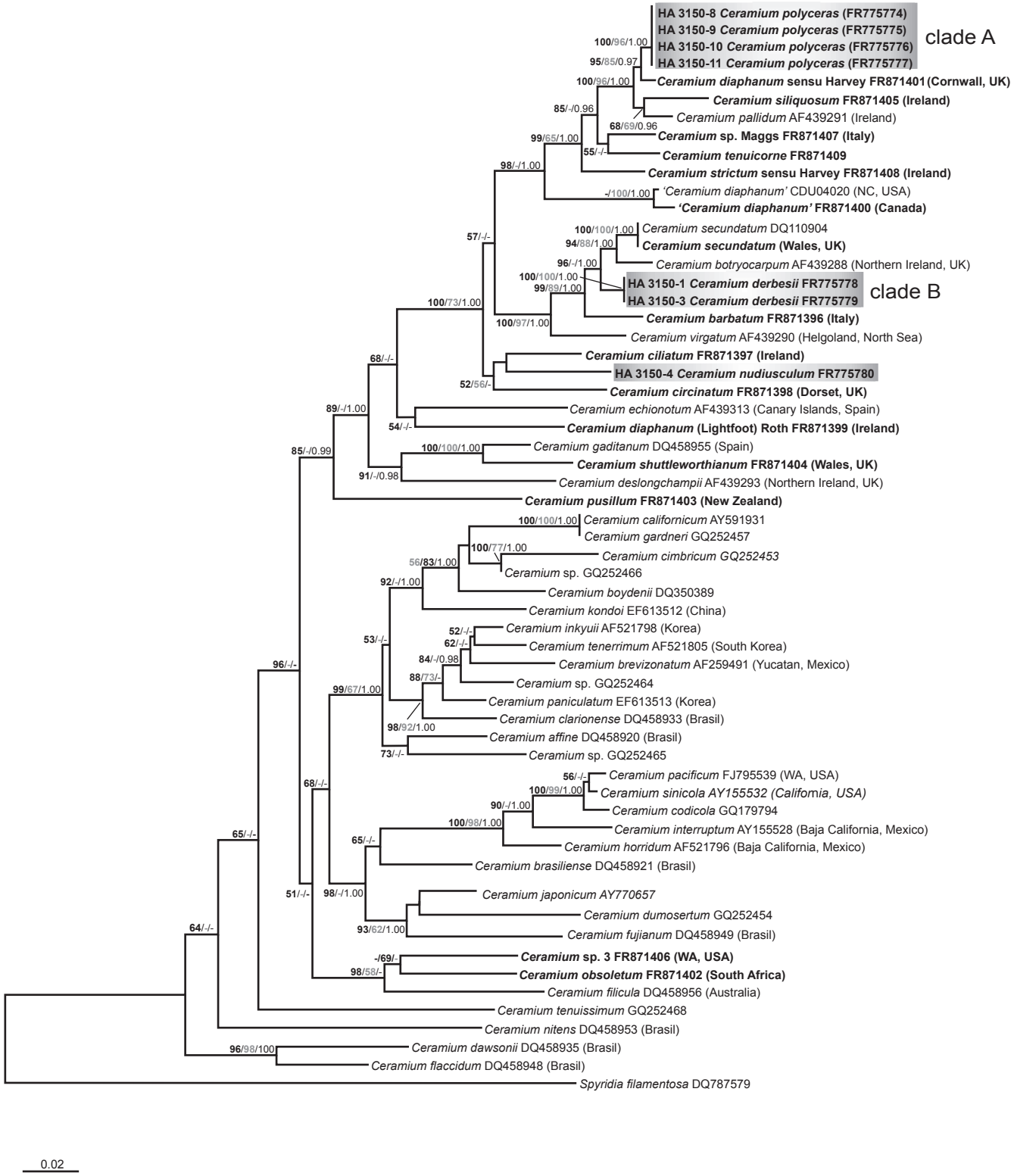


Fig. 4. ML tree inferred from *rbcL* gene sequences, calculated using the GTR+I+G model of evolution. Numbers near nodes indicate bootstrap values (>50%) for ML analysis (in bold black font), bootstrap values (>50%) for MP analysis (in bold grey font), and posterior probabilities (>0.95, in normal font). New sequences are in bold and for each one the GenBank accession number and collection locality of the sample are supplied. The Venice lagoon samples are highlighted by grey boxes. Bar represents 0.02 nucleotide substitutions per site.

partially corticated samples from the Venice lagoon should be *Ceramium polyceras* (Kützing) Zanardini (1847: 223).

The name *Ceramium diaphanum* (Lightfoot) Roth has been applied erroneously over the past two centuries to a wide range of taxa in the genus *Ceramium*. The demonstration by Maggs & Hommersand (1993) that the type of Lightfoot's (1777) *Conferva diaphana* represents a morphologically distinctive, and relatively invariant, species was an important step in beginning to unravel the tangled nomenclatural and taxonomic situation. Our discovery that English material provisionally identified as *Ceramium diaphanum* sensu Harvey is probably conspecific with *Ceramium polyceras* from Venice (on the basis of only 0.80% nucleotide divergence between their *rbcL* sequences) is another major advance. Maggs & Hommersand (1993) erroneously included several morphologically variable species under the name *Ceramium cimbricum* H. Petersen in Rosenvinge. One of these taxa, illustrated by Maggs & Hommersand (1993: figs. 15A, C, I), appears to be the entity treated by Harvey (1848: pl. 193) as *Ceramium diaphanum* (Ward, 1999) and recognized here as *C. polyceras*.

***Ceramium derbesii* Solier ex Kützing (clade B). —** We initially identified these fully corticated specimens as *Ceramium secundatum* Lyngbye (1819) based on published literature. *Ceramium secundatum* belongs to the "*C. rubrum* complex", a group of species in which the axial cells are typically fully covered by a cortex that lacks spines (Boo & Lee, 1994). All the members of this group were, in fact, formerly considered to be a single species, under the illegitimate name of *C. rubrum* (Hudson) C. Agardh (1811), but a survey by Maggs & al. (2002), based on molecular markers, led to the identification of four distinct species for the British Isles: *C. virgatum*, *C. pallidum*, *C. secundatum*, and *C. botryocarpum*. Each of these species shows a wide range of morphological variation, which seems to be related to environmental factors. For example, as reported by Maggs & Hommersand (1993), whereas most *C. virgatum* thalli are fully corticated, occasional populations growing in shallow pools become banded, with long ecorticate internodes. Curvature of the apices also varies greatly. Moreover, culture studies on other isolates of the *C. rubrum* complex from Nova Scotia, Wales, and Italy showed that the development of cortical

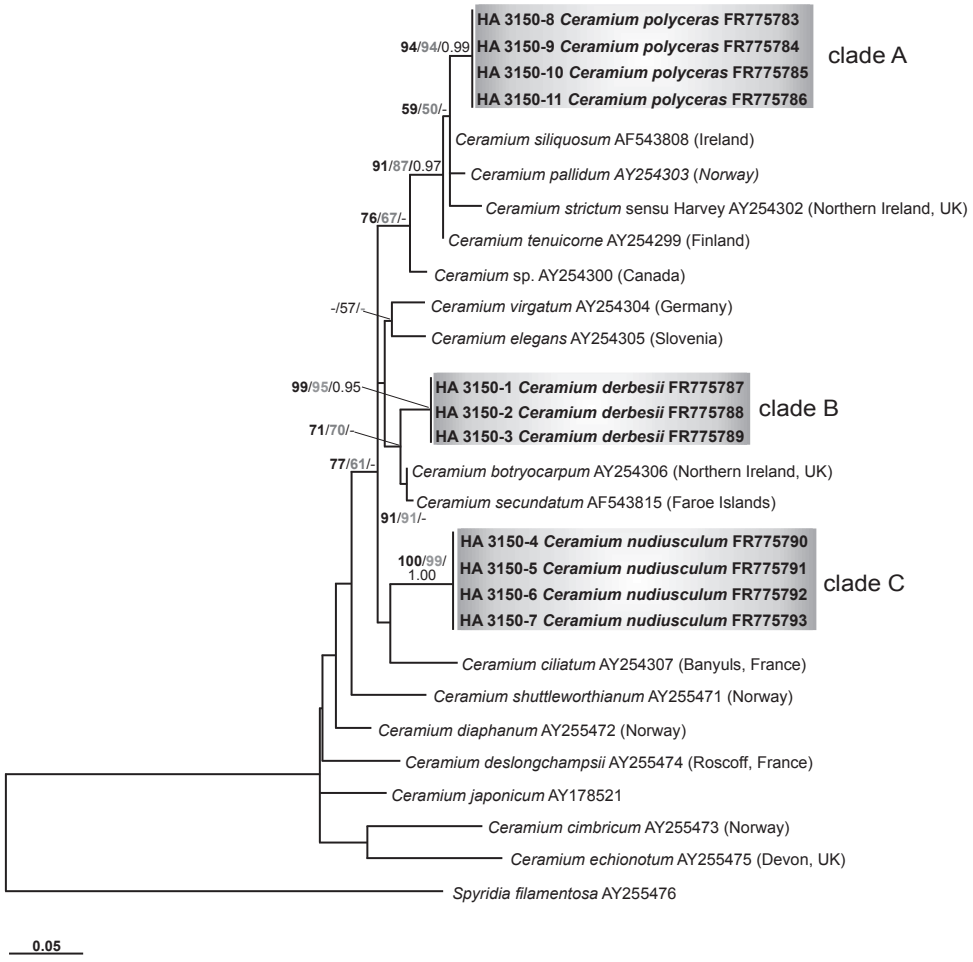


Fig. 5. ML tree inferred from the *rbcL-rbcS* spacer region sequence, calculated using the TIM3+G model of evolution. Numbers near nodes indicate bootstrap values (≥50%) for ML analysis (in bold black font), bootstrap values (≥50%) for MP analysis (in bold grey font), and posterior probabilities (≥0.95, in normal font). New sequences are in bold and for each one the GenBank accession number is supplied. The clades formed by the Venice lagoon samples are highlighted by grey boxes. Bar represents 0.05 nucleotide substitutions per site.

bands and ecorticate internodes is affected by both day length and temperature (Garbary & al., 1978; Cormaci & Motta, 1987). All these observations underline, once again, the limits of morphology-based identifications and the requirement for molecular analyses in species recognition.

The position of our fully corticated samples in both phylogenetic trees confirms their identification as members of the *C. rubrum* complex. They are resolved as the sister taxon to *C. secundatum* and *C. botryocarpum* and less closely related to *C. virgatum*. This result and the nucleotide divergences calculated for the *rbcL-rbcS* spacer region confirm that the Venice specimens are not conspecific with Atlantic *C. secundatum*. *Ceramium secundatum* was originally described from the Faroe Islands and is widely distributed in north-west Europe (Maggs & al., 2002). Kützing (1842) named an entity from Croatia (Mediterranean Sea), morphologically similar to *C. secundatum*, as *Ceramium barbatum*. *Ceramium barbatum* Kützing is now regarded as a variety in the *Ceramium rubrum* complex (e.g., *Ceramium nodulosum* var. *barbatum* (Kützing) Furnari & Serio) or as a synonym of *C. secundatum* (Gómez Garreta & al., 2001).

It is therefore likely that our samples represent *C. barbatum* Kützing (1842: 740), of which we have examined type material in Leiden, including the lectotype (L 940.265.17). However, this name is illegitimate, being pre-dated by *Ceramium barbatum* (J.E. Smith) Duby (1833), now known as *Anotrichium barbatum* (C. Agardh) Nägeli. Nevertheless, type material of another Mediterranean species, *Ceramium derbesii* Solier ex Kützing (1847: 33), also resembles our material from Venice. The lectotype in Leiden (L 940.142.361), collected by Solier at Marseilles, Mediterranean France, was illustrated by Kützing (1863: pl. 14). The specimen is fully corticated, with main axes up to 500 µm in diameter, tightly hooked apices, a branching interval of 13 to 14 segments, abundant adventitious branching, and immersed tetrasporangia. In conclusion, we propose that the correct name for our Adriatic samples resembling the Atlantic *C. secundatum* Lyngbye could be *Ceramium derbesii* Solier ex Kützing. *Ceramium derbesii* has previously been reported from a few localities in Italy (Cormaci & al., 2001; Gómez Garreta & al., 2001) although caution has been employed due to morphological variation in this group. It is likely that *C. derbesii* is common and widespread in the Mediterranean Sea. The distribution of this entity in the Mediterranean Sea will need to be determined using molecular markers.

***Ceramium nudiusculum* (Kützing) Rabenhorst (clade C).** — We initially identified this species as *Ceramium ciliatum* (Ellis) Ducluzeau var. *robustum* (J. Agardh) Mazoyer, 1938 based on published literature. *Ceramium ciliatum* (J. Ellis) Ducluzeau (1806) is such a polymorphic species that Kützing (1842, 1847, 1849, 1862) alone described some 20 species that were later reduced to synonymy or varietal status (Dixon, 1962). Two varieties are currently recorded in the Adriatic Sea, *C. ciliatum* var. *ciliatum* and *C. ciliatum* var. *robustum* Feldmann-Mazoyer (Furnari & al., 1999; Gómez Garreta & al., 2001). *Ceramium ciliatum* is based on *Conferva ciliata* J. Ellis (1768), lectotypified by an illustration (Ellis, 1768: pl. 18, fig. H), probably based on material from England (Maggs & Hommersand, 1993). *Ceramium ciliatum* has 3-celled spines that are formed in whorls and has 6–7 periaxial cells (Maggs & Hommersand, 1993). *Ceramium robustum* J. Agardh (1894: 35), based on an illustration by Kützing (1862: pl. 86) of Mediterranean material, was recognized as a distinct species because of its 4-celled spines borne in whorls. Feldmann-Mazoyer (1941) reduced *C. robustum* to a variety of *C. ciliatum*, as *C. ciliatum* var. *robustum* (J. Agardh) Feldmann-Mazoyer. The larger number of cells in the spines has been considered the main diagnostic character of var. *robustum* (Coppejans, 1983), which is very common in the Mediterranean Sea (Feldmann-Mazoyer, 1941). However, Dixon (1962) showed that 4-celled spines could be found in England, and a population with 3-celled spines occurred at Banyuls (Mediterranean France).

Subsequently, *C. ciliatum* var. *robustum* was reported from southern Japan by Itono (1972, 1977), who described samples with 6 periaxial cells and spines of up to 4 cells, borne in whorls. Our specimens from the Venice lagoon were similar to Itono's, having 6 periaxial cells and 2–4-celled spines. However, Japanese specimens had whorled spines whereas the spines of our Venice material were borne singly or paired on the axes, rather than being whorled. Furthermore, whereas Atlantic and Mediterranean *Ceramium ciliatum* thalli are up to 15 cm high (Feldmann-Mazoyer, 1941; Maggs & Hommersand, 1993), the Japanese ones were much smaller, about 0.4–0.5 cm high (Nakamura, 1965).

In the *rbcL* analysis our spiny material was placed near *C. ciliatum* from Ireland, but with little support, confirming that the Adriatic samples are not conspecific with *C. ciliatum*. The nucleotide divergence (5.27%) between the sequences of the spiny Adriatic sample HA 3150-4 and *C. ciliatum* from Ireland is higher than the interspecific *rbcL* sequence divergence reported for other *Ceramium* species (2.5%–3.8%, Cho & al., 2003) and within the interspecific range in other red algae, for example between *Grateloupia* species (2%–10%, Gavio & Fredericq, 2002).

The *rbcL-rbcS* spacer tree topology confirms that our spiny specimens are related to *C. ciliatum* from Mediterranean France, but are very probably distinct at the species level. Also the nucleotide divergence between the spacer sequence of our samples and that of the published sequence of *C. ciliatum* from Banyuls (Skage & al., 2005) strongly suggests that they should be assigned to separate species. In fact, the spacer divergence (8.21%) is much higher than interspecific divergences reported for other *Ceramium* species (e.g., 2.9% between *C. interruptum* Setchell & Gardner and *C. sinicola* Setchell & Gardner, see Cho & al., 2003). The *rbcL-rbcS* spacer of our material was only 91 nucleotides long, the shortest known for the genus. In other *Ceramium* species the *rbcL-rbcS* spacer ranges from 93 to 105 bp, mainly because of an 11 bp indel found in *C. cimbricum* H.E. Petersen, *C. diaphanum* (Lightfoot) Roth, *C. deslongchampsii* Chauvin ex Duby, *C. echionotum* J. Agardh, and *C. shuttleworthianum* (Kützing) Rabenhorst (Skage & al., 2005).

