Journal of Biological Research-Thessaloniki **13**: 47 – 57, 2010
J. Biol. Res.-Thessalon. is available online at http://www.jbr.gr
Indexed in: WoS (Web of Science, ISI Thomson), SCOPUS, CAS (Chemical Abstracts Service) and DOAJ (Directory of Open Access Journals)

Identification of cryptic *Ulva* species (Chlorophyta, Ulvales) transported by ballast water

Maria Monia FLAGELLA[1†], Nikos ANDREAKIS[1*], Masanori HIRAOKA[2], Marc VERLAQUE[3] and Maria Cristina BUIA[1]

[1] *Stazione Zoologica "A. Dohrn", 80121 Naples, Italy*
[2] *Usa Marine Biological Institute, Kochi University, Usa, Tosa, Kochi 781-1164, Japan*
[3] *UMR 6540 DIMAR, COM, Université de la Méditerranée, 13288 Marseille cedex 9, France*
[†] *Present address: Centre d'Estudis Avançats de Blanes-CSIC, Acc. Cala St. Francesc 14, 17300 Blanes, Spain*

Received: 25 July 2009 Accepted after revision: 7 December 2009

Ballast waters of ships coming from Egyptian (Port Said) and Lebanese (Beirut) harbours have been collected in the West Mediterranean harbour of Naples (Italy). Samples have been cultured for one month in order to identify seaweed species by means of morphological characters and molecular markers. At the morphological level, two *Ulva* species have been recognized. At the molecular level, the same isolates have been identified as *Ulva ohnoi* Hiraoka et Shimada, a green-tide-forming alga described from Japan, and *Ulva fasciata* Delile, a species described from the eastern Mediterranean (Egypt) and reported from all tropical and warm temperate seas. Since *U. ohnoi* has never been reported from the Mediterranean coasts before, crossing tests were performed between Mediterranean and Japanese isolates corroborating results obtained by molecular markers. In light of these findings, the role of ballast water in the introduction and secondary dispersal of marine macrophytes over long distances is discussed.

Key words: ballast water, cryptic introduction, invasive algae, *Ulva*, Mediterranean.

INTRODUCTION

In the recently updated list of introduced marine organisms in the Mediterranean Sea, annotated through to the end of 2005, Zenetos *et al.* (2005) presented a total of 963 morphologically distinct species, classifying thereafter 745 of them as "valid aliens". Today, it is unquestionably accepted that both the number of introduced species and their effective impact on the native Mediterranean communities have been largely underestimated due to the co-occurrence of genetically distinct but morphologically cryptic species or species complexes (Boudouresque & Verlaque, 2002; Bickford *et al.*, 2007). This hidden marine diversity poses a challenge for taxonomists since the robust delineation of taxa is of primary importance for several reasons. 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 these purposes, the contribution of molecular techniques in marine ecology is significant. In several cases, molecular phylogenies were able to discriminate among morphologically similar species, elucidate cryptic invasion processes, and trace migration and dispersal routes throughout continents and biogeographical provinces (Wattier & Maggs, 2001; Gabrielson *et al.*, 2003; Andreakis *et al.*, 2004, 2007).

Human activities such as aquaculture, aquariology, opening of inter-oceanic canals, and maritime traffic have been widely accepted as causes and vectors of biological introductions (Carlton, 1987; Boudouresque & Verlaque, 2002; Ribera Siguan, 2003). Among them, maritime traffic has been intensively investigated since it is considered one of the major causes of species relocation and introductions. Mar-

* Corresponding author: Present address: Australian Institute of Marine Science, PMB No. 3, QLD, 4810, Australia, tel.: +61-7-47534124, fax: +61-7-47725852, e-mail: n.andreakis@aims.gov.au

itime traffic alone is able to promote a massive transport of organisms in several ways, namely, hull fouling, ballast waters, and sea-chests (Carlton, 1985; Leppäkoski *et al.*, 2002; Davis, 2004). Ship's sea-chests are here referred to as "recesses built into a ship's hull located beneath the waterline on the side and/or on the bottom near the engine room" as in Coutts *et al.* (2003). More than 10,000 different species can travel weekly, transported with the seawater in ballast water tanks worldwide (Streftaris *et al.*, 2005; Flagella *et al.*, 2006).