Although our initial identification of the material from the Venice lagoon was as *C. ciliatum* var. *robustum*, as noted above, there were important morphological differences from J. Agardh's *Ceramium robustum*, particularly the occurrence of

spines in a single row rather than in whorls as in *C. robustum*. However, one of Kützing's taxa that has long been considered a synonym of *C. ciliatum*, *Echinoceras nudiusculum* Kützing (1842: 739), is very similar to our material from the Venice lagoon. *Echinoceras nudiusculum* is based on a specimen that was collected at Venice. Kützing (1862: pl. 94) shows 4-celled spines borne in a single adaxial row or paired rather than in whorls. Some lengths of the axes lack spines (*nudiusculum* is the Latin diminutive of the word *nudus*, naked). Between branches the axes are swollen, as seen in our material (Fig. 3B). *Echinoceras nudiusculum* was subsequently transferred to *Ceramium* by both Rabenhorst and Zanardini in 1847, but the Rabenhorst combination has priority (Furnari & al., 1999). We therefore propose that the correct name for our Venice lagoon samples initially identified as *C. ciliatum* var. *robustum* is *C. nudiusculum* (Kützing) Rabenhorst (1847: 143). The distribution of this species has yet to be established, but it is likely that in future other spiny *Ceramium* specimens from the Mediterranean currently considered conspecific with *C. ciliatum* will be recognized as this species or could be assigned to other undescribed or unrecognized spiny species. Similarly, in the genus *Centroceras*, *C. clavulatum* was formerly considered to be cosmopolitan, but has recently been shown to consist of at least six cryptic or semi-cryptic species (Won & al., 2009).

Conclusions. — Our results confirm the necessity of combining morphological observations with molecular analyses in order to correctly identify species belonging to the genus *Ceramium*. The *rbcL-rbcS* spacer region has proved to be a good marker for this purpose because, as previously pointed out by other studies (Cho & al., 2003; Skage & al., 2005), the spacer region is essentially invariant within a species, but is extremely variable at the interspecific level. Our comparative study of Mediterranean and Atlantic European *Ceramium* samples has resolved some longstanding intractable nomenclatural problems in this complex genus and has led to the (re)recognition of *Ceramium polyceras* (Kützing) Zanardini, *Ceramium derbesii* Solier ex Kützing, and *Ceramium nudiusculum* (Kützing) Rabenhorst.

■ ACKNOWLEDGMENTS

This work was supported by grants provided by Progetto di Ateneo of the University of Padova.

■ LITERATURE CITED

Agardh, C.A. 1811. *Dispositio algarum sueciae*, part. 2. Lund: Berling.

Agardh, J.G. 1894. *Analecta algologica*, continuatio 1. Acta Universitatis Lundensis 29. Lund: Berling.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990. Basic local alignment search tool. *J. Molec. Biol.* 215: 403–410.

Barros-Barreto, M.B., McIvor, L., Maggs, C.A. & Ferreira, P.C.G. 2006. Molecular systematics of *Ceramium* and *Centroceras* (Ceramiaceae, Rhodophyta) from Brazil. *J. Phycol.* 42: 905–921.

Boo, S.M. & Lee, I.K. 1994. *Ceramium* and *Campylaephora* (Ceramiaceae, Rhodophyta). Pp. 1–33 in: Akatsuka, I. (ed.), *Biology of economic algae*. The Hague: SPB Academic Publishing bv.

Cho, T.O., Boo, S.M., Hommersand, M.H., Maggs, C.A., McIvor, L.J. & Fredericq, S. 2008. *Gayliella* gen. nov. in the tribe Ceramieae (Ceramiaceae, Rhodophyta) based on molecular and morphological evidence. *J. Phycol.* 44: 721–738.

Cho, T.O., Fredericq, S. & Boo, S.M. 2003. *Ceramium inkyuii* sp. nov. (Ceramiaceae, Rhodophyta) from Korea: A new species based on morphological and molecular evidence. *J. Phycol.* 39: 237–247.

Coppejans, E. 1983. Iconographie d'Algues Méditerranéennes: Chlorophyta, Phaeophyta, Rhodophyta. *Biblioth. Phycol.* 63: 1–317.

Cormaci, M. & Motta, G. 1987. Osservazioni sulla morfologia di *Ceramium rubrum* (Hudson) C. Agardh (Rhodophyta, Ceramiales) in coltura e considerazioni sulla sua tassonomia. *Boll. Accad. Gioenia Sci. Nat. Catania* 20: 239–251.

Cormaci, M., Furnari, G., Alongi, G., Serio, D., Petrocelli, A., Cecere, E., 2001. Censimento delle macroalghe marine bentoniche delle coste pugliesi. *Thalassia Salentina* 25: 75–158.

Dixon, P.S. 1960. Studies on marine algae of the British Isles: The genus *Ceramium*. *J. Mar. Biol. Assoc. U.K.* 39: 331–74.

Dixon, P.S. 1962. Taxonomic and nomenclatural notes on the Florideae, III. *Bot. Not.* 115: 245–260.

Duby, J.E. 1833. Second mémoire sur le groupe des Céramiées, VI. *Mém. Soc. Phys. Genève* 6: 1–25.

Ducluzeau, J.A.P. 1806 ('1805'). *Essai sur l'histoire naturelle des conferves des environs de Montpellier.* Montpellier: Auguste Ricard.

Ellis, J. 1768. Extract of a letter from John Ellis, Esquire, F.R.S. to Dr. Linnaeus of Upsala, F.R.S. on the animal nature of the genus of zoophytes, called *Corallina*. *Philos. Trans.* 57: 404–425.

Feldmann-Mazoyer, G. 1941 ('1940'). *Recherches sur les Céramiacées de la Mediterranée Occidentale*. Algiers: Imprimerie Minerva.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using bootstrap. *Evolution* 39: 783–791.

Fredericq, S., Hommersand, M. & Freshwater, D.W. 1996. The molecular systematics of some agar- and carrageenan-containing marine red algae based on *rbcL* sequence analysis. *Hydrobiologia* 326/327: 125–135.

Freshwater, D.W. & Rueness, J. 1994. Phylogenetic relationships of some European *Gelidium* (Gelidiales, Rhodophyta) species, based on *rbcL* nucleotide sequence analysis. *Phycologia* 33: 187–94.

Freshwater, D.W., Fredericq, S., Butler, B.S., Hommersand, M.H. & Chase, M.W. 1994. A gene phylogeny of the red algae (Rhodophyta) based on plastid *rbcL*. *Proc. Natl. Acad. Sci. U.S.A.* 91: 7281–7285.

Freshwater, D.W., Montgomery, F., Greene, K.J., Hamner, M.R., Williams, M. & Whitfield, E.P. 2006. Distribution and identification of an invasive *Gracilaria* species that is hampering commercial fishing operations in south-eastern North Carolina, USA. *Biol. Invas.* 8: 631–637.

Furnari, G., Cormaci, M. & Serio, D. 1999. Catalogue of the benthic marine macroalgae of the Italian coast of the Adriatic Sea. *Bocconea* 12: 1–214.

Gabrielsen, T.M., Brochmann, C. & Rueness, J. 2003. Phylogeny and interfertility of North Atlantic populations of '*Ceramium strictum*' (Ceramiales, Rhodophyta): How many species? *Eur. J. Phycol.* 38: 1–13.

Garbary, D.J., Grund, D. & McLachlan, J. 1978. The taxonomic status of *Ceramium rubrum* (Huds.) C. Ag. (Ceramiales, Rhodophyta) based on culture experiments. *Phycologia* 17: 85–94.

Gavio, B. & Fredericq, S. 2002. *Grateloupia turuturu* (Halymeniaceae, Rhodophyta) is the correct name of the non-native species in the Atlantic known as *Grateloupia doryphora*. *Eur. J. Phycol.* 37: 349–360.

Gómez-Garreta, A., Gallardo, T., Ribera, M.A., Cormaci, M., Furnari, G., Giaccone, G. & Boudouresque, C.F. 2001. Checklist of Mediterranean seaweeds. III. Rhodophyceae Rabenh. 1. Ceramiales Oltm. *Bot. Mar.* 44: 425–460.

Guindon, S. & Gascuel, O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol*. 52: 696–704.

Guiry, M.D. & Guiry, G.M. 2011. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org.

Gurgel, C.F.D. & Fredericq, S. 2004. Systematics of the Gracilariaceae (Gracilariales, Rhodophyta): A critical assessment based on *rbcL* sequence analyses. *J. Phycol*. 40: 138–159.

Harvey, W.H. 1848. *Phycologia Britannica*, pls. 145–216. London: Reeve & Benham.

Hommersand, M.H., Fredericq, S., Freshwater, D.W. & Hughey, J. 1999. Recent developments in the systematics of the Gigartinaceae (Gigartinales, Rhodophyta) based on *rbcL* sequence analysis and morphological evidence. *Phycol. Res*. 47: 139–151.

Itono, H. 1972. The genus *Ceramium* (Ceramiaceae, Rhodophyta) in southern Japan. *Bot. Mar*. 15: 74–86.

Itono, H. 1977. Studies on the ceramiaceous algae (Rhodophyta) from southern parts of Japan. *Biblioth. Phycol*. 35: 1–499.

Kützing, F.T. 1842 ('1841'). Ueber *Ceramium* Ag. *Linnaea* 15: 727–746.

Kützing, F.T. 1843. *Phycologia generalis (oder Anatomie, Physiologie und Systemkunde der Tange)*. Leipzig: Brockhaus.

Kützing, F.T. 1847. Diagnosen und Bemerkungen zu neuen oder kritischen Algen (Fortsetzung). *Bot. Zeitung (Berlin)* 5: 33–38.

Kützing, F.T. 1849. *Species algarum*. Leipzig: Brockhaus.

Kützing, F.T. 1862. *Tabulae phycologicae*, vol. 12. Nordhausen.

Kützing, F.T. 1863. *Tabulae phycologicae*, vol. 13. Nordhausen.

Lightfoot, J. 1777. *Flora scotica: or, A systematic arrangement, in the Linnaean method, of the native plants of Scotland and the Hebrides*, vol. 2. London: B. White.

Lyngbye, H.C. 1819. *Tentamen hydrophytologiae danicae continens omnia hydrophyta cryptogama Daniae, Holsatiae, Faeroae, Islandiae, Groenlandiae hucusque cognita, systematice disposita, descripta et iconibus illustrata, adjectis simul speciebus norvegicis*. Copenhagen: typis Schultzianis.

Maddison, W.P. & Maddison, D.R. 2009. Mesquite: A modular system for evolutionary analysis, version 2.71. http://mesquiteproject.org.

Maggs, C.A., McIvor, L.M., Evans, C.E., Rueness, J. & Stanhope, M.J. 2002. Molecular analyses elucidate the taxonomy of fully corticated, nonspiny species of *Ceramium* (Ceramiaceae, Rhodophyta) in the British Isles. *Phycologia* 41: 409–420.

Maggs, C.A. & Hommersand, M.H. 1993. *Seaweeds of the British Isles*, vol. 1, *Rhodophyta*, part 3A, *Ceramiales*. London: HMSO.

Mazoyer, G. 1938. Les Céramiacées de l'Afrique du Nord. *Bull. Soc. Hist. Nat. Afrique N.* 29: 317–331.

Nakamura, Y. 1965. Species of the genera *Ceramium* and *Campylaephora,* especially those of northern Japan. *Sci. Pap. Inst. Algol. Res., Fac. Sci. Hokkaido Imp. Univ.* 5: 119–180.

Perrière, G. & Gouy, M. 1996. WWW-Query: An on-line retrieval system for biological sequence banks. *Biochimie* 78: 364–369.

Posada, D. & Buckley, T.R. 2004. Model selection and model averaging in phylogenetics: Advantages of the AIC and Bayesian approaches over likelihood ratio tests. *Syst. Biol*. 53: 793–808.

Posada, D. & Crandall, K.A. 1998. Modeltest: Testing the model of DNA substitution. *Bioinformatics* 14: 817–818.

Rabenhorst, G.L. 1847. *Deutschlands Kryptogamen-Flora*, vol. 2(2). Leipzig: Kummer.

Rambaut, A. & Drummond, A.J. 2007. Tracer, version 1.5. http://beast.bio.ed.ac.uk/Tracer.

Ronquist, F. & Huelsenbeck, J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

Roth, A.W. 1797. *Catalecta botanica*, fasc. 1. Leipzig: in Bibliopolo I.G. Mülleriano.

Skage, M., Gabrielsen, T.M. & Rueness, J. 2005. A molecular approach to investigate the phylogenetic basis of three widely used species groups in the red algal genus *Ceramium* (Ceramiales, Rhodophyta). *Phycologia* 44: 353–360.

South, G.R. & Skelton, P.A. 2000. A review of *Ceramium* (Rhodophyceae, Ceramiales) from Fiji and Samoa, South Pacific. *Micronesica* 33: 45–98.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molec. Biol. Evol*. 24: 1596–1599.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* 22: 4673–4680.

Ward, B.A. 1999. *The systematics of* Ceramium: *A molecular and morphological approach*. Ph.D. thesis, Queen's University of Belfast, U.K.

Won, B.Y., Cho, T.O. & Fredericq, S. 2009. Morphological and molecular characterization of species of the genus *Centroceras* (Ceramiaceae, Ceramiales), including two new species. *J. Phycol*. 45: 227–250.

Zanardini, G. 1847. Notizie intorno alle cellulari marine delle lagune de' litorali di Venezia. *Atti Reale Ist. Veneto Sci. Lett. Arti* 6: 185–262.

Appendix. List of taxa for which *rbcL* gene sequences have been newly produced in this study. Available voucher codes (in parentheses), collection data.

Ceramium barbatum Kützing (CAM 1528), Lerici, Liguria, Italy, *C.A. Maggs*; ***Ceramium ciliatum*** (J. Ellis) Ducluzeau, Finavarra, Co. Clare, Ireland, *L. McIvor*; ***Ceramium circinatum*** (Kützing) J. Agardh (CAM 181), Kimmeridge, Dorset, England, U.K., *C.A. Maggs*; ***Ceramium diaphanum*** (Lightfoot) Roth (CAM 136), St John's Point, West Donegal, Ireland, *C.A. Maggs*; **'*Ceramium diaphanum*'** (CAM 249), Pomquet Harbour, Antigonish Co., Nova Scotia, Canada, *C. Bird*; ***Ceramium diaphanum*** sensu Harvey (CAM 199), Cornwall, England, U.K., *C.A. Maggs*; ***Ceramium obsoletum*** C. Agardh, Houk Bay, Cape Town, South Africa, *O. DeClerck*; ***Ceramium pusillum*** Harvey (CAM 1542), Christchurch, New Zealand, *M.P. Johnson*; ***Ceramium shuttleworthianum*** (Kützing) Rabenhorst (CAM 1049), Broad Haven Beach, Wales, U.K., *C.A. Maggs*; ***Ceramium siliquosum*** (Kützing) Maggs & Hommersand (CAM 172), Fanore, Co. Clare, Ireland, *C.A. Maggs*; ***Ceramium* sp. 3** (CAM 414), Neah Bay, Puget Sound, Washington, U.S.A., *M.H. Hommersand*; ***Ceramium* sp.** (CAM 1130), Ischia, Italy, culture, *C.A. Maggs*; ***Ceramium strictum*** sensu Harvey, Skerries, Co. Dublin, Ireland, *C.A. Maggs*; ***Ceramium tenuicorne*** (Kützing) Waern (Rueness 9817), culture, *B. Eklund*.

Phycologia

MORPHOLOGY, REPRODUCTION AND TAXONOMY OF GRACILARIA VIRIDIS SP. NOV. (RHODOPHYTA, GIGARTINALES): A NEW RED ALGAL SPECIES FOR THE MEDITERRANEAN SEA

--Manuscript Draft--

Manuscript Number:	
Full Title:	MORPHOLOGY, REPRODUCTION AND TAXONOMY OF GRACILARIA VIRIDIS SP. NOV. (RHODOPHYTA, GIGARTINALES): A NEW RED ALGAL SPECIES FOR THE MEDITERRANEAN SEA
Article Type:	Original Article
Keywords:	Gracilaria viridis sp. nov.; Gracilariaceae; Mediterranean Sea; new species; rbcL; rbcL-rbcS spacer.
Corresponding Author:	Isabella Moro, Ph.D. University of Padova Padova, ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Padova
Corresponding Author's Secondary Institution:	
First Author:	Adriano Sfriso, Prof.
First Author Secondary Information:	
Order of Authors:	Adriano Sfriso, Prof.
	Marion Adelheid Wolf, Ph.D.
	Katia Sciuto, Ph.D.
	Marina Morabito, Ph.D.
	Carlo Andreoli, Prof.
	Isabella Moro, Ph.D.
Order of Authors Secondary Information:	
Abstract:	Here we report the characterization of a new Gracilaria species from the Venice Lagoon, determined through morphological features and molecular analyses, based on the plastid large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase gene (rbcL) and the intergenic Rubisco spacer (rbcL-rbcS), combined with morphology and pigment composition data. This new entity, recorded in the artificial substrata of the Venice Gulf and never found in other localities of the Adriatic Sea, has not been described by local or international literature up to now and adds to the other 12 Gracilaria taxa recorded in the Mediterranean Sea. Thalli exhibit a green-yellowish pigmentation with pink shades and dense bushy and spiny branching in the distal portions. Tetrasporangial plants show abundant short spinelike uni-tribranched branchlets throughout the thallus. This species grows attached on artificial rocky substrata of the low mid-littoral and upper sub-littoral zone in spring and early summer. Molecular analyses based on the plastidial rbcL gene showed a 99.68 % nucleotide identity with another Gracilaria sp. (named Gracilaria-LI02, GenBank AY651040) from southern Sicily reported by Gargiulo and collaborators in 2006 and not identifiable with any other taxa described for the Mediterranean Sea until now. Moreover the comparison of our rbcL-rbcS spacer sequences with those of these two cryptic species and the phylogenetic analyses confirmed that the Venice specimen is to consider a new distinct species. Probably the recent finding of this new species is not due to an extra-Mediterranean introduction, but is the consequence of its misidentification with other specimens whose features are very similar: Gracilaria gracilis and Gracilaria conferta.