The Mediterranean Sea is particularly susceptible to biological invasions due to its central position between the Atlantic and the Indo-Pacific seaways (connected *via* the Strait of Gibraltar and the Suez Canal, respectively) and to an increasing amount of maritime traffic. World sea-borne trade increased by 77% from 1985 to 2001 and is expected to continue growing in the next years (Dobler, 2002). Despite its modest size, the Mediterranean Sea hosts nearly 12,000 species; almost 30% of them are reported to be endemic (Bianchi & Morri, 2000; Galil & Zenetos, 2002; Boudouresque, 2003). Yet, it represents a major recipient of exotic species with an estimated average of one introduction event every four weeks over the past five years (Streftaris *et al.*, 2005).

The cities of Naples and Salerno, situated in the Tyrrhenian coastline of Italy, represent two main maritime bays of national and international commercial interchange. The two harbours are characterised by additional intense traffic due to numerous connections with the nearby islands (e.g. Capri, Ischia, and Procida) (Flagella *et al.*, 2006). Two rapidly spreading invasive seaweeds occur along the aforementioned localities: the green alga *Caulerpa racemosa* var. *cylindracea* (Sonder) Verlaque, Huisman & Boudouresque and the invasive lineage 2 of the red seaweed *Asparagopsis taxiformis* (Delile) Trevisan de Saint-Léon, reported since 1996 and 2001, respectively (Gambi & Terlizzi, 1998; Buia *et al.*, 2001; Flagella *et al.*, 2005; Andreakis *et al.*, 2007).

The cosmopolitan genus *Ulva* Linnaeus, commonly known as the "sea lettuce", is represented by species distributed in all oceans and estuaries of the world (Guiry & Guiry, 2008). Seventeen *Ulva* species have been recorded in the Mediterranean Sea: *U. clathrata* (Roth) C. Agardh, *U. compressa* Linnaeus, *U. curvata* (Kütz.) De Toni, *U. fasciata* Delile, *U. flexuosa* Wulfen, *U. intestinalis* Linnaeus, *U. kylinii* (Bliding) Hayden, Blomster, Maggs, P.C. Silva, M.J. Stanhope & J.R. Waaland, *U. laetevirens* J.E. Areschoug, *U. linza* Linnaeus, *U. neapolitana* Bliding, *U. olivascens* P. Dang, *U. pertusa* Kjellman, *U. prolifera* O.F. Müller, *U. pseudolinza* (R.P.T. Koeman & Hoek) Hayden, Blomster, Maggs, P.C. Silva, M.J. Stanhope & J.R. Waaland, *U. rotundata* Bliding, *U. scandinavica* Bliding, and *U. simplex* (K.L. Vinogradova) Hayden, Blomster, Maggs, P.C. Silva, M.J. Stanhope & J.R. Waaland (Gallardo *et al.*, 1993; Furnari *et al.*, 2003). The identification of species within the genus, if based solely on gross morphology, is often incorrect due to the extreme phenotypic plasticity induced by diverse environmental conditions (Tanner, 1986; Malta *et al.*, 1999; Hayden & Waaland, 2004). Furthermore, molecular analyses and culture experiments provided strong evidence that *Ulva* and the previously reported sister genus *Enteromorpha* Link are not reciprocally monophyletic assemblages of species and should not be recognized as separate genera (Tan *et al.*, 1999; Hayden *et al.*, 2003). Since radical morphological differences as well as complete phenotypic similarities may co-occur among closely related *Ulva* species, even within a monophyletic clade (Tan *et al.*, 1999), molecular phylogenetic analysis becomes of primary importance for species discrimination. Several molecular markers have been deployed; the nuclear-encoded ribosomal DNA internal transcribed spacer regions (ITS1, ITS2) and the intervening 5.8S rDNA have been used successfully to identify *Ulva* species responsible for green tide phenomena (Coat *et al.*, 1998; Malta *et al.*, 1999; Tan *et al.*, 1999; Shimada *et al.*, 2003).

In a pioneer study, Flagella *et al.* (2007) tested the hypothesis that ballast water could represent a potential vector for transport of marine macrophytes. The authors confirmed the presence of living macrophytes in the collected water and morphologically identified most of the recovered specimens as species belonging to the stress-tolerant genus *Ulva* (Flagella *et al.*, 2007). In the present study, we combine morphological, reproductive, and molecular evidence in order to discriminate among previously unrecognisable and morphologically similar *Ulva* species found in ballast water tanks. In addition, we re-evaluate the role of the ballast water as a potential carrier for the dissemination of marine macrophytes.

MATERIALS AND METHODS

Sampling procedure and culture conditions

Ballast water samples were collected from a container ship and a roll on/roll off (RORO) carrier in the har-

bour of Naples in March and May 2003, respectively. The last harbours of ballast water loading were Port Said (Egypt: isolate 1) and Beirut (Lebanon: isolate 2) and the time in transit was four and seven days, respectively. Water was extracted from ship ballast pumps, stored in dark plastic bottles and carried to the laboratory for further analyses within six hours from sampling. The collected water (7.5 l per ballast tank) was filtered by a hand vacuum pump through 0.45 μm pore size filters (0.47 mm diameter) and each filter was placed in Petri dishes with 50 ml of autoclaved culture medium (f/2 Guillard's 20 ml l^{-1}) as in Flagella *et al*. (2007). The culture medium was replaced weekly. To test the possibility of laboratory contamination, a clean filter was used as control. Filters were inspected under fluorescent microscopy (blue light) and then kept in culture for a month at conditions matching closely those of the Mediterranean Sea (37‰, 18°C, 12:12 hrs LD and 60 μmol photons $m^{-2} s^{-1}$; Flagella *et al.*, 2007).

DNA extraction and PCR

Approximately 200 mg of fresh unialgal tissue was ground in liquid nitrogen and incubated in 700 μl of DNA extraction buffer containing 2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8, 0.2% w/v PVP, 0.01% w/v SDS and 0.2% ß-mercaptoethanol at 65°C for 45 min. Homogenates were extracted twice with an equal volume of chloroform: isoamyl alcohol (24:1 v/v) and centrifuged in a tabletop Eppendorf microfuge (Eppendorf AG, Hamburg, Germany) at maximum speed (14000 rpm) for 10 min. The aqueous phase was then mixed thoroughly with NaCl to 1.66 M, mixed with an equal volume of ice-cold 100% isopropanol, left on ice for 5 min and centrifuged subsequently in a pre-cooled Eppendorf microfuge under maximum speed for 15 min. DNA pellets were washed in 300 μl 70% v/v ethanol and after decanting the ethanol, allowed to dry in air. Quantity and quality of DNA were examined against known DNA standards by means of 1% agarose TAE buffer gel electrophoresis. The polymerase chain reaction (PCR) was used to amplify the nuclear ribosomal internal transcribed spacer 1 (ITS1, approximately 265 bp) of four specimens grown from ballast water samples. Primers for the PCR reaction were the ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) located in the 18S and the 5.8S rDNA genes, respectively and described in White *et al.* (1990). Amplification reactions were performed as described in Coat *et al*. (1998). The resulting PCR products were purified using the QIAEX II Gel Extraction kit 500 (Qiagen GmbH, Hilden, Germany) following manufacturer's instructions. Purified products were directly sequenced on a Beckman CEQ 2000, using a Dye-terminator cycle sequencing kit (Beckman) according to manufacturer's instructions. Products were analysed on a 3730 DNA Analyzer Sequencer (Applied Biosystems).

Sequence analysis and phylogenetic reconstruction

Sequences were assembled using the DNASTAR computer package (Lasergene) supplied with the Beckman sequencer and aligned using the ClustalW (Thompson *et al.*, 1994) algorithm as implemented in Bioedit v. 4.8.5 (Hall, 1999) with an additional 19 ITS1 sequences downloaded from GenBank (Table 1). The only sequence of presumably *U. rotundata* Bliding available in the literature (Brittany, France; Coat *et al.*, 1998) has been produced from a misidentified specimen of *U. pertusa* (Baamonde López *et al.*, 2007) and has been included in this study. This is to point out the strong incongruence between molecular and morphological identification of *Ulva* species. The alignment was refined by eye. Maximum likelihood (ML) and maximum parsimony (MP) phylogenies were inferred using PAUP* 4.0b10 version for Windows (Swofford, 2002). Prior to ML reconstruction, hierarchical likelihood ratio tests (hLRTs) were performed using Modeltest version 3.06 (Posada & Crandall, 1998) to find the best-fitting parameters (substitution model, gamma distribution, proportion of invariable sites, transition-transversion ratio) for the computation given the alignment. Then, ML analyses were performed using heuristic searches and 10 random sequence additions. For MP, trees were inferred using the heuristic search option, tree bisection-reconnection (TBR) branch swapping and 1000 sequence addition replicates. All sites were equally weighted and gaps were treated as missing data. Bootstrap support for individual clades (Felsenstein, 1985) was calculated on 1000 replicates using the same methods, options and constraints as used in the tree inferences but with identical sequences represented only once.