MORPHOLOGY, REPRODUCTION AND TAXONOMY OF *GRACILARIA VIRIDIS* SP. NOV. (RHODOPHYTA, GIGARTINALES): A NEW RED ALGAL SPECIES FOR THE MEDITERRANEAN SEA

Adriano Sfriso[1§]*, Marion Adelheid Wolf[2§], Katia Sciuto[2], Marina Morabito[3], Carlo Andreoli[2] and Isabella Moro[2]

[1]Department of Environmental Sciences, Informatics & Statistics,
University of Venice, Calle Larga 2137, 30123-Venice, Italy

[2]Department of Biology, University of Padova
Via U. Bassi, 58/B 35131 Padova, Italy

[3]Department of Life Sciences "Marcello Malpighi", University of Messina
V. Ferdinando Stagno d'Alcontres, 31 98166 Messina, Italy

[§]These authors contributed equally to this work

*Corresponding authors: adriano.sfriso@unive.it
isabella.moro@unipd.it

Abstract

Here we report the characterization of a new *Gracilaria* species from the Venice Lagoon, determined through morphological features and molecular analyses, based on the plastid large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase gene (*rbc*L) and the intergenic Rubisco spacer (*rbc*L-*rbc*S), combined with morphology and pigment composition data. This new entity, recorded in the artificial substrata of the Venice Gulf and never found in other localities of the Adriatic Sea, has not been described by local or international literature up to now and adds to the other 12 *Gracilaria* taxa recorded in the Mediterranean Sea. Thalli exhibit a green-yellowish pigmentation with pink shades and dense bushy and spiny branching in the distal portions. Tetrasporangial plants show abundant short spinelike uni-tribranched branchlets throughout the thallus. This species grows attached on artificial rocky substrata of the low mid-littoral and upper sub-littoral zone in spring and early summer. Molecular analyses based on the plastidial *rbc*L gene showed a 99.68 % nucleotide identity with another *Gracilaria* sp. (named *Gracilaria*-LI02, GenBank AY651040)

from southern Sicily reported by Gargiulo and collaborators in 2006 and not identifiable with any other taxa described for the Mediterranean Sea until now. Moreover the comparison of our *rbc*L-*rbc*S spacer sequences with those of these two cryptic species and the phylogenetic analyses confirmed that the Venice specimen is to consider a new distinct species. Probably the recent finding of this new species is not due to an extra-Mediterranean introduction, but is the consequence of its misidentification with other specimens whose features are very similar: *Gracilaria gracilis* and *Gracilaria conferta*.

Running title: *Gracilaria viridis* sp. nov.

Keywords: *Gracilaria viridis* sp. nov.; Gracilariaceae; Mediterranean Sea; new species; *rbc*L; *rbc*L-*rbc*S spacer.

INTRODUCTION

Gracilaria Greville (1830) is an important economic red algal genus being the major agarophyte resource in the world (Troell *et al.* 2003). It is characterized by a wide phenotypic variability and great species diversity. Thalli of this genus range from erect to prostrate and from terete to broadly flattened, with species forming articulated fronds composed of cylindrical or irregularly shaped units (Guiry & Guiry 2011).

Gross morphological characters, considering vegetative/reproductive anatomy, allowed to distinguish many genera and provided the basis of Mediterranean seaweeds identification for years (Fredericq & Hommersand 1989a, 1989b, 1990; Gargiulo *et al.* 1987, 1992). Unfortunately, these features are often limited and ambiguous, leading to misidentifications of different species in the case of convergent evolution or environmental influences in the form of plasticity. This makes the systematics of this group very problematic (Bird 1995).

Progress in molecular techniques has solved problems in species identification and led to revise the systematics of many algal groups including the genus *Gracilaria* (Bellorin *et al.* 2002; Gurgel & Fredericq 2004). Several markers of different DNA regions have been used to study Rhodophyta phylogenetic relationships at the species level: the plastid large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase gene (*rbc*L) (Freshwater *et al.* 1994; Fredericq *et al.* 1996; Hommersand *et al.* 1999; Gurgel & Fredericq 2004), the *rbc*L-*rbc*S intergenic spacer region, the mitochondrial *cox*1 gene, and the nuclear internal transcribed spacer ITS.

At present Guiry & Guiry (2011) reports 170 specific and intraspecific currently accepted taxa of *Gracilaria* in the world and out of these 11 (Tab. 1**)** are present in the Mediterranean Sea (Gargiulo *et al.* 1992; Furnari & Cormaci 1999, 2003; Sfriso & Curiel 2007; Cecere & Petrocelli 2009; Sfriso *et al.* 2009). However, the development of taxonomy, especially on molecular basis, and the introduction of allochthnous taxa (Sfriso *et al.* 2010) are increasing this number every year.

Here we report the characterization of specimens collected in the Venice Lagoon, identified through molecular analyses using the chloroplast encoded *rbc*L gene, , and the more variable intergenic *rbc*L-*rbc*S spacer, useful for distinguishing between cryptic species (strictly correlated species) (Maggs *et al.* 2002), combined with morphological observations and pigment composition data.

*rbc*L results showed that the Venice specimens are conspecific with another *Gracilaria* species (named *Gracilaria*-LI02) sampled in the southern Sicily and reported by Gargiulo and collaborators (2006) and not identifiable with any other taxa described for the Mediterranean Sea until now. All the results lead us to consider our strain as well as the Sicily strain a new species of *Gracilaria* genus for which we propose the name *Gracilaria viridis* Sfriso *et al.*

MATERIALS AND METHODS

Samplings

Samples of *Gracilaria viridis* sp. nov. were collected on the artificial stone breakwaters perpendicular to the shore coastline of the Lido and Pellestrina islands, along the Venice sea coastline and in the jetty "dikes" formed by limestone blocks, placed between the sea and the Venice Lagoon to protect the wide sea inlets of Lido, Malamocco and Chioggia (Fig. 1, Lido island: 45° 23' N, 12° 21'E; Pellestrina island: 45° 17' N, 12° 18' E). Moreover, thalli of *G. viridis* were sampled in Licata (Agrigento, Sicily) (37° 05' N, 13°55' E), at Marianello beach, west to the fishing harbour.

Usually, thalli are only present from March to July, just below the water surface, where they formed a belt of scattered specimens assembled together with many epiphytic and/or filamentous taxa.

Morphology

Morphological observations were carried out on fresh samples and on specimens fixed in 4% buffered formaldehyde-seawater solution by a stereo zoom microscope E-1654ZT45 (Euromex microscopes, Holland) and a light microscope-353Ph (Optika microscopes, Italy).

Spermatangium details were observed on semi-thin sections. Parts of thalli were fixed overnight in 6% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9), and post-fixed overnight in 1% OsO_4 in the same buffer. The post-fixed samples were dehydrated in a graded ethanol series followed by propylene oxide and then they were embedded in araldite. Semi-thin sections were cut with an ultramicrotome (Reichert Ultracut S), stained with 1% toluidine blue and examined with a DMR Leica (Wetzlar, Germany) microscope, equipped with a digital image acquisition system.

Hydrosoluble and liposoluble photosynthetic pigment analyses

Hydrosoluble pigment analyses were carried out on phosphate buffer extracts from fresh samples, finely ground in a mortar with liquid nitrogen according to Costa & Plastino (2011). The absorbance values for R-phycoerithrin, phycocyanin and allophycocyanin were measured with a spectrophotometer DU530 Beckman Coulter spectrophotometer (Fullerton, California, USA). Phycobiliprotein contents were calculated as µg/ml using the equations reported by Kursar *et al.* (1983). Chlorophyll *a* and carotenoid analyses were carried out on acetone:hexane (80:20) extracts by a high performance liquid chromatograph (HPLC) (Agilent, Waldbronn), composed by a Rheodyne valve (Rohnert Park, CA, USA), a reversed-phase column (5 µm particle size; 25 x 0.4 cm; 250/4 RP 18 Lichrocart), a binary pump, and an Agilent 1100 series diode array detector. The analyses were performed according to Färber & Jahns (1998).

Molecular procedures

Genomic DNA was extracted from dried samples using the Genomic DNA purification kit (Fermentas International Inc., Burlington, Ontario, Canada) following manufacturer's instructions. For amplification and sequencing reactions of the plastid *rbc*L gene two primer pairs were used: F7-R753 and F577-R1381, as described by Freshwater & Rueness (1994).

For the *rbc*L-*rbc*S spacer amplification and sequencing the forward primer F1320 designed by Freshwater *et al.* (2006) was used in combination with the reverse primer RrbcSstart (Freshwater & Rueness 1994). PCR reactions were performed as follows: an initial denaturation step of 2 min at 94°C; 30 cycles at 94°C for 40 sec, at 50°C for 40 sec, and at 72°C for 50 sec; and a final extension step at 72°C for 5 min. The amplification products were cleaned with ExoSAP-ITTM kit (GE Healthcare, Uppsala, Sweden, Europe), following the manufacturer's protocol. DNA sequencing was

performed at the BMR Genomics Sequencing Service (Padova University, Italy) on automated ABI DNA sequencers (Applied Biosystem, Foster City, CA, USA). Final consensus sequences were assembled using the SeqMan II program from the Lasergene software package (DNAStar©, Madison, WI, USA). Identity of new sequences was checked with the BLAST program (Altschul *et al.* 1990) available at the USA National Center for Biotechnology Information (NCBI) web server (http://www.ncbi.nlm.nih.gov) and the sequence alignments were obtained by the ClustalW computer program (Thompson *et al.* 1994).

For the phylogenetic analyses of the *rbc*L gene a dataset of 71 sequences was constructed including the obtained sequences plus other *Gracilaria* ones accessible in the DDBJ/GenBank™/EBI Data Bank. Analyses were performed according to maximum likelihood (ML) method with the PHYML 2.4.4 program (Guindon & Gascuel 2003) applying the GTR+I+ Γ evolutionary model (Lanave *et al*. 1984). Non parametric bootstrap re-sampling (Felsenstein 1985) was performed to test the robustness of the tree topology (1000 replicates). The models that best fitted our data were found using the program jMODELTEST under the Akaike Information Criterion (AIC) (Posada & Crandall 1998; Posada & Buckley 2004).

Bayesian Inference analyses were carried out too, using MrBayes version 3.1 (Ronquist & Huelsenbeck 2003). The substitution model was the GTR+I+ Γ and the Bayesian analyses were performed with four search chains for 3,000,000 generations, sampling trees every 100 generations. The first 7,500 trees were discarded as burn-in. Parameter stability was estimated by plotting log-likelihood values against generation time, and a consensus tree with posterior probabilities was then produced. The nexus files for the Bayesian analyses were generated with the Mesquite 2.71 software package (Maddison & Maddison 2009).

The *rbc*L gene sequence and the *rbc*L-*rbc*S spacer sequence of the Venice isolate were deposited in DDBJ/GenBank™/EBI Data Bank with the accession numbers HE614144 and HE614145 respectively.

RESULTS

Gracilaria viridis Sfriso *et al.* sp. nov (Figs 2-12)

Diagnosis: Thalli tereti 0.5-1.3 mm diametro, 5-35 cm alti, saxicolae, irregularis, fere horizontaliter effusi, haptero parvo discoideo affixi. Color viridis-flavidus, aliquando purpureus. Rami alterni, dense fructicosis, ad apices angustati et saepe incurvati.
In transversa sectio frondes multistrato constitutas: ab exterior parte cellulae corticalis per 1-2 stratos; mediae cellulae medullariae subcorticalesque, 10-20 μm diametro, per 2-3 stratos; interioris cellulae medullariae magnae, 100-200 μm diametro, parietibus tenuibus.
Cystocarpia matura ellipsoidea, ~900 μm diametro, basi constricta, per totem fronda sparsa. Gonimoblasti per cellulas nutrices tubulares ad cellulas basales pericarpii affixi.
Tetrasporangia, ~30 μm longa ~13 μm lata, filamentis corticalibus circumcinctis, cruciatum divisa, in brevis ramulis, per totam frondem sparsa.
Spermatangia in concectaculis, ~35 μm diametro ~75 μm altis, in cellula corticalis et subcorticalibus mersi.

Thalli terete 0.5-1.3 mm thick, 5-35 cm high, green-yellowish, sometimes partially reddish, attached on rocky substrata by a small discoid holdfast, strongly irregular with dense distal branching. Branching arising alternately, whorly or on all sides at irregular intervals and bushy toward apices. Branches and branchlets sigmoid, frequently recurvate, divergent with open angles or inserted in perpendicular manner.

In cross-section filaments present 1-2 layers of pigmented elongated cortical cells, subcortical cells (10-20 µm in diameter) arranged in 2-3 strata and large medullary cells (100-200 µm in diameter). Sub-spherical cystocarps (~ 900 µm in diameter) constricted at the basis spread throughout the main axis and branches. Gonimoblasts connected to the inner pericarp by tubular nutritive cells. Tetrasporangia (~ 30 µm long by 13 µm broad) cruciately divided and immersed in the cortex of the main branches and in spine-like uni-tribranched branchlets produced in the whole thallus. Spermatangial conceptacles (~ 35 µm in diameter, ~ 75 µm deep) are "*Verrucosa*-type", deeply immersed in the cortical and subcortical cells.

HOLOTYPE: Tetrasporophytic plant with spine-like uni-tribranched branchlets. Voucher specimen (n° A000008) has been deposited at the Herbarium Patavinum (PAD) of the University of Padova, Italy (Fig. 2).

TYPE LOCALITY: Breakwaters and jetties in the marine coasts of Lido (45° 23' N, 12° 21' E) and Pellestrina (45° 17' N, 12° 18' E) islands in the gulf of Venice.

ETYMOLOGY: referring to the green-yellowish thallus colour.

HABITAT: artificial rocky substrata of the low midlittoral and upper sublittoral zones.

DISTRIBUTION: Adriatic Sea, Gulf of Venice and southern Sicily, Agrigento, Licata.

THALLUS MORPHOLOGY: Green-yellowish, sometimes partially reddish thalli (Figs 2-3) attached by a small discoid holdfast bearing erect fronds, 5-35 cm high and 0.5-1.3 mm thick, terete throughout, irregularly branched at the basal and median regions, with

dense entangled branching in the apical parts, especially in the oldest specimens. Branches and branchlets of any order arise disorderly, mostly in a perpendicular manner, and are frequently sigmoid and recurvate (Fig. 3); branching insertions are feebly restricted at the base.

The same thallus morphology is observed also in the Sicily strain, collected in 2002 (Fig. 4).

Cortical cells are rounded-polyhedric, ca. 7-10 µm in surface view. In cross-section they are 3-6 µm width and 7-10 µm long. Below, 1-2 strata of subcortical cells, 10-20 µm in diameter, surround larger colourless thin-walled medullary cells, ca. (150)-200-300 µm in diameter (Fig. 5). Subspherical tetrasporangia, ca. 12-15 x 20-35 µm, are produced throughout the main branches (Fig. 6) and in short spine-like uni-tribranched branchlets (Fig. 7), 0.3-0.45 µm in diameter and 1-2-(3) mm long, frequently enlarged in the median region, scattered in the main ramifications of the thallus and provided with 2-3 short twigs; tetraspores are cruciately arranged (Fig. 8).

Dark red mature ostiolate cystocarps, ca. 800-1050 µm in diameter, restricted at the base, are borne throughout the main axis and branches (Figs 9-10). Their dark-red pigmentation stands out on the green-yellowish plants. The inner pericarp is connected to the gonimoblast by tubular nutritive cells 5-10 µm thick and 50-100 µm long (Fig. 11). The gonimoblast tissue consists of cellular rows producing ovoid carposporangia of 15-18 x 20-30 µm.