Morphological identification

Specimens of *Ulva* grown in Petri dishes and F1 progeny from crossing experiments were morphologically identified by taking into account taxonomical characters such as the shape of the thallus, the number of

TABLE 1. List of *Ulva* species, GenBank accession numbers and collection localities of the sequences involved in the analysis

Species	Accession	Locality	Reference
Isolate 1	GU121401	Naples, Italy	This study
Isolate 2a	GU121402	Naples, Italy	This study
Isolate 2b	GU121403	Naples, Italy	This study
Isolate 2c	GU121404	Naples, Italy	This study
U. armoricana, Dion, de Reviers & Coat	AB097662	Yokohama, Kanagawa Prefecture, Japan	Shimada *et al.* (2003)
U. californica Wille	AY422514	Monterey County, California, USA	Hayden & Waaland (2004)
U. fasciata Delile	AY422523	Nueces County, Texas, USA	Hayden & Waaland (2004)
U. fasciata Delile	AB097664	Miyajima, Hiroshima Prefecture, Japan	Shimada *et al.* (2003)
U. fenestrata Postels & Ruprecht	AJ234316	North Boardman St. Park, Oregon, USA	Tan *et al.* (1999)
U. flexuosa Wulfen	AB097647	Thessaloniki, Greece	Shimada *et al.* (2003)
U. intestinalis Linnaeus	AY422508	Valdez-Cordova County, Alaska, USA	Hayden & Waaland (2004)
U. linza Linnaeus	AB097649	Yoshino River, Tokushima Prefecture, Japan	Shimada *et al.* (2003)
U. ohnoi Hiraoka & Shimada	AB116034	Tosa, Kochi Prefecture, Japan	Hiraoka *et al.* (2004)
U. ohnoi Hiraoka & Shimada	AB116031	Tosa, Kochi Prefecture, Japan	Hiraoka *et al.* (2004)
Ulvaria obscura var. *blyttii* (JE. Areschoug) Bliding	AY260571	Skagit County, Washington, USA	Hayden & Waaland (2004)
U. pertusa Kjellman	AY422504	Hyogo Prefecture, Japan	Hayden & Waaland (2004)
*U. rotundata**	–	Plestin-les-Greves, France	Coat *et al.* (1998)
U. reticulata Forsskål	AB097665	Cebu Island, The Philippines	Shimada *et al.* (2003)
U. rigida C. Agardh	AY422522	SE of Puerto Montt, Chile	Hayden & Waaland (2004)
U. rigida C. Agardh	AJ000208	Redpoint, Wester Ross, Scotland	Tan *et al.* (1999)
U. scandinavica Bliding	AB097659	Wadden Sea, The Netherlands	Shimada *et al.* (2003)
U. spinulosa Okamura & Segawa	AB097666	Fubenhama, Kochi, Japan	Shimada *et al.* (2003)
U. stenophylla Setchell & Gardner	AY260569	King County, Washington, USA	Hayden & Waaland (2004)
U. taeniata (Setchell) Setchell & Gardner	AJ234320	Seal Rock, Oregon, USA	Tan *et al.* (1999)

Ulva spp. 1 to 2c: specimens analyzed in this study
* = misidentification of *Ulva pertusa*; the sequence is found in Coat *et al.* (1998)

pyrenoids, the presence/absence of marginal teeth, and only for the "*U. rotundata*" phenotype, the thickness of the cross section.

Crossing experiments

Crossing experiments were performed between gametes of isolate 1 (see Results section) and the KF22 strain of *U. ohnoi*, kept in Usa Marine Biological Institute, Kochi University, Japan (Hiraoka *et al.*, 2004). Release of zoids in isolate 1 was induced using the punching method described in Hiraoka & Enomoto (1998). Released biflagellate zoids were mixed with KF22 male or female gametes on a glass slide and the successful mating of the gametes was determined under a photomicroscope. Control crossing tests were performed only between known (KM22 × KM22) *U. ohnoi* isolates and results were compared against crosses from isolate KM22 and putative *U. ohnoi* isolate 1 to evaluate differences in reproductive success. Zygotes were cultured in PES medium (Provasoli, 1968) at 20 °C, with a 12:12 hrs LD cycle under fluorescent light at 100 µmol photons $m^{-2} s^{-1}$.

RESULTS

After one month under culture, only one and three specimens of *Ulva*-shape morphology were found on the filters from the ship ballast water originating from Egyptian (Port Said) and Lebanese (Beirut) harbours, respectively while no growth occurred on the control filters. Thalli were collected and named as isolate 1 and isolates 2a, b and c for further experiments.

Sequence analysis

Although of restricted length, the ITS1 region was able to resolve adequately the species engaged in the phylogenetic reconstruction shown in Figure 1. This marker was therefore appropriate in identifying the *Ulva* specimens reported in this study. Isolate 1, morphologically identified as *U. rotundata*, possessed an ITS1 sequence distinct from isolates 2a, b and c and identical to sequences belonging to *U. ohnoi* collected in Japan (AB116031, AB116034; Tosa, Kochi Prefecture, Japan). Isolates 2a, b and c, morphologically identified as *U. bifrons*, were found to share the same ITS1 sequence and clustered together with the two *U. fasciata* Delile GenBank sequences collected from the western Atlantic and the northern Pacific oceans (AY422523, Port Aransas, Nueces County, Texas; AB097664, Miyajima, Hiroshima Prefecture, Japan).

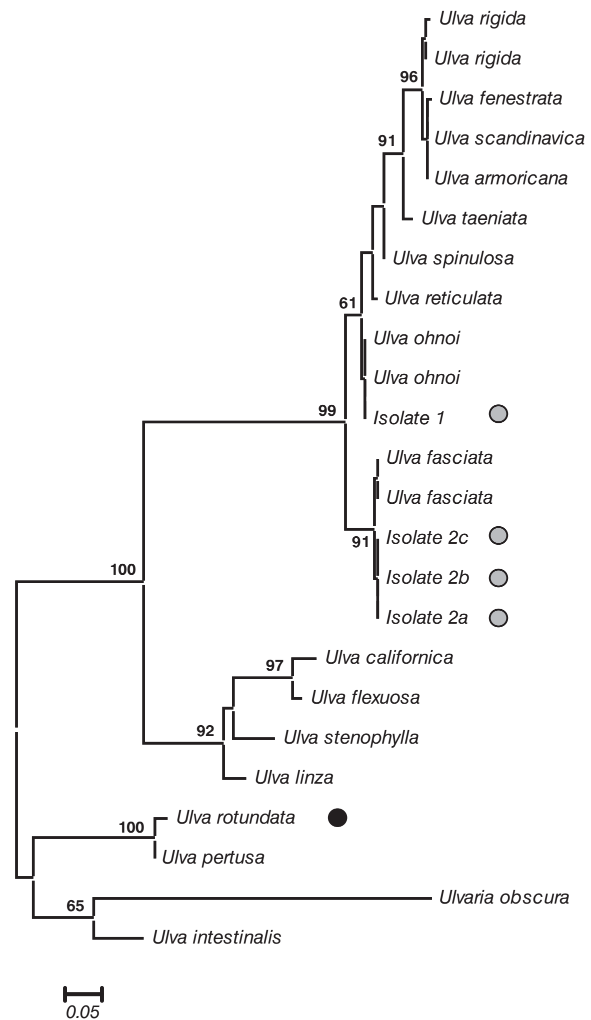

FIG. 1. Maximum likelihood phylogram of *Ulva* spp. ITS1 sequences. Numbers on nodes indicate bootstrap support. Grey circles indicate the four *Ulva* isolates collected from ballast water in this study. Black circle denotes misidentified specimen of *U. pertusa* previously identified as *U. rotundata*.

The latter sequences were identical but differed from our *U. fasciata* isolates 2a, 2b and 2c by two bp positions. Maximum likelihood phylogenies, constrained with the model that fits the data best (F81 + Γ) and optimal parameters obtained from Modeltest analysis (nucleotide frequencies: A = 0.1418, C = 0.3773, G = 0.2782, and T = 0.2026; equal substitution rate; proportion of variable sites assumed to follow a gamma distribution with shape parameter $\alpha = 0.2327$) resulted in a single ML tree (-lnL = 1313.8666; Fig. 1). Maximum parsimony recovered an identical tree topology with similar nodal support (data not shown).