The male gametophytic plants form round-elliptical spermatangial *Verrucosa*-type conceptacles (Fig. 12) scattered on the cortical layer. Sometimes they are confluent showing a cristated shape in surface view. Conceptacle cavities ~ 20-30 µm diameter by 25-35 µm deep.

Habitat and seasonality: Thalli are common from March to July in the artificial stone breakwaters, along the Lido and Pellestrina island artificial substrata, and in the first

part of the limestone block jetties (dikes), placed between the sea-side and the Lagoon-side to protect the wide Lagoon inlets of Lido, Malamocco and Chioggia (Fig. 1). They colonize the lower intertidal and the upper sublittoral zones only in the first shallow part of breakwaters and jetties, forming a fringe of isolated thalli strongly entangled with many epiphytic taxa, especially belonging to the genera *Ceramium*, *Polysiphonia* and *Centroceras* and other smaller species. Thalli are always haptophytic and are not able to grow in the free floating form.

Pigment composition: Spectrophotometric measurements of the phycobiliprotein content of the Venice Lagoon specimens showed a predominance of R-phycoerythrin (53%) and allophycocyanin (30%) over phycocyanin (17%). The lipid-soluble pigment analyses, carried out at the HPLC, showed the presence of antheraxanthin (29%), zeaxanthin (33%), β-cryptoxanthin (5%), chlorophyll-*a* and β-carotene.

Molecular analyses. The *rbc*L and *rbc*L-*rbc*S spacer sequences, obtained for the 5 isolates sampled from the different collection sites, were all identical among them. Comparisons of the *rbc*L gene and *rbc*L-*rbc*S spacer sequences of the Venice Lagoon specimens with published data available in GenBank were performed. The partial plastid gene sequence showed 99.68% identity with the sequence of another *rbc*L sequence of *Gracilaria* sp. reported by Gargiulo and coworkers in Sicily (GenBank AY651040). For the *rbc*L-*rbc*S spacer the highest similarity of our sequences was with those of *Gracilaria conferta* (Schousboe *ex* Montagne) Montagne and *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine *et* Farnham. The comparison of the *rbc*L-*rbc*S spacer region sequences (220 bp long), including the *rbc*L-*rbc*S spacer (116 bp) and the *rbc*L (85 bp) and *rbc*S (19 bp) coding regions, showed a nucleotide divergence of 2.27% with *G. conferta* and of 5.45 % with *G. gracilis*.

The *rbc*L dataset (1263 positions) used for the phylogenetic analyses included one of the identical sequences obtained for the Venice specimens plus other 69 *Gracilaria*

sequences available in public databases, and the outgroup sequence of *Hypnea spinella* (C. Agardh) Kützing (AF385635). The GTR+I+Γ model, as selected by MODELTEST, was used in both ML and BI analyses. The *rbc*L phylogenetic tree (Fig. 13) showed the Venice specimen in a well supported clade with *Gracilaria* sp. from Sicily (100% of bootstrap support and 1.00 of posterior probability). This clade placed as sister taxon to different *G. gracilis* specimens from France (AY049399), Italy (EF434946) and United Kingdom (AY049400) (72BT/0.99PP) and *G. pacifica* from Indian Island (U.S.A.) was basal to all of them (98BT/1.00PP).

DISCUSSION

The molecular results combined with the morphological features strongly suggest that our strain represents a new species, here called *Gracilaria viridis* sp. nov., never noticed before in the Mediterranean Sea. In fact the *rbc*L phylogenetic analyses (Fig. 13) show that our strain is not conspecific with the cryptic species *Gracilaria gracilis*, but it forms a separate clade with *Gracilaria* sp. LI02 from Sicily (GenBank AY651040) supported by a 100% bootstrap value. Moreover, the small nucleotide divergence between the Sicilian and the Venice specimens (0.32%) strongly suggests that they could be considered the same species. Contrariwise, the genetic distance found between Venice *G. viridis* and *G. gracilis* (4.83%) is higher than the range of intraspecific distance (0.00–1.89%) described for the genus *Gracilaria* by Gurgel *et al.* (2001). The comparison of our *rbc*L-*rbc*S spacer sequence with those of the two cryptic species *G. gracilis* and *G. conferta* shows nucleotide divergences (5.45 % and 2.27 % respectively) that, although small, are inside the interspecific range reported for this genus (1.05-17.07%) and are comparable with the values found among other *Gracilaria* species using the same marker (1.94% for *G. gracilis*-*G. dura*; 2.72% for *G. conferta*-*G. dura* and 3.92% for *G. conferta*-*G. gracilis*) (Iyer *et al.* 2005). Moreover our results fit

with those of other authors, showing that no variation was found between individuals assigned to the same morphospecies (Guillemin, M.-L *et al.* 2008; Hommersand & Freshwater 2009).

Thalli of *G. viridis* sp. nov. have never been recorded by literature about the Venice Lagoon (Sfriso & Curiel 2007; Sfriso *et al.* 2009), but this species could be already present in the Venice sea-coastline. In fact, it is easily misidentifiable with *G. gracilis*, whose gross morphology is very similar, except the spiny branching and for the colour of the thalli, green-yellow in *G. viridis* and dark red to purple in *G. gracilis*. However, closer examination can question the belonging of the green-yellowish samples to *G. gracilis* because of differences in dimensions of both cortical and subcortical cells and tetrasporangia. In fact, *G. gracilis* is characterized by bigger structures: cortical cells are 10 µm width and 14 µm long; subcortical cells are 40-80 in diameter while tetrasporangia are 30 x 44 µm (Iyer *et al*. 2004).

Regarding the green-yellowish colour of the Venice Lagoon specimens, the pigment composition analysis evidences percentages of phycobiliproteins common to other red algae. The xanthophyll percentages show zeaxanthin as the dominant xanthophyll, followed by antheraxanthin and β-cryptoxanthin. This situation leads to ascribe our species to the XC group, as reported by Schubert *et al.* (2006) for *Gracilaria gracilis*. The accumulation of zeaxanthin is probably an acclimation response to light stress (Skriptsova & Yakovleva 2002; Schubert *et al*. 2006).

In 1992 Gargiulo and collaborators identified an exsiccata from Chioggia (Levi, no date, MS-33076-04) , characterized by some morphological features similar to that of *G. viridis* sp. nov., as conspecific with *G. conferta*. In fact, it presented terete filaments with a diameter of the main axis <1.5 µm and a dense bushy and spiny branching in the distal portions. However, our specimens are bigger, reaching an height of 25-30 cm, instead of the 8 cm reported in the description by Gargiulo *et al.* (1992), and thalli are

never wine-red in colour, but always green-yellowish, with at most some reddish parts. Unfortunately, there is only a morphological description for this entity and no molecular data are available for a comparison with our samples.

This research highlights the importance of using molecular data for the identification of red algal species without clear morphological diagnostic criteria, such those belonging to the genus *Gracilaria* whose taxonomy is still controversial.

ACKNOWLEDGMENTS

The authors would like to thank Prof. G.M. Gargiulo and Dr. G. Genovese for their helpful comments on the observations on material from Licata. This work was supported by grants provided by Progetto di Ateneo of the University of Padova.

REFERENCES

ALTSCHUL S.F., GISH W., MILLER W., MYERS E.W. & LIPMAN D.J. 1990. Basic Local Alignment Search Tool. *Journal of Molecular Biology* 215: 403-410.

BELLORIN A.M., OLIVEIRA M.C. & OLIVEIRA E.C. 2002 Phylogeny and systematics of the marine algal family Gracilariaceae (Gracilariales, Rhodophyta) based on small subunit rDNA and ITS sequences of Atlantic and Pacific species. *Journal of Phycology* 38: 551–563.

BIRD C. J. 1995. A review of recent taxonomic concepts and developments in the Gracilariaceae (Rhodophyta). *Journal of Applied Phycology* 7: 255-267.

CECERE E. & PETROCELLI A. 2009. The Mar Piccolo of Taranto. In: *Flora and Vegetation of the Italian Transitional Water Systems* (Ed. by Cecere E., Petrocelli A., Izzo G. & Sfriso A.), pp. 195-228. CORILA, Spinea, Italy.

COSTA V.L. & PLASTINO M.E. 2011. Color inheritance and pigment characterization of red (wild-type), greenish-brown, and green strains of *Gracilaria birdiae* (Gracilariales, Rhodophyta). *Journal of Applied Phycology* (in press).

FÄRBER A. & JAHNS P. 1998. The xanthophyll cycle of higher plants: influence of antenna size and membrane organization. *Biochimica et Biophysica Acta* 1363: 47-58.

FELSENSTEIN J. 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evoution* 39: 783-791.

FREDERICQ S. & HOMMERSAND M. H. 1989a. Proposal of the Gracilariales ord. nov. (Rhodophyta) based on analysis of the reproductive development of *Gracilaria verrucosa*. *Journal of Phycology* 25: 213-227.

FREDERICQ S. & HOMMERSAND M.H. 1989b. Comparative morphology and taxonomic status of *Gracilariopsis* (Gracilariales, Rhodophyta*). Journal of Phycology* 25: 228-241.

FREDERICQ S. & HOMMERSAND M.H. 1990. Diagnoses and key to the genera of the Gracilariaceae (Gracilariales, Rhodophyta). *Hydrobiologia* 204/5: 172-178.

FREDERICQ S., HOMMERSAND M. & FRESHWATER D.W. 1996. The molecular systematics of some agar- and carrageenan-containing marine red algae based on *rbc*L sequence analysis. *Hydrobiologia* 326/327: 125-135.

FRESHWATER D.W., FREDERICQ S., BUTLER B.S., HOMMERSAND M.H. & CHASE M.W. 1994. A gene phylogeny of the red algae (Rhodophyta) based on plastid *rbc*L. *Proceedings of the National Academy of Scence* USA 91: 7281-7285.

FRESHWATER D. W. & RUENESS J. 1994. Phylogenetic relationships of some European *Gelidium* (Gelidiales, Rhodophyta) species, based on *rbc*L nucleotide sequence analysis. *Phycologia* 33: 187-94.

FRESHWATER D.W., MONTGOMERY F., GREENE K.J., HAMNER M.R., WILLIAMS M. & WHITFIELD E.P. 2006. Distribution and identification of an invasive *Gracilaria* species that is hampering commercial fishing operations in southeastern North Carolina, USA. *Biological Invasions* 8: 631-637

FURNARI G., CORMACI M. & SERIO D. 1999. Catalogue of the benthic marine macroalgae of the Italian coast of the Adriatic Sea. *Bocconea* 12: 5-214.

FURNARI G., GIACCONE G., CORMACI M., ALONGI G. & SERIO, D. 2003. Biodiversità marina delle coste italiane: catalogo del macrofitobenthos. *Biologia Marina Mediterranea* 10: 1-484.

GARGIULO G.M., DE MASI F. & TRIPODI G. 1987. Structure and reproduction of *Gracilaria longa* sp. nov. (Rhodophyta, Gigartinales) from the Mediterranean Sea. *Giornale Botanico Italiano* 121: 247-257.

GARGIULO G.M., DE MASI F. & TRIPODI G. 1992. Morphology, reproduction and taxonomy of the Mediterranean species of *Gracilaria* (Gracilariales, Rhodophyta*)*. *Phycologia* 31 (1): 53-80.

GARGIULO G. M., MORABITO M., GENOVESE G. & DE MASI F. 2006. Molecular systematics and phylogenetics of gracilariacean species from the Mediterranean Sea. *Journal of Applied Phycology* 18: 497-504.

GUILLEMIN M.-L., AIT AKKI S., GIVERNAUD T., MOURADI A., VALERO M. & DESTOMBE C. 2008. Molecular characterisation and development of rapid molecular methods to identify species of Gracilariaceae from the Atlantic coast of Morocco. *Aquatic Botany* 89(3): 324-330.

GUINDON S. & GASCUEL O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52: 696-704.

GUIRY M.D. & GUIRY, G.M. 2011. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org.

GURGEL C.F.D., FREDERICQ S. & NORRIS J.N. 2001. Phylogeny, taxonomy and biogeography of the Gracilariaceae (Gracilariales, Rhodophyta) with emphasis on the northwestern Atlantic. *Phycologia* 40: 21.

GURGEL C.F.D. & FREDERICQ S. 2004. Systematics of the Gracilariaceae (Gracilariales, Rhodophyta): a critical assessment based on *rbc*L sequence analyses. *Journal of Phycology* 40: 138-159.

HOMMERSAND M.H., FREDERICQ S., FRESHWATER D.W. & HUGHEY J. 1999. Recent developments in the systematics of the Gigartinaceae (Gigartinales, Rhodophyta) based on *rbc*L sequence analysis and morphological evidence. *Phycological Research* 47: 139-151.

HOMMERSAND M.H. & FRESHWATER D.W. 2009. *Gracilaria hummii* sp. nov. (Gracilariales, Rhodophyta), a new name for the agarophyte "*Gracilaria confervoides*" harvested in North Carolina during World War II. *Journal of Phycology* 45(2): 503-516.

KURSAR T.A., VAN DER MEER J. & ALBERTE R.S. 1983. Light-Harvesting System of Red Alga *Gracilaria tikvahiae*. 1. Biochemical analyses of pigment mutations. *Plant Physiology* **73**: 353-360.

IYER R., TRONCHIN E.M., BOLTON J.J. & COYNE V.E. 2005. Molecular systematics of the Gracilariaceae (Gracilariales, Rhodophyta) with emphasis on Southern Africa. *Journal of Phycology* 41: 672-84.

LANAVE C., PREPARATA G., SACCONE C. & SERIO G. 1984 A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution* 20: 86–93.

MADDISON W.P. & MADDISON D.R. 2009. Mesquite: a modular system for evolutionary analysis. Version 2.71. http://mesquiteproject.org.

MAGGS C.A., MCIVOR L.M., EVANS C.E., RUENESS J. & STANHOPE M.J. 2002. Molecular analyses elucidate the taxonomy of fully corticated, nonspiny species of *Ceramium* (Ceramiaceae, Rhodophyta) in the British Isles. *Phycologia* 41: 409-420.

POSADA D. & CRANDALL K.A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817-818.

POSADA D. & BUCKLEY T.R. 2004. Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. *Systematic Biology* 53: 793-808.

RONQUIST F. & HUELSENBECK J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572-1574.

SCHUBERT N., MENDOZA E. G. & RUIZ I. P. 2006. Carotenoid composition of marine red algae. *Journal of Phycology* 42: 1208-1216.

SFRISO A. & CURIEL D. 2007. Check-list of marine seaweeds recorded in the last 20 years in Venice lagoon and a comparison with the previous records. *Botanica Marina* 50: 22-58. SFRISO A., CURIEL D. & RISMONDO A. 2009. The Venice Lagoon. In: *Flora and Vegetation of the Italian Transitional Water Systems* (Ed by Cecere E., Petrocelli A., Izzo G. & Sfriso A.), pp. 17-80. CORILA, Spinea, Italy.

SFRISO A., MAISTRO S., ANDREOLI C. & MORO I. 2010. First record of *Gracilaria vermiculophylla* (Gracilariales, Rhodophyta) in the Po Delta lagoons, Mediterranean Sea (Italy). *Journal of Phycology* 46: 1024-1027.

SKRIPTSOVA A.V. & YAKOVLEVA I.M. 2002. The influence of variations in irradiance upon morphology in an unattached form of *Gracilaria gracilis* (Stackhouse) Steentoft during field cultivation, South Primorye, Russia. *Aquatic Ecology* 36: 511-518.

THOMPSON J.D., HIGGINS D.G. & GIBSON T.J. 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-

specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673-4680.

TROELL M., HALLING C., NEORI A., CHOPIN T., BUSCHMANN A.H., KAUTSKY N. & YARISH C. 2003. Integrated mariculture: asking the right questions. *Aquaculture* 226: 69-90.

FIGURE CAPTIONS

Fig. 1. Growth areas of *G. viridis*. The asterisks indicate the sampling sites. However the species is present in the whole artificial rocky littoral of the Venice gulf.

Fig. 2. Habitus of the thallus of *G. viridis* collected in the Venice Lagoon (holotype). Scale bar, 2 cm.

Fig. 3. View of branches and branchlets of thallus. Scale bar, 0.5 cm.

Fig. 4. Habitus of the thallus of *G. viridis* collected in Sicily. Scale bar, 2 cm.

Fig. 5. Cross-section of an axis showing cortical, sub-cortical and medullar cells. Scale bar, 200 µm.

Fig. 6. Tetrasporophytic branches with spine-like uni-tribranched branchlets. Scale bar, 1 mm.

Fig. 7. Tetrasporophytic branches with spine-like bi-branched branchlets. Scale bar, 0.5 mm.