Morphological observations

On the basis of preliminary analysis of morphological characters compared with Mediterranean *Ulva* spe-

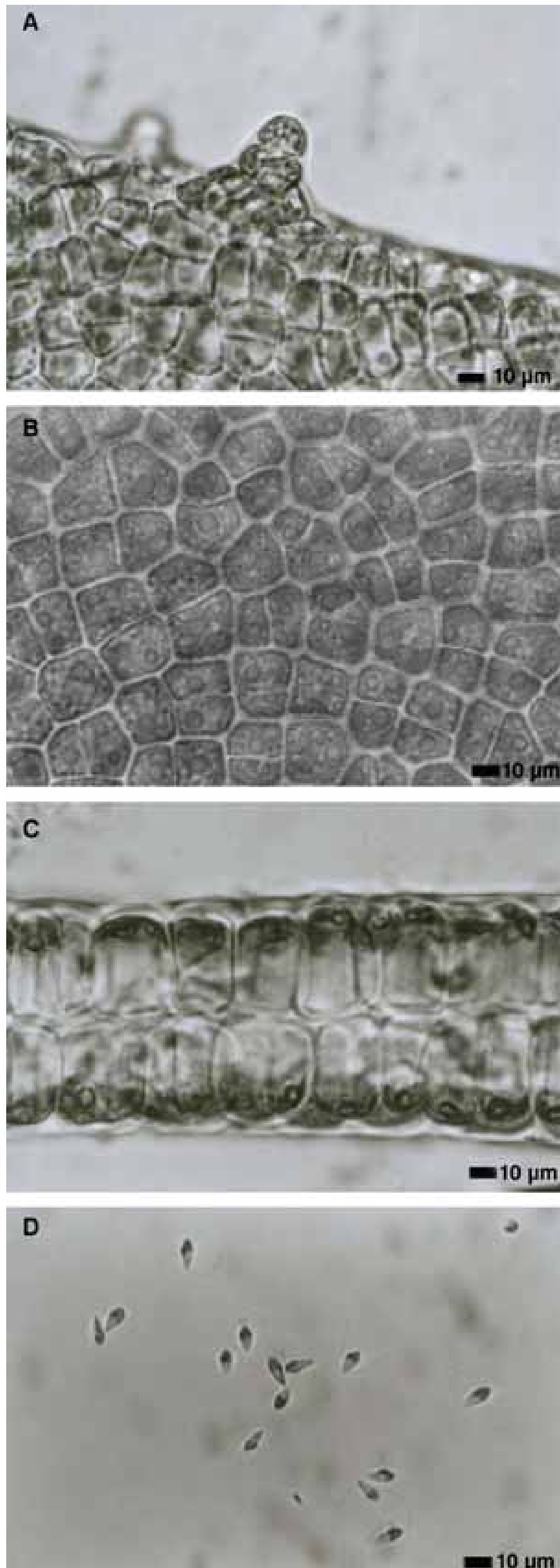

FIG. 2. Isolate 1 from ballast water of a container ship from Port Said: A) margin of blade with microscopic teeth, B) surface view of the vegetative thallus, C) transverse section of the vegetative thallus, D) male gametes.

cies, isolate 1 (Fig. 2) and isolates 2a, b, c, were provisionally identified as *U. rotundata* (due to the cell size, the presence of marginal teeth, and the number of pyrenoids) and *U. bifrons* Ardré (due to the asymmetrical cell layers, the cell size, the presence of marginal teeth, and the number of pyrenoids) (Table 2). Thallus cells from a surface view were polygonal for all the specimens and of variable size (Table 2).

Crossing experiments

Crossing experiments were performed only between isolate 1 and *U. ohnoi* isolate KM22 since a) molecular analysis indicated isolate 1 to be *U. ohnoi* and b) recent investigations on the genus *Ulva* showed that *U. rotundata* cannot be considered as a valid species (Frédéric Mineur, personal communication). Isolate 1, morphologically identified as *U. rotundata*, released biflagellate zooids (Fig. 2D) following induced zoid formation. These gametes demonstrated sexual compatibility with female gametes of the Japanese *U. ohnoi* (isolate KM22) and viable zygotes were successfully formed. From the zygotes, sporophytes developed and from these, quadriflagellate zoospores were released and developed into gametophytes (Fig. 3). From the aforementioned observations, isolate 1 was identified to be a male gametophyte of *U. ohnoi*. Many clusters of male and female gametes were aggregated into clumps and numerous zygotes were produced also in test crosses. Aggregation and copulation were thus similar during both hybrid (isolate1/KM22) and non-hybrid (KM22/KM22) zygote formation as observed under a photomicroscope. Zygotes developed into sporophytes, they released

FIG. 3. Young gametophytes grown from quadriflagellate zooids that were produced by sporophytes after a cross between female gametes of the Japanese KM22 *Ulva ohnoi* isolate and male gametes of isolate 1.

TABLE 2. Morphological comparisons between isolate 1, isolates 2a, b, c and the closest species of *Ulva*

	Blade	**Thickness (µm; A, M regions)**	**Cell layers**	**Cell size (µm; surface view)**	**MT**	**P**	**Origin**	**Source**
Isolate 1	lobate	60	symmetrical	A (11-19×12-17) M (11-20×16-22) B (22-33×22-32)	yes	1-3	Port Said (Egypt)	this study
U. rotundata	lobate	56-95	symmetrical	young plants, longest dimensions: 30-38 (45) adult plants: *ca* 20×26	yes	1-3	*Gulf of Naples (Italy)	Bliding (1968)
U. ohnoi	orbicular to irregularly expanded	30-55	symmetrical	A (14-20×7-15)° M (14-20×7-15)° B (14-30×12-20)°	yes	1-3	Tosa Bay (Japan)	Hiraoka *et al.* (2004)
Isolates 2	lanceolate	na	slightly asymmetrical	A (13-20×9-13) M (19-23×13-18) B (15-30×16-30)	yes	1-3	Beirut (Lebanon)	this study
U. bifrons	asymmetrical to lobate	65-90	clearly asymmetrical	A_1 (9-17×14-22)° A_2 (7-13×7-18)° M_1 (10-23×10-35)° M_2 (8-15×10-20)° B_1 (9-30×15-40)° B_2 (9-23×10-32)°	yes	1-3	*Sesimbra (Portugal)	Ardré (1967)
U. fasciata	divided from near the base	88-100	symmetrical	A (10-14×8-22) M (7-12×10-20) B (12-18×10-26)	yes	1-3	Thau Lagoon (France)	M. Verlaque (unpublished data)

na: not available
A = apical, M = middle, B = basal regions; MT = marginal teeth, P = pyrenoids
° = deduced from illustrations; * = type locality

zoospores which developed into mature gametophytes and, in their turn, they released gametes. Reproduction success rates were not calculated between the two crosses. Yet, we have not observed differences in aggregation activity of gametes and copulation process indicating that all of the isolates belong to the same *Ulva* species. Indeed, in *Ulva* crosses, when the engaged isolates represent two biologically distinct *Ulva* species, dissimilarities during aggregation and copulation processes occur regularly (Bliding, 1963; Hiraoka *et al.*, 2004).

DISCUSSION

Ballast-water-mediated transport of organisms increases the possibility of biological introductions. Nevertheless, it is generally accepted that, in the case of macroalgal species, ballast water cannot be considered as an efficient carrier since algal propagules have been reported only rarely from ballast water samples (Carlton & Geller, 1993; Gollasch *et al.*, 2002; Lewis *et al.*, 2003). Contrary to this opinion, our results provide evidence that *U. ohnoi*, described from Japan (Hiraoka *et al.*, 2004) and *U. fasciata*, a cosmopolitan species described from the Mediterranean Sea (Gallardo *et al.*, 1993; Furnari *et al.*, 2003), can both survive in ballast tanks and can be transported via ballast water. In the present study, both species recovered promptly from filtered ballast water and developed into adult gametophytes after one month in the culture medium. These results indicate that microscopic forms of stress-tolerant marine macrophytes can be efficiently transported in ballast tanks, and propagules are able to recover immediately in the laboratory or regularly in the wild under favourable conditions for survival and growth (Flagella *et al.*, 2007).