Fig. 8. Tetrasporangium. Scale bar, 20 µm.

Fig. 9. Dark red ostiolate cystocarps (arrows) on green-yellowish thalli. Scale bar, 2mm.

Fig. 10. Longitudinal section of cystocarp with tubular nutritive cells. Scale bar, 200 µm.

Fig. 11. Longitudinal section of cystocarp. Scale bar, 250 µm

Fig. 12. Cross-section of a male gametophytic plant showing round-elliptical spermatangial *Verrucosa*-type conceptacles (arrow) scattered on the cortical layer. Scale bar, 50 µm.

Fig. 13. ML tree inferred from *rbc*L gene sequences, calculated using the GTR+I+Γ model of evolution. Numbers near nodes indicate bootstrap values (>50%) for ML analysis (in bold black font), and posterior probabilities (in normal font). The new species sequence determined in this work is indicated in bold. The Mediterranean

species sequences are evidenced in blue. Bar represents 0.02 nucleotide substitutions per site.

Fig. 1
Click here to download Figure: Fig1.pdf

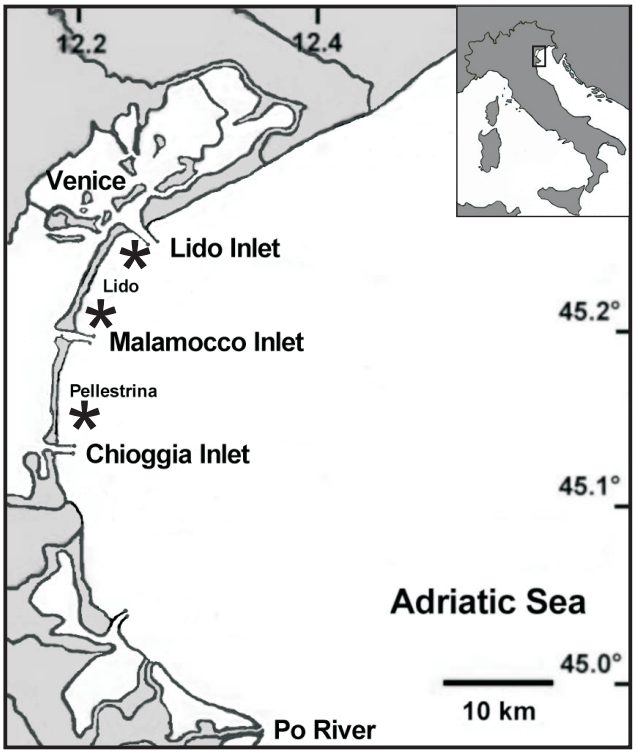
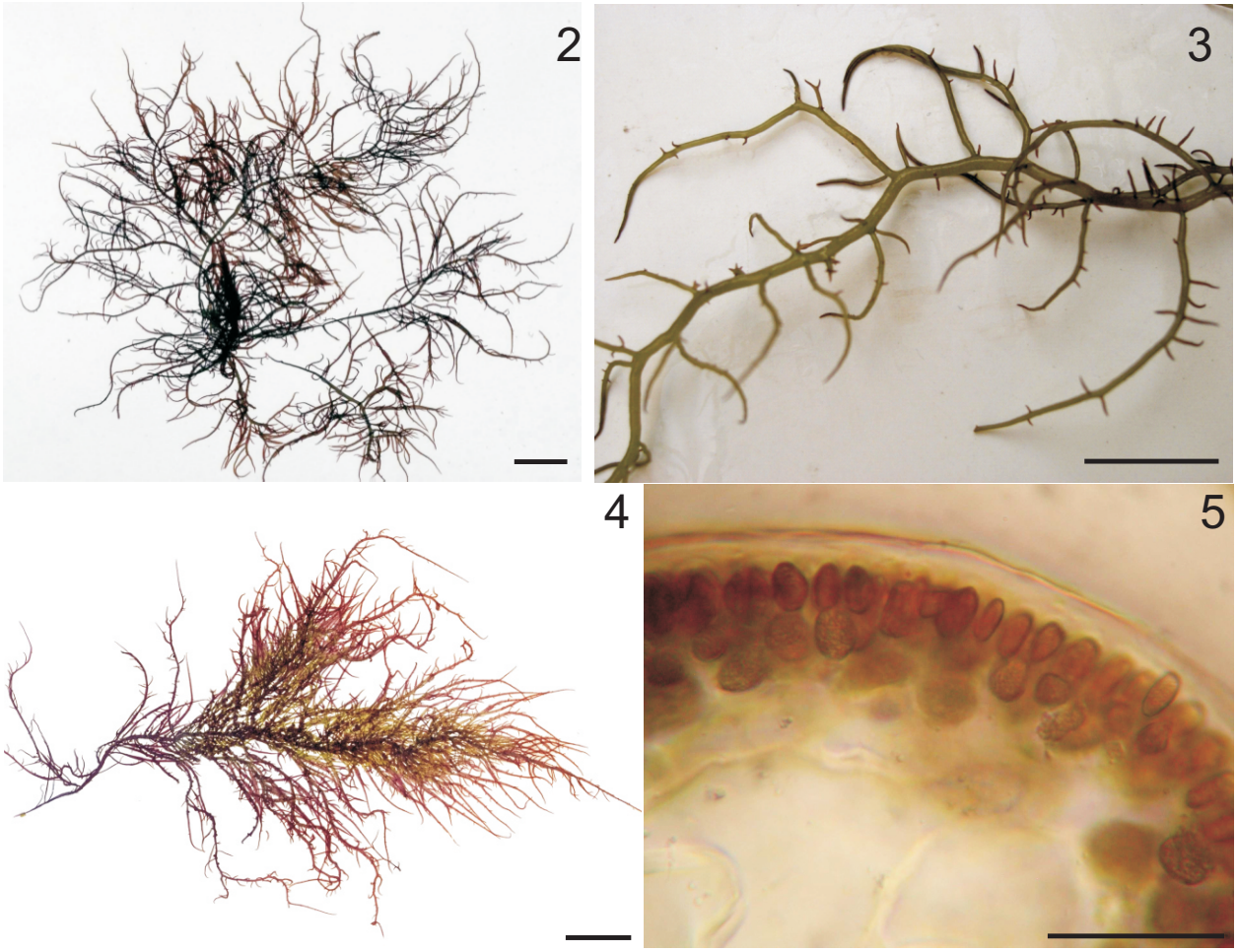


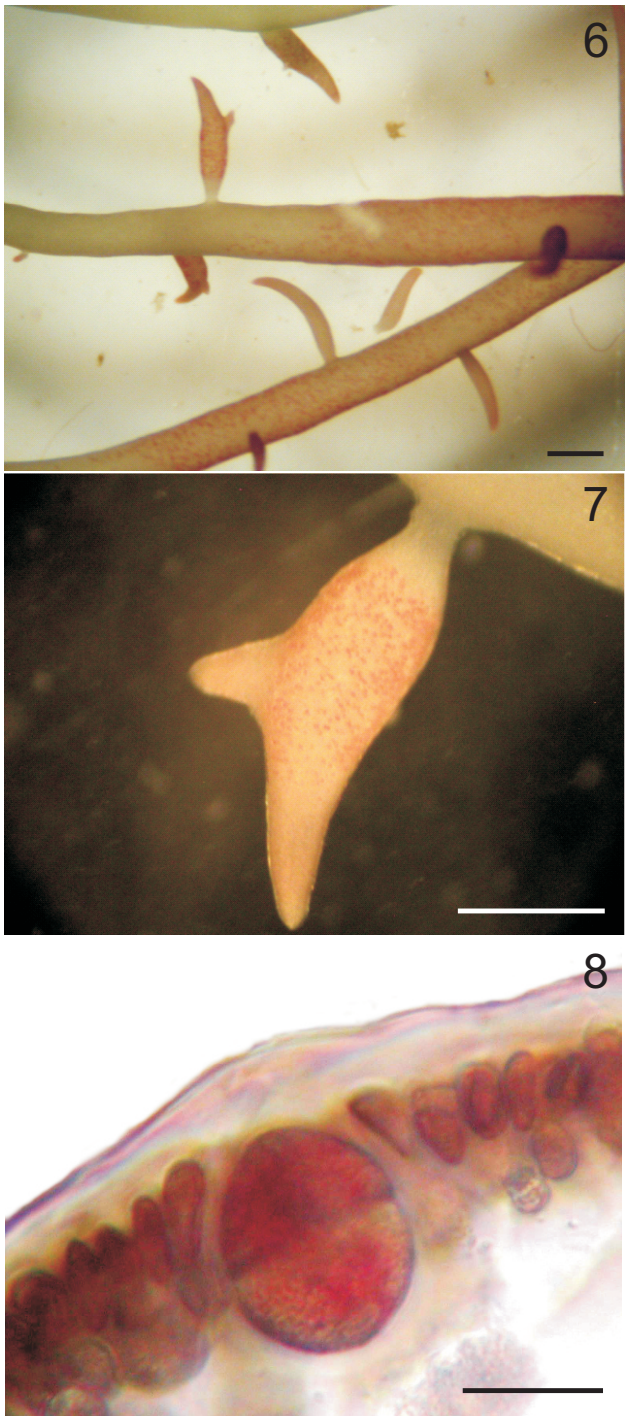
Fig. 1

Figs 2-5
Click here to download Figure: Figs2-5.pdf



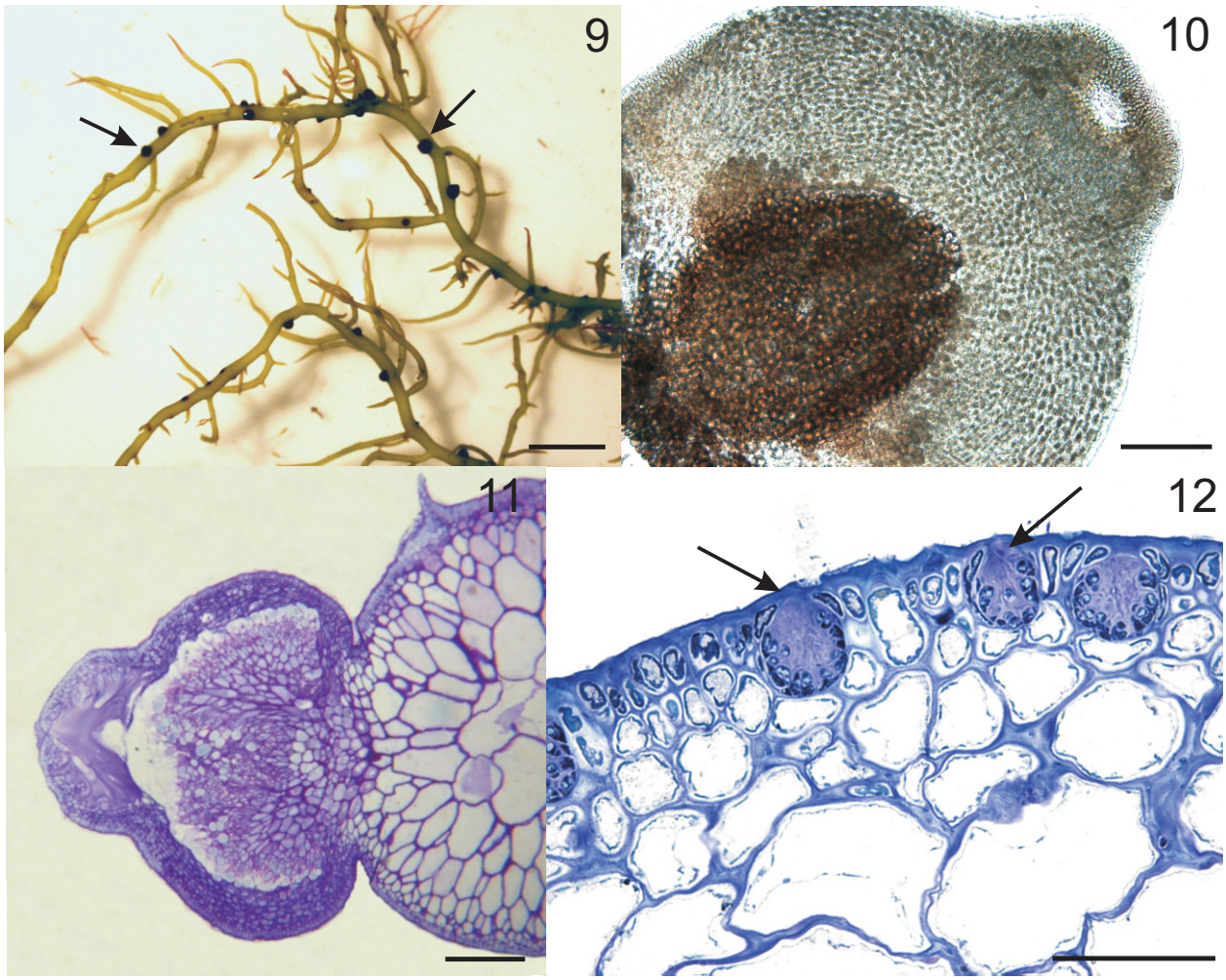
Figs 2-5

Figs 6-8
Click here to download Figure: Figs6-8.pdf



Figs 6-8

Figs 9-12
Click here to download Figure: Figs9-12.pdf



Figs 9-12

Fig. 13

Click here to download Figure: Fig13.pdf

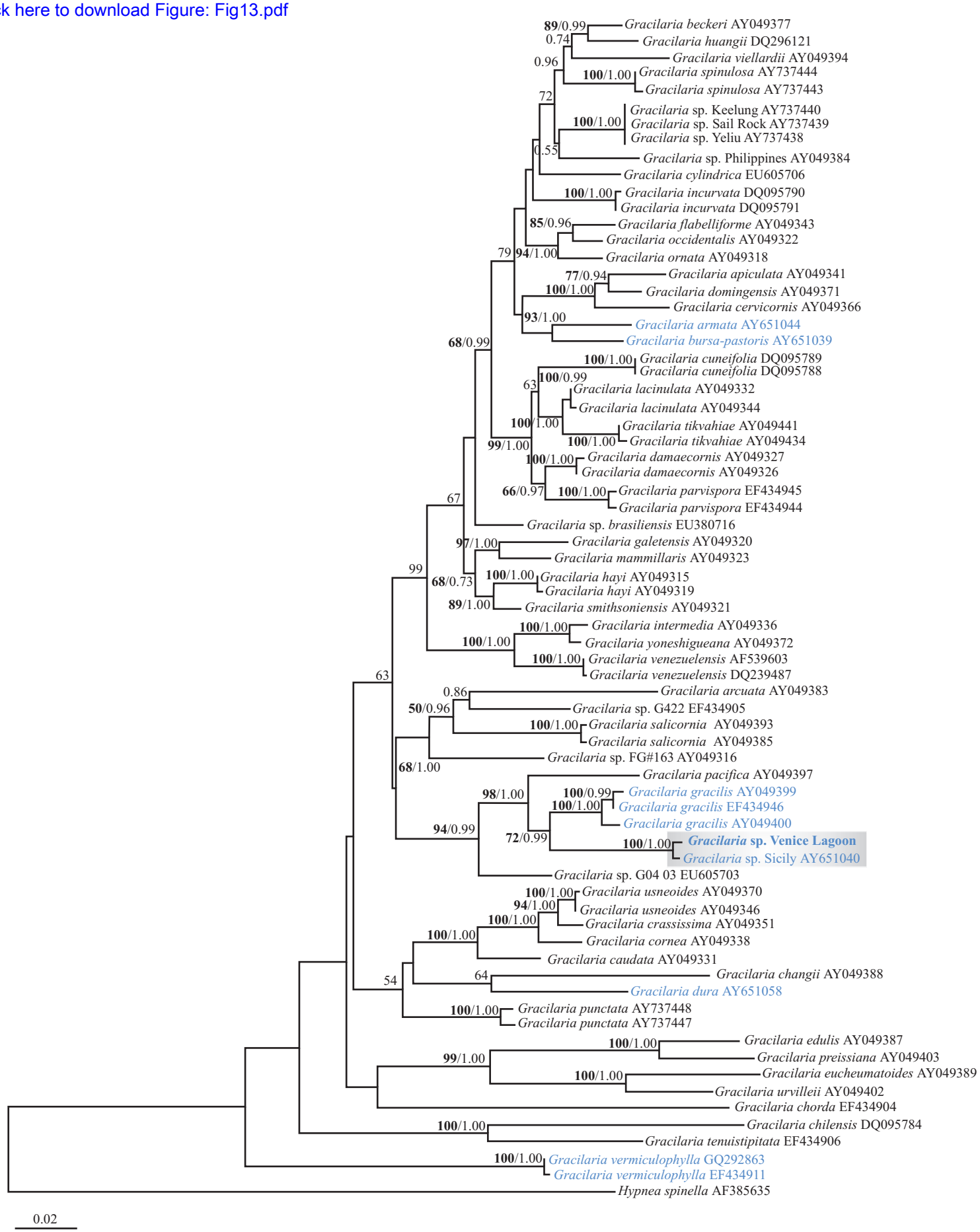


Fig. 13

Tab. 1- *Gracilaria* species recorded in the Mediterranean Sea

Gracilaria armata (C. Agardh) Greville
Gracilaria bursa-pastoris (S.G. Gmelin) P.C. Silva
Gracilaria conferta (Schousboe *ex* Montagne) Montagne
Gracilaria corallicola Zanardini
Gracilaria dendroides Gargiulo, De Masi, Tripodi
Gracilaria dura (C. Agardh) J. Agardh
Gracilaria gracilis (Stackhouse) Steentoft, Irvine *et* Farham
Gracilaria heteroclada (Montagne) Feldmannn *et* Feldmann-Mazoyer
Gracilaria longa Gargiulo, De Masi *et* Tripodi.
Gracilaria multipartita (Clemente) Harvey
Gracilaria vermiculophylla (Ohmi) Papenfuss

PROBLEMS AND SOLUTIONS IN *GRACILARIA* (RHODOPHYTA, GRACILARIALES) SYSTEMATICS: AN EXAMPLE FROM *G. BURSA-PASTORIS* (GMELIN) SILVA

K. Sciuto[1]*, M.A. Wolf[1]*, A. Sfriso[2], A. Petrocelli[3], E. Cecere[3], I. Moro[1]

[1]*Department of Biology, University of Padova, via U. Bassi 58/B, Padova, 35131, Italy*

[2]*Department of Environmental Sciences, University of Venice, Dorsoduro 2137, Italy*

[3]*Institute for Marine Coastal Environment C.N.R. v. Roma 3 – 74100 Taranto Italy*

*equal contributors

ABSTRACT

Type specimens, on which names of species are based, are necessary for comparison in systematic studies, as stated by the International Code of Botanical Nomenclature. Unfortunately, very often the types lack or are illustrations, so not suitable for modern systematics. In other case, instead, herbarium types are still available.

With the spread of the DNA barcoding method, molecular techniques have developed to extract DNA and to amplify molecular markers also from historical material, making herbaria a precious source of information.

Here we focus on the type species of the genus *Gracilaria,* a taxonomically challenging group because of structural simplicity, high morphological plasticity, and great species diversity. Classically, the identification of *Gracilaria* species has been based on gross morphological characters and incorrect applications have often led to misidentifications. Moreover, species outlining is problematic for the limitations of distinct morphological

and reproductive characteristics.

In this study we analyzed some Mediterranean entities, both preserved in herbarium collections of the University of Padova (Italy) and recently sampled, that have been attributed or are morphologically ascribable, respectively, to the species *Gracilaria bursa-pastoris* (Gmelin) Silva. Long since, it is known that *G. bursa-pastoris*, the current type species of the genus, is characterized by the presence of several morphotypes. This can lead to two possible errors: assignment of different morphotypes to distinct species or identification as *G. bursa-pastoris* of species actually different, thinking that they represent diverse morphotypes.

The comparison of the *rbc*L-*rbc*S sequences of the different samples considered in this study with that obtained from the lectotype (*Sphaerococcus compressus* Cabrera; herb. alg. Agardh, LD 28991) revealed the presence of diverse morphotypes and the existence of a species, collected in 1869 and preserved in the PAD historical herbarium, never sequenced before.

INTRODUCTION

Members of the genus *Gracilaria* are economically valuable algae, both as direct foodstuffs (Abbott, 1985) and for their agar content. For this reason they have been intensively investigated in the last 40 years and comprehensive information about biology (Oliveira and Plastino, 1994), cultivation (Oliveira et al., 2000), and utilization (cf. Critchley and Ohno, 1998) have been reported. However, there is still much to be done to solve the many remaining taxonomic problems (e.g. Bird, 1995). In fact, species of the genus *Gracilaria* are difficult to identify because of structural simplicity, high morphological plasticity (morphotypes), great species diversity, and presence of cryptic species (convergent evolution). Plants of the largest species can reach 60 cm in length.

Thalli range from erect to prostrate and from terete to broadly flattened. Some species form articulated fronds composed of cylindrical or irregularly shaped units (Guiry and Guiry, 2012).

Gracilaria was established by Greville in 1830, and of the14 species that he assigned to this genus only four are currently retained under this taxon: *G. compressa* (C. *Ag.)* Grev. (now *G. bursa-pastris* (Gmel.) Silva), *G. confervoides* (L.) Grev. (now *G. verrucosa* (Huds.) Papenf.), *G. lernanaeformis* (Bory) Grev., and *G. zichenoides* (Turn.) Grev. (now *G. edulis* (Gmel.) Silva).

Different approaches have been attempted to clarify the taxonomy of the genus: early concepts of the species of *Gracilaria* were mainly based on external form; however, the nature of the reproductive organs has been considered in the classification of species since 1926.

There are 316 species (and intraspecific) names in the database at present, of which 167 have been flagged as currently accepted taxonomically. Many of them are poorly known and with very limited distributions (Guiry and Guiry, 2012). On the other hand, some species, for a long time considered to be widely distributed, appear to be complexes of distinct taxa, difficult to separate merely by habit differences.

Taxonomists are united in stressing the difficulties in identifying and characterizing many of the described species only by anatomical features.

In the last two decades the use of DNA barcoding has enabled the identification of new species and lead to revise the systematics of many seaweed groups. In fact, for the high degree of morphological plasticity exhibited by many members of these taxa, several characters traditionally considered diagnostic have revealed ambiguous and so useless. This is true also for members of the genus *Gracilaria* Greville (Saunders, 2009; Destombe et al., 2010).

Here we focus on some Mediterranean entities, both preserved in herbarium collections of the University of Padova (Italy) and recently sampled, that have been attributed or are morphologically ascribable, respectively, to the species *Gracilaria bursa-pastoris* (Gmelin) Silva. Long since, it is known that *G. bursa-pastoris*, the current type species of the genus, is characterized by the presence of several morphotypes. This can lead to two possible errors: assignment of different morphotypes to distinct species or identification as *G. bursa-pastoris* of species actually different, thinking that they represent diverse morphotypes.

Systematic studies to solve this kind of problems involve comparison with type specimens (on which species names are based), typically stored in collections or described in the scientific literature. Recently, molecular techniques have developed to extract DNA and to amplify short, but informative, portions of the genome, like the intergenic spacers *rbc*L-*rbc*S and *cox*2-*cox*3.

The plastid *rbc*L-*rbc*S spacer region is proven to be a good marker for species discrimination, being essentially invariant within a species, but extremely variable at the interspecific level (Cho et al., 2003; Skage et al., 2005, Wolf et al., 2011). The more variable mitochondrial *cox*2-*cox*3 spacer is useful for population studies (Zuccarello et al., 1999). DNA barcoding can be a good way to have an objective comparison between newly sampled organisms and historical ones. This is important, for example, to compare seaweed biodiversity patterns and to summarize the occurrence and the distribution of introduced species (Provan et al., 2008; Lister et al., 2010). In the light of this, historical herbarium collections, like that of the University of Padua (Italy), can play an important role in modern researches on biodiversity, systematics, and phylogeny.

The comparison of the *rbc*L-*rbc*S sequences of the different samples considered in this

study and that obtained from the lectotype of *G. compressa* = *G. bursa.pastoris* (*Sphaerococcus compressus* Cabrera; herb. alg. Agardh, LD 28991; Steentoft et al., 1991) revealed both the presence of diverse morphotypes of this taxa and the existence of a species collected in 1869, misidentified with *G. bursa-pastoris* according to the morphological characters and never sequenced before.

MATERIALS AND METHODS

Samples. Fresh samples, morphologically ascribable to the species *G. bursa-pastoris*, were collected in the Mar Piccolo of Taranto (40°28'N; 17°15'E) and along the coastline of Venice (45°24'N; 12°17'E). Two different morphotypes were found in both the Mar Piccolo and the marine sites of Alberoni-Faro Rocchetta (Lido of Venice; geographical coordinates: Lido island: 45° 23' N, 12° 21'E) (morphotypes 1 and 2). A third morphotype was collected only in the lagoon sites of Malamocco (Lido of Venice) (morphotype 3).

The historical samples analyzed in this study, attributed to the species *G. compressa* (now *G. bursa-pastoris*), are stores in folders and preserved in the PAD herbarium (University of Padova, Italy) as part of the collection A. Forti 1937.

The lectotype of *G. compressa* (*Sphaerococcus compressus* Cabrera, LD 28991) is conserved in the historical Agardh collection (19th century) of the Botanical Museum Lund University (Sweden).

Morphology. The internal structure of the fresh samples was observed on semi-thin sections. Parts of thalli were fixed overnight in 6% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9), and post-fixed overnight in 1% OsO4 in the same buffer. The post-fixed samples were dehydrated in a graded ethanol series, followed by propylene oxide, and then were embedded in araldite. Semi-thin sections were cut with an

ultramicrotome (Reichert Ultracut S), stained with 1% toluidine blue and examined with a DMR Leica (Sweden) microscope, equipped with a digital image acquisition system.

Ultrastructural features were observed with transmission electron microscopy (TEM). The samples were prepared as described in Moro and collaborators (2010). TEM analyses were carried out with an Hitachi HS9 (Tokyo, Japan) transmission electron microscope operating at 75 kV.

Molecular procedures. Genomic DNA was extracted from fresh samples using the Genomic DNA purification kit (Fermentas International Inc., Burlington, Ontario, Canada) following manufacturer's instructions.

For the herbarium samples we used a modified protocol based on Hughey et al. (2001). Fragments (about 5 mm^2) of the exiccata were taken for DNA extraction.

The *rbc*L-*rbc*S spacer was amplified using the forward primer F1320, designed by Freshwater et al. (2006), in combination with the reverse primer RrbcSstart (Freshwater and Rueness, 1994). PCR reactions were performed as follows: an initial denaturation step of 2 min at 94°C; 30 cycles at 94°C for 40 sec, at 50°C for 40 sec, and at 72°C for 50 sec; and a final extension step at 72°C for 5 min. The amplification products were cleaned with ExoSAP-ITTM kit (GE Healthcare, Uppsala, Sweden, Europe), following the manufacturer's protocol.

For the lectotype the amplification was carried out using the primer F1467 (Hughey et al., 2001) and the primer RrbcSstart. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen).

DNA sequencing was performed at the BMR Genomics Sequencing Service (Padova University) on automated ABI DNA sequencers (Applied Biosystem, Foster City, CA, USA), with the same primer pairs used in the amplification reactions. Final consensus sequences were assembled using the SeqMan II program from the Lasergene software

package (DNAStar©, Madison, WI, USA). Identity of the obtained sequences was checked by using the BLAST program (Altschul et al., 1990) available at the USA National Center for Biotechnology Information (NCBI) web server (http://www.ncbi.nlm.nih.gov) and the sequence alignments were obtained by the ClustalW computer program (Thompson et al., 1994).

For the phylogenetic analyses of the *rbc*L-*rbc*S spacer region, a dataset of 42 sequences was constructed including the obtained sequences plus other *Gracilaria* ones accessible in the DDBJ/GenBankTM/EBI Data Bank. The analyses were performed according to maximum likelihood (ML) method with the PHYML 2.4.4 program (Guindon and Gascuel, 2003) by applying the TPM1uf+G evolutionary model, as found by the program MODELTEST under the Akaike Information Criterion (AIC) (Posada and Crandall, 1998; Posada and Buckley, 2004). Non parametric bootstrap re-sampling (Felsenstein, 1985) was performed to test the robustness of the tree topology (1000 replicates).

Bayesian Inference analyses were carried out too, using MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003). The substitution model was the TPM1uf+G and the Bayesian analyses were performed with four search chains for 3,000,000 generations, sampling trees every 100 generations. The first 7,500 trees were discarded as burn-in. Parameter stability was estimated by plotting log-likelihood values against generation time and a consensus tree with posterior probabilities was then produced. The nexus file for the Bayesian analyses was generated with the Mesquite 2.71 software package (Maddison and Maddison, 2009).

Tree topologies were visualized with the NJplot computer program (Perrière and Gouy 1996).

RESULTS AND DISCUSSION

We analyzed different specimens, both fresh collected (Fig. 1a,b,c) and preserved in herbarium collection (PAD) (Fig. 2a,b,c), in addition to the lectotype of *G. compressa* = *G. bursa.pastoris* (*Sphaerococcus compressus* Cabrera; herb. alg. Agardh, LD 28991) (Fig. 3a). The choice of the lectotype of *G. compressa* as type specimen was due to the lack of an herbarium sample of *G. bursa-pastoris*. In fact**,** the lectotype of this species is represented only by an original illustration by S.G. Gmelin (1768, pl. VIII, fig. 3) (Fig. 3b).

MORPHOLOGY

Habit and vegetative structure. The habitus of the freshly collected samples was very different among the diverse specimens: less branched, more compressed with elongated branches, and dull red in colour in morphotypes 1 and 2 (Fig. 1a,b); more bushy, with recurvate branches ending in acuminate tips, and yellowish in colour in morphotype 3 (Fig. 1c). Thalli presented a discoid holdfast in both morphotypes 1 and 2, found in Venice (Alberoni-Faro Rocchetta) and Taranto marine sites. The specimens from the lagoon sites of Venice (Malamocco), showing the morphotype 3, frequently grow unattached without hold structures.

Inner structure of the vegetative axis (Fig. 4a), instead, was similar in all samples and presented an outer cortex, consisting of one or two layers of small pigmented cortical cells, two layers of subcortical cells (20 μm to 100 μm in diameter) with a large amount of starch grains (Fig. 4b), and an inner medullary layer composed by bigger cells ranging from 150 to 250 μm in diameter.

Tetrasporangia. Tetrasporangia (Fig. 4c) (16-20 µm x 25-29 µm) were cruciately divided, originated from terminal cortical cells, and occurred on branches of any order of tetrasporangial thalli.

Spermatangia. Spermatangium conceptacles (Fig. 4d,e) were present in shallow sunken patches (27 µm in diameter and 25 µm deep), characteristic of the 'Textorii' type, and were separated by unmodified cortical cells.

According to Liao and Hommersand (2003) 10 species groups are identified in the Gracilariaceae, based on spermatangial type and cystocarp development:

1) *abscissa* group (*Melanthalia*), 2) *flabellata* group (*Curdiea*), 3) *lemaneiformis* group (*Gracilariopsis*), 4) *chilensis* group, 5) *edulis* group (*Plocaria/Polycavernosa*), 6) *urvillei* group (*Hydropuntia*), 7) *crassissima* group, 8) *salicornia* group (*Corallopsis*), 9) *gracilis* group, and 10) *bursa-pastoris* group (*Gracilaria*).

The *bursa-pastoris* group comprises a large morphologically diverse assemblage of species, including *Gracilaria beckeri*, the type species of *Tyleiphora* J. Agardh. At the present, two characters distinguish most members of the group: spermatangia borne in shallow *Textorii*-type conceptacles and gonimoblasts producing tubular cells, that link to cells of the outer pericarp with the formation of tubular fusion cells.

Ultrastructural features – pit-connections. Pit-connections (septal pores between two cells, useful for cellular continuity) are characterized by structures which occlude the septal pore. These are called "pit-plugs" and are formed by an internal plug-core eventually surrounded by one or two plug-caps. Investigations, aimed at discovering ultrastructural features useful in systematic considerations on red algae, have concerned the presence or absence of plug-caps bracketing the plug-core (Pueshel and Cole, 1982).

All our fresh collected samples presented the same pit-plug structure (Fig. 5a,b): absence of plug-caps, naked cap membrane usually present at either end of the plug-

core. This type of pit-plug is typical of all species of the genus *Gracilaria* (Pueshel and Cole, 1982). Pit-plug is not suitable in species discrimination, but it is a useful systematic character at family or genus level. In fact, it represents a totally independent and conserved feature, which can be used to test present concepts of familial and ordinal relationships.

MOLECULAR ANALYSES

The *rbc*L-*rbc*S dataset included 42 sequences, 34 of which were retrieved from DDBJ/GenBankTM/EBI Data Bank. Two sequences of *Gracilariopsis longissima* were chosen as outgroup. The alignment consisted of a total of 235 positions, including the *rbc*L-*rbc*S spacer (variable in relation to the different species), the *rbc*L (84 bp) and the *rbc*S (17 bp) coding regions.

In the genus *Gracilaria* the Rubisco spacer lengths vary from 65 (*G. multipartita*) to 169 nucleotides (*G. hummi*) (Hommersand and Freshwater, 2009).

Alignment data showed that the *rbc*L-*rbc*S spacer region sequences of our Mediterranean samples (166 bp long) have 0.60% nucleotide divergence with other sequences of *G. bursa-pastoris* available in GenBank. This genetic distance is inside the intraspecific range reported for this genus (0-1.89% Gurgel et al., 2001).

No differences were found between the sequences of the collected specimens and the sequence obtained from the herbarium lectotype, considering only the intergenic spacer (65 bp in length) (Fig. 6).

In the phylogenetic *rbc*L-*rbc*S tree (Fig. 7) all freshly collected samples and two herbarium specimens (*G. compressa* #4 and *G. compressa* #5) formed a clade with two sequences of *G. bursa-pastoris* from Canada (DQ984678 and DQ984688). *G.*

compressa #6, instead, was sister taxon to this clade, with 5.23% of nucleotide divergence.

This genetic distance suggests that G. compressa #6 (Savona, Italy, 1869) represents a different species, never sequenced before. In fact, this result is congruent with the interspecific range of nucleotide divergence reported for this genus (1.05-17.07%) and is comparable with the values found between other *Gracilaria* species using the same *rbc*L-*rbc*S spacer marker (1.94% for *G. gracilis*-*G. dura*; 2.72% for *G. conferta*-*G. dura* and 3.92% for *G. conferta*-*G. gracilis*) (Iyer et al., 2005).

The molecular data produced in this study fit with those of other authors showing that no variation in the *rbc*L-*rbc*S spacer is found between individuals assigned to the same species (Guillemin,M.-L et al., 2008; Hommersand and Freshwater, 2009).

Our survey confirms the existence of different morphotypes for the species *G. bursa-pastoris*, which can be easily, but erroneously, assigned to distinct species, basing merely on morphology. Probably this phenotypic plasticity is due to the different environmental conditions, as observed in previous studies based on morphological characters (Gargiulo et al., 2006). In fact, in sheltered sites thalli become more bushy, whereas they are less branched and more compressed if collected in marine sites or in wave exposed lagoon areas.

Moreover the molecular analyses on historical material allowed us to recognize a putative new species, the herbarium specimen named *G. compressa* #6, at the time misidentified with *G. compressa* based on morphology. Further studies will be necessary to understand if this entity is still present in the Mediterranean Sea.

In addition, the *rbc*L-*rbc*S spacer sequence of *G. bursa-pastoris* lectotype, for which no other molecular sequences were available up to now, can constitute a valid reference for future researches.

CONCLUSIONS

This study highlights the importance of using a molecular approach to solve taxonomic problems of complex species or species-groups in which morphological data are insufficient or ambiguous. Moreover it underlines the importance of involving significant historical specimens to obtain a correct taxonomic collocation of uncertain fresh samples and to solve historical debates like the one presented in this work.

ACKNOWLEDGEMENTS

This work was supported by grants provided by Progetto di Ateneo of the University of Padova.

REFERENCES

Abbott, I. A., and Hollenberg G. J. 1976. *Marine algae of California*. Stanford University Press, Stanford, California, USA.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990. Basic Local Alignment Search Tool. *J. .Mol. Biol.* 215: 403-410.

Bird, C. J. 1995. A review of recent taxonomic concepts and development in the Gracilariaceae (Rhodophyta). *J. Appl. Phycol*. 7: 255-267.

Cho, T. O., Fredericq, S. & Boo, S. M. 2003. *Ceramium inkyuii* sp. nov. (Ceramiaceae, Rhodophyta) from Korea; a new species based on morphological and molecular evidence. *J. Phycol*. 39:237–47.

Critchley, A. T. & Ohno, M. 1998. *Seaweed Resources of the World*. Japan International Cooperation Agency, Nagai, Japan, pp. 431.

Destombe, C., Valero, M. & Guillemin, M.L. 2010. Delineation of two sibling red algal species, *Gracilaria gracilis* and *Gracilaria dura* (Gracilariales, Rhodophyta),

using multiple DNA markers: resurrection of the species *G. dura* previously described in the northern Atlantic 200 years ago. *J. Phycol.* 46: 720-727.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evoution* 39: 783-791.

Freshwater, D. W. & Rueness, J. 1994. Phylogenetic relationships of some European *Gelidium* (Gelidiales, Rhodophyta) species, based on *rbc*L nucleotide sequence analysis. *Phycologia* 33:187–94.

Freshwater D. W., Montgomery F., Greene K. J., Hamner M. R.,Williams M. & Whitfield E. P. 2006. Distribution and identification of an invasive Gracilaria species that is hampering commercial fishing operations in southeastern North Carolina, USA. *Biological Invasions* 8: 631–637

Gargiulo G. M., Morabito M., Genovese G. & De Masi F. 2006. Molecular systematics and phylogenetics of gracilariacean species from the Mediterranean Sea. *Journal of Applied Phycology* 18: 497-504.

Gmelin, S.G. 1768. *Historia fucorum*. pp. [i-xii], [i]-239, [i]-6 expl. tab., 35 plates [1A, IB, IIA, IIB, III-XXXIII]. Petropoli [St. Petersburg]: Ex typographia Academiae scientiarum.

Guillemin, M.-L., Ait Akki, S., Givernaud, T., Mouradi, A., Valero, M. & Destombe, C. 2008. Molecular characterisation and development of rapid molecular methods to identify species of Gracilariaceae from the Atlantic coast of Morocco. *Aquatic Botany* 89: 324-330.

Guindon, S. & Gascuel, O. 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52: 696-704.

Guiry, M.D. & Guiry, G.M. 2012. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org.

Hommersand, M.H. & Freshwater, D.W. 2009. *Gracilaria hummii* sp. nov. (Gracilariales, Rhodophyta), a new name for the agarophyte "*Gracilaria confervoides*" harvested in North Carolina during World War II. *Journal of Phycology* 45(2): 503-516.

Hughey, J.R., Silva, P.C. & Hommersand, M.H. 2001 Solving taxonomic and nomenclatural problems in Pacific Gigartinaceae (Rhodophyta) using DNA from type material. *J. Phycol.* 37: 1091–1109.

Liao, L.M. & Hommersand, M.H. 2003. A morphological study and taxonomic reassessment of the generitype species in the Gracilariaceae. *J. Phycol.* 39: 1207-1232.

Lister D.L., Bower, M.A. & Jones, M.K., 2010. Herbarium specimens expand the geographical and temporal range of germplasm data in phylogeographic studies. *Taxon* 59: 1321-1323

Maddison, W.P. & Maddison, D.R. 2009. Mesquite: a modular system for evolutionary analysis. Version 2.71. http://mesquiteproject.org.

Moro, I., Rascio, N., La Rocca, N., Sciuto, K., Albertano, P.,Bruno, L., Andreoli, C., 2010. Polyphasic characterization of a thermo-tolerant filamentous cyanobacterium isolated from the Euganean thermal muds (Padova, Italy). *Eur. J. Phycol.* 45: 143–154.

Oliveira, E. C., Alveal, K. & Anderson, R. J. 2000. Mariculture of the agar-producing gracilarioid red algae. *Rev. Fish. Sci*. 8: 345-377.

Oliveira, E. C. & Plastino, E. M. 1994. Gracilariaceae, *in:* Akatsuka, I. (ed.) *Biology of Economic Algae*. SPB Academic Publishing bv, The Hague, The Netherlands, pp. 185-226.

Perrière, G. & Gouy, M. 1996. WWW-Query: An on-line retrieval system for biological sequence banks. *Biochimie* 78: 364-369.

Posada, D. & Crandall, K.A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817-818.

Posada, D. & Buckley, T.R. 2004. Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. *Systematic Biology* 53: 793-808.

Provan, J., Booth, D., Todd, N.P., Beatty, G.E. & Maggs, C.A., 2008. Tracking biological invasions in space and time: elucidating the invasive history of the green alga *Codium fragile* using old DNA. *Diversity and Distributions A Journal of Conservation Biogeography* 14: 343-354.

Pueshel, C. M. and Cole, K. M. 1982. Rhodophycean pit plugs: an ultrastructural survey with taxonomic implications. *Amer. J. Bot*. 69: 703-720.

Ronquist, F. & Huelsenbeck, J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572-1574

Saunders, G.W., 2009. Routine DNA barcoding of Canadian Gracilariales (Rhodophyta) reveals the invasive species *Gracilaria vermiculophilla* in British Columbia. *Molecular Ecology Resources*. 9: 140-150.

Skage M., Gabrielsen T. M. & Rueness J. 2005. A molecular approach to investigate the phylogenetic basis of three widely used species groups in the red algal genus *Ceramium* (Ceramiales, Rhodophyta). *Phycologia* 44: 353-360.

Steentoft, M.,. Irvine, L. M & Bird, C. J. 1991. Proposal (1015) to conserve the type of *Gracilaria*, nom. cons., as *G. compressa* and its lectotypification (Rhodophyta: Gracilariaceae), *in:* Nicolson, D. H. (ed.), *Proposals to Conserve or Reject*. *Taxon 40*: 663-666.

Thompson J.D., Higgins D.G. & Gibson T.J. 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673-4680.

Wolf, M. A., Sciuto, K., Maggs, C. A., Barros-Barreto, M.B.B., Andreoli, C. & Moro, I., 2011. *Ceramium* Roth (Ceramiales, Rhodophyta) from Venice lagoon (Adriatic Sea, Italy): comparative studies of Mediterranean and Atlantic taxa. *Taxon* 60: 1584-1595.

Zuccarello, G.C., Burger, G., West, J.A. & King, R.J., 1999. A mitochondrial marker for red algal intraspecific relationships. *Mol. Ecol.* 8: 1443-1447.

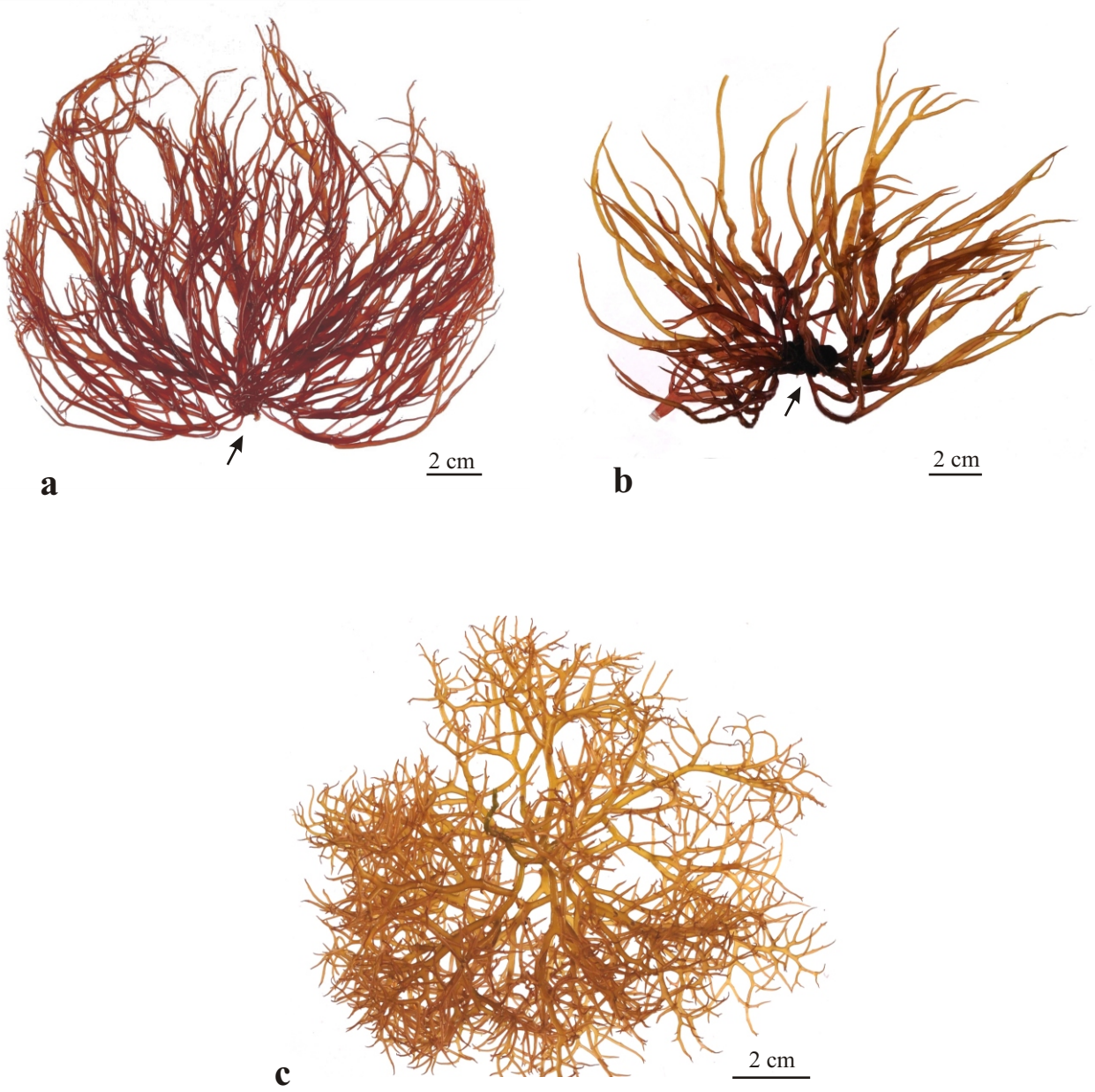


Fig. 1. Habitus of the different fresh sampled specimens. Morphotypes 1 (**a**) and 2 (**b**) less branched, more compressed with elongated branches and dull red in colour. Collected in the marine sites of Venice and Taranto (Italy) at 3 m depth. Thalli presented a discoid holdfast (arrows). Morphotype 3 (**c**) more bushy and yellowish in colour. Collected in the lagoon sites of Venice (50 cm deep). Absence of a hold structure.

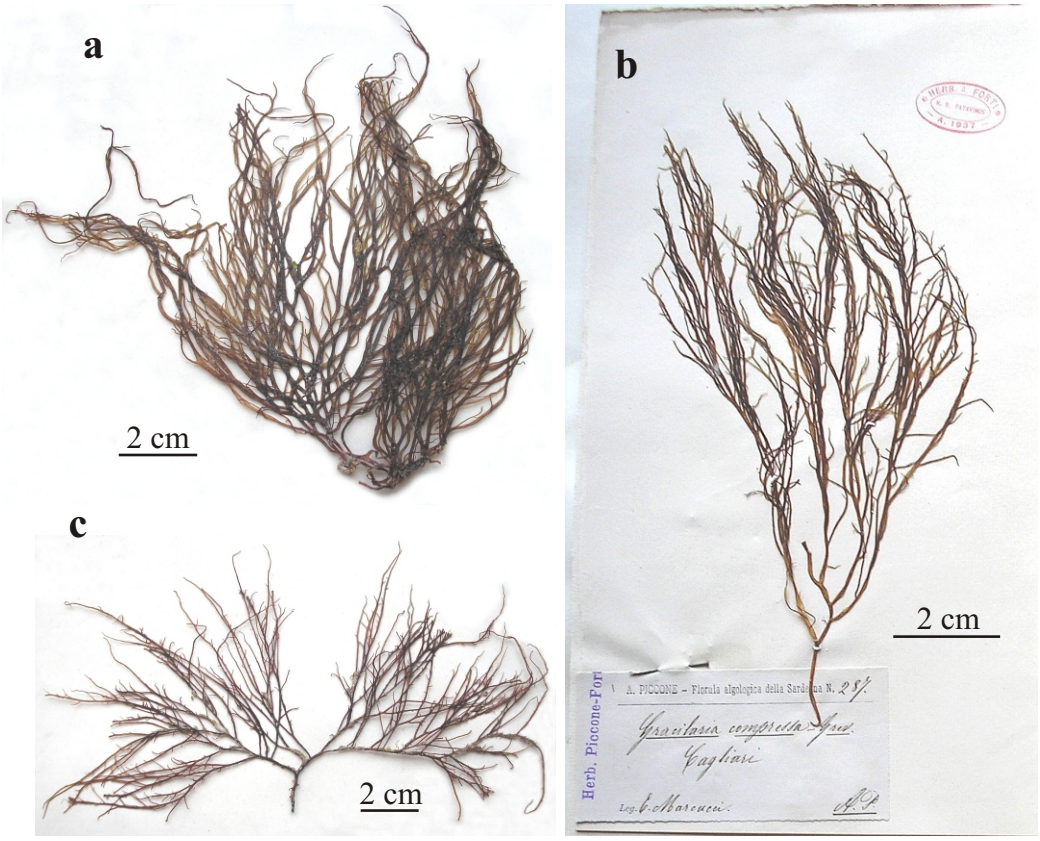


Fig. 2. Specimens of the historical collection A. Forti 1937 preserved in the PAD historical Herbarium of the University of Padova (Italy). **a**) *Gracilaria compressa* #4 Palermo 1903; **b**) *Gracilaria compressa* #5 Cagliari; **c**) *Gracilaria compressa* #6 Savona 1869.

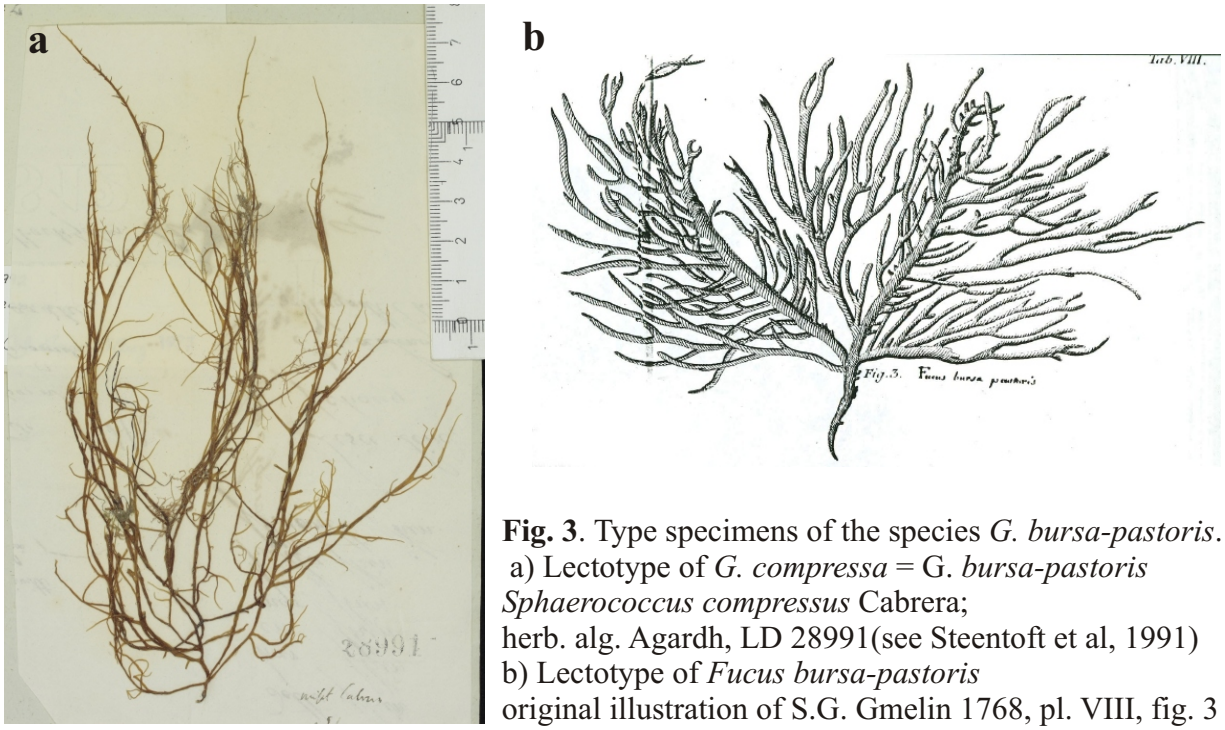


Fig. 3. Type specimens of the species *G. bursa-pastoris*.
a) Lectotype of *G. compressa* = G. *bursa-pastoris* *Sphaerococcus compressus* Cabrera;
herb. alg. Agardh, LD 28991(see Steentoft et al, 1991)
b) Lectotype of *Fucus bursa-pastoris*
original illustration of S.G. Gmelin 1768, pl. VIII, fig. 3

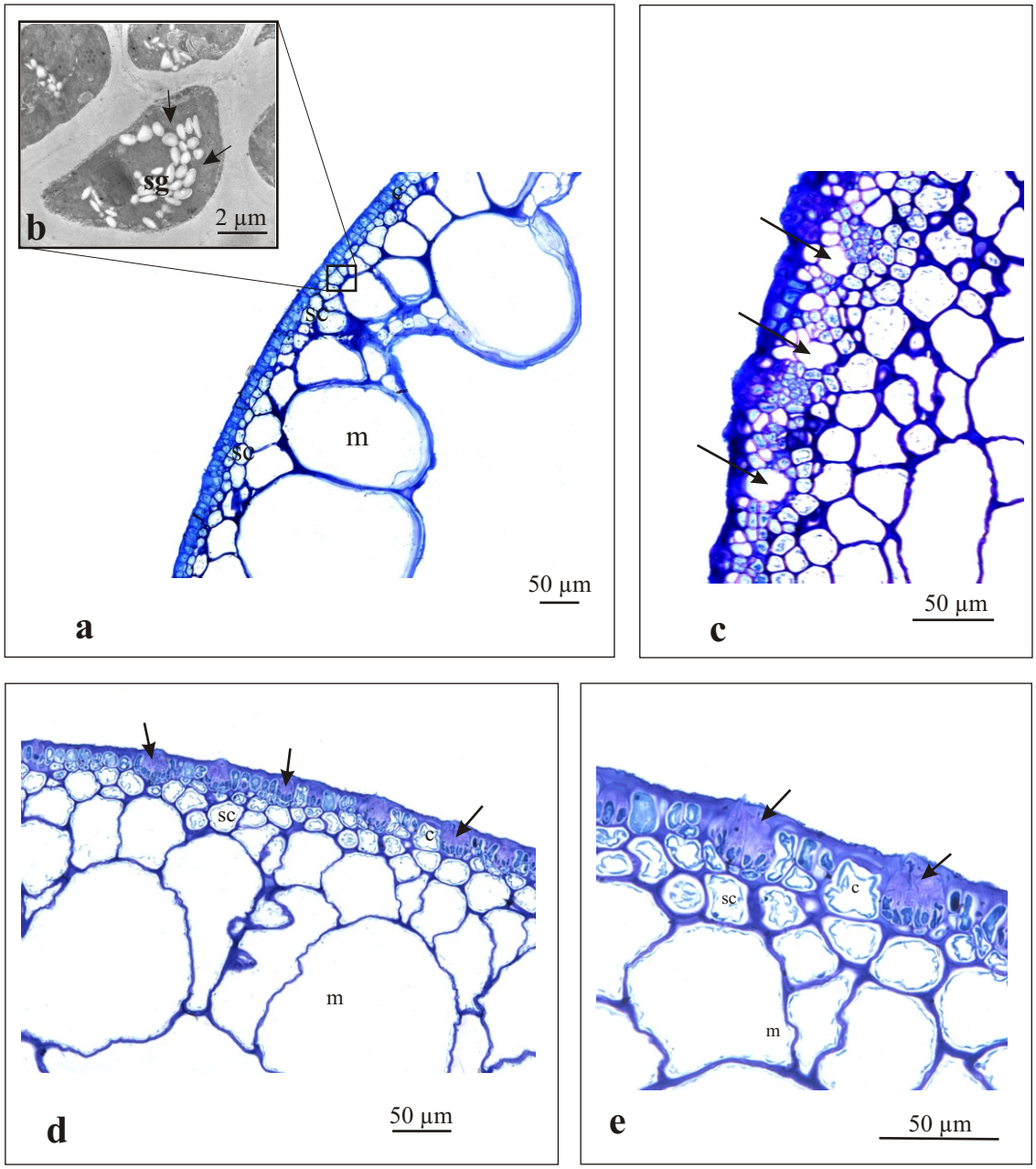


Fig. 4. Semi-thin cross-sections showing the inner structure of the fresh samples.
a) vegetative axis composed of: cortical (**c**), subcortical (**sc**) and medullar (**m**) cells;
b) ultrastructure (TEM) of a subcortical cell containing a large amount of starch grains (**sg**);
c) tetrasporangia (arrows) originate from terminal cortical cells;
d,e) fertile axis showing spermatangial conceptacles of the 'textorii' type (arrows) separated by unmodified cortical cells.

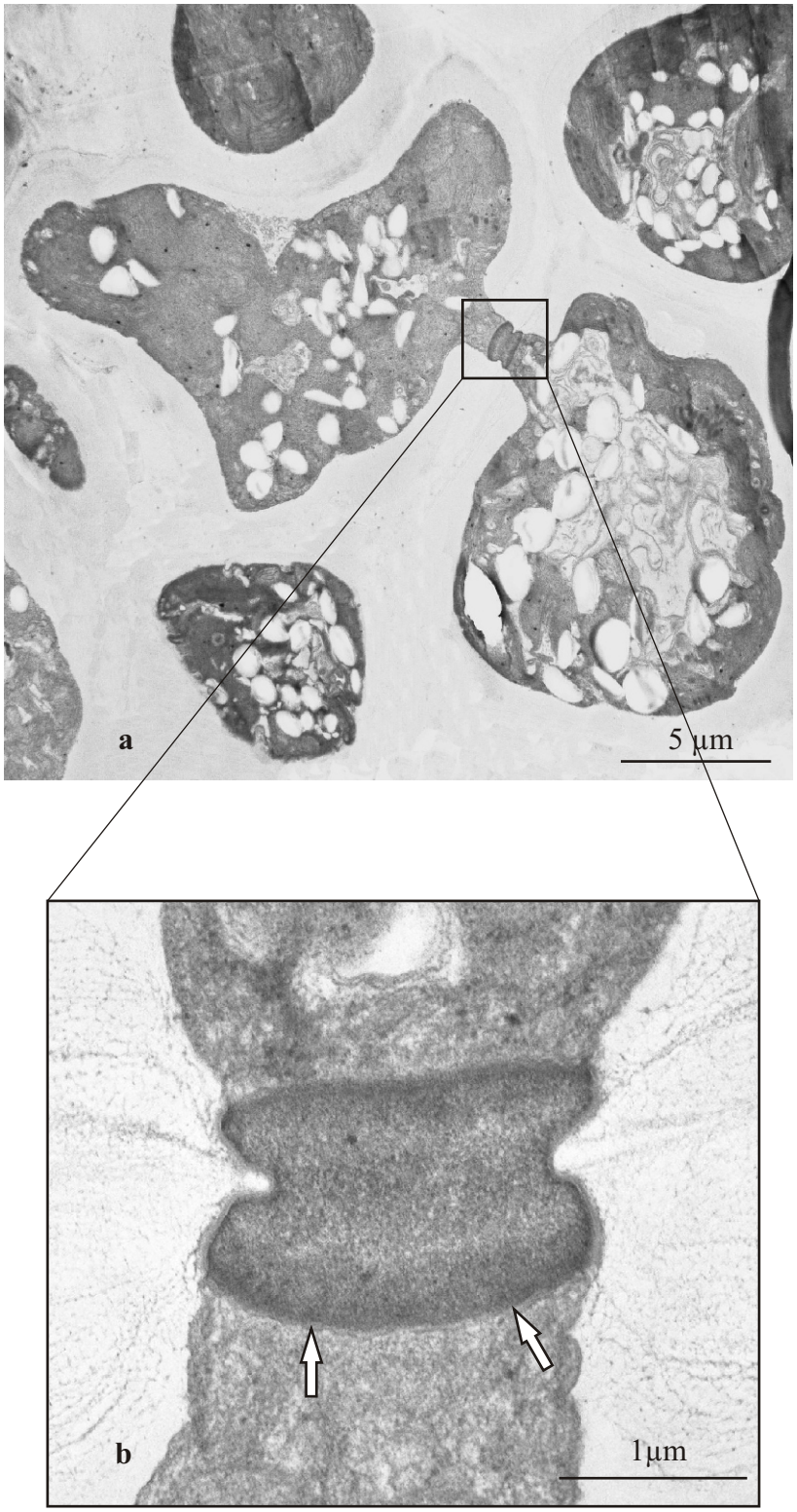


Figura 5 a,b. Ultrastructure (TEM) of a pit connection. b) plug caps absent, naked cap membrane is indicated by arrows.

```
                rbcL gene|
Samples VE, TA     TAGTATATATCTATTCTTTATCTTGTGATCAGATTTATTTTCA
Lectotype 28991    TAGTATATATCTATTCTTTATCTTGTGATCAGATTTATTTTCA
                   ****************************************

                                                  |rbcS gene
Samples VE, TA     TAATATTATTAAGGAGTTTTTAGTAGTGAGACTAACACAAGG
Lectotype 28991    TAATATTATTAAGGAGTTTTTAGTAGTGAGACTAACACAAGG
                   ****************************************
```

Fig 6. ClustalW alignment between the Rubisco spacer sequence of the fresh collected samples and that of the herbarium lectotype of *G. compressa* = *G. bursa-pastoris* (*Sphaerococcus compressus* Cabrera; herb. alg. Agardh, LD 28991).

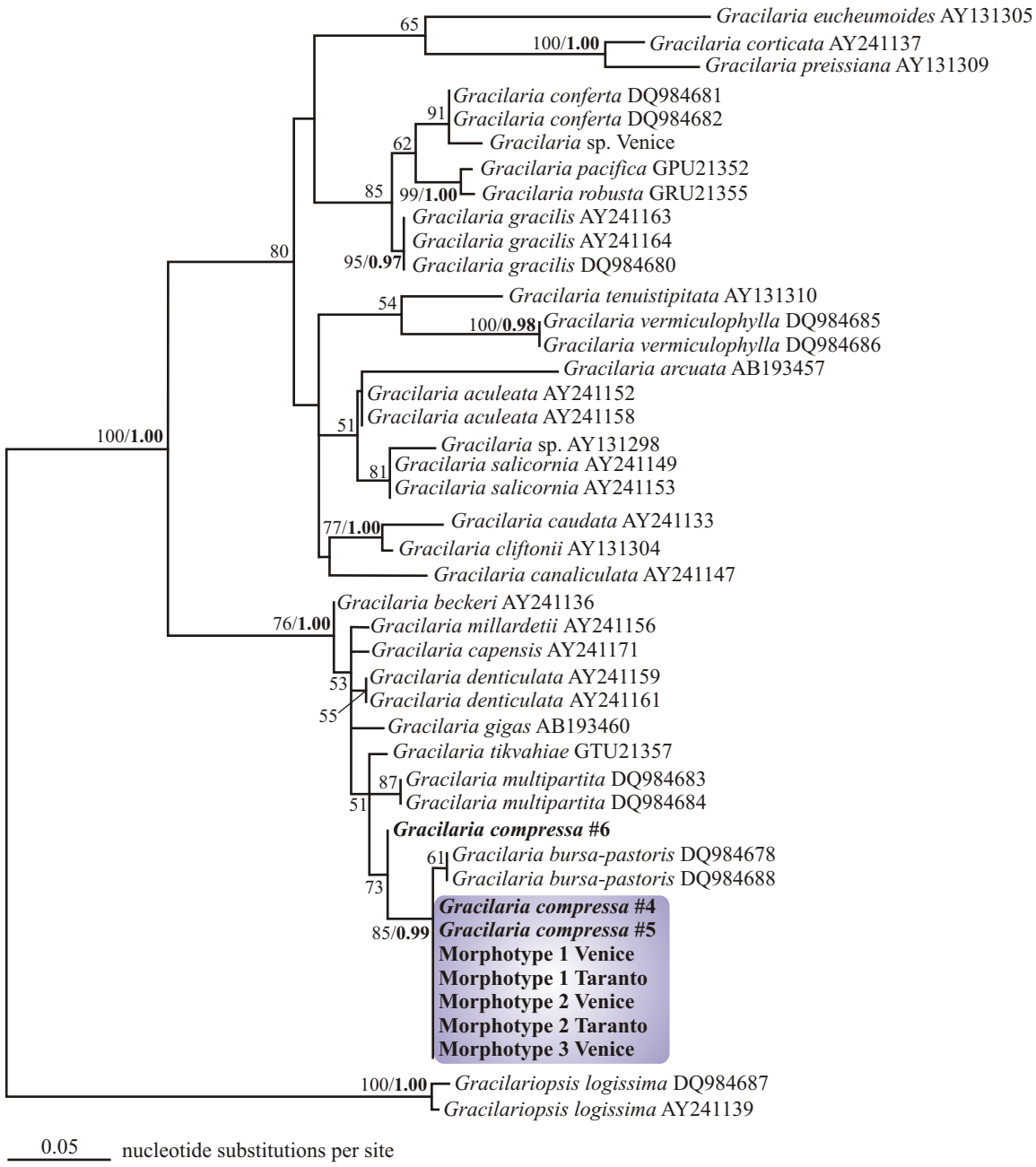


Fig. 7. ML tree inferred from *rbc*L-rbcS spacer region sequences, calculated using the model TPM1uf + G of evolution. Numbers near nodes indicate bootstrap values >50% for ML analysis (in normal font), and >0.95 for posterior probabilities (in bold black font) . The sequences determined in this work are indicated in bold. The specimens identified as morphotypes of *G. bursa-pastoris* are highlighted in violette.