Ulva ohnoi

Ulva spp. can survive more than 10 months in the darkness (Worm *et al.*, 2001; Santelices *et al.*, 2002). Accordingly, *U. ohnoi* propagules could have been loaded from other harbours of tropical and subtropical Indo-Pacific waters and not necessarily from Port Said, since the ship followed a Singapore-Malaysia-India-Egypt course that finally ended in Italy. The high-temperature tolerance of the species in Japan (*ca* 30°C during the summer for the formation of the green tide phenomenon; Ohno, 1988; Hiraoka *et al.*, 2004), the sexual compatibility, and the sequence similarity with the Japanese isolate KM22 favour this hypothesis. In any case, the Port Said harbour, due to its pivotal position on the entrance of the Suez Canal, represents an important receiving area of alien species in which dispersal is ensured by the intense maritime traffic throughout the Mediterranean (Dobler, 2002; Flagella *et al.*, 2006). For instance, 14,379 ships sailed through the canal in 2001 alone (Informare, http://www.informare.it/news/gennews/2001/20011609.asp). Our results indicate that *U. ohnoi*, a species responsible for the formation of green tides in warm temperate regions of southern and western Japan (Hiraoka *et al.*, 2004), is a potentially new, cryptic macroalga in the Mediterranean Sea introduced *via* ballast water. Natural populations of *U. ohnoi* within the basin have not been reported before and therefore a further survey is needed to assess whether populations of the species have already been established. For this reason, a periodical screening for potential invaders in ballast water collected from key Mediterranean harbours is essential for timely alien species recognition, prevention, and anticipated management.

Ulva fasciata

Ulva fasciata has been morphologically identified and reported in Italy from Naples, Sicily, and the Venetian Lagoon (Funk, 1955; Furnari *et al.*, 1999) and within the Mediterranean basin from Algeria, Egypt, France (introduced), Greece, Libya, Spain, and Turkey (Gallardo *et al.*, 1993; Verlaque, 2001). The species is considered as introduced by shipping (fouling and/or ballast water) in Hawaii and Australia, and it has been recently reported from the west coast of Baja California (Aguilar-Rosas *et al.*, 2005). Without doubt, ballast water can act as a valid dispersal mechanism for the circulation and dispersal of *U. fasciata* over long distances and even across biogeographical barriers worldwide. Yet, the finding of this species in the harbour of Naples is particularly significant given the strategic position of the latter within the Mediterranean Sea, the high commercial exchanges, and the frequent maritime connections with the islands (Flagella *et al.*, 2006). This finding suggests a dual role with the Neapolitan harbour acting both as a recipient for the arrival of introduced biota *via* the Strait of Gibraltar and the Suez Canal and as a source for the local dissemination of species within the basin.

Introduced species are able to interfere with the local ecological regime in several undesirable ways, e.g. by space and resource competition (niche displacement, exclusion, predation) and by genetic competition (hybridization, introgression), as reviewed in Mooney & Cleland (2001). Molecular analyses have demonstrated that the effective number of introductions and their impact is still underestimated due to the existence of cryptic (morphologically indistinguishable) or cryptogenic (of not demonstrable native or introduced origin) species since widely dispersed taxa are often simply stated as being cosmopolitan (Carlton, 1996).

Species delineation within Ulva

Despite the numerous bibliographic records, species delineation within the genus *Ulva* is complicated by the characteristically high phenotypic plasticity in response to ecological factors (usually observed in specimens grown under laboratory conditions; see Results section), and the lack of valid taxonomical characters (Hayden & Waaland, 2004). For example, Mineur *et al.* (2007), by analyzing the macroalgal hull fouling composition from ships entering the Mediterranean harbour of Sète (France), uncovered an unrecognised *Ulva* sp. morphotype. The specimen was thereafter genetically identified as a mix of *U. ohnoi* and *U. fasciata* but morphologically corresponded to *U. rotundata* (Mineur *et al.*, 2007). In the present study, preliminary morphological analyses identified isolate 1 and isolates 2a, b and c as *U. rotundata* and *U. bifrons*, respectively, yet isolate 1 shared identical ITS1 sequences with the Japanese *U. ohnoi* and isolates 2a, b, c exhibited ITS1 sequence similarity with *U. fasciata* collected from the western Atlantic and the northern Pacific oceans. As a matter of fact, *U. fasciata, U. rotundata*, and *U. ohnoi* share very similar anatomical features (Table 2) and the identification of small specimens on the basis of only these characters appears to be unreliable. Seemingly, other records of *Ulva* spp. may have been misidentified in the past (Fig.

1, *U. pertusa* as *U. rotundata*). These observations emphasise the incongruence between morphological and molecular characters in *Ulva* species delineation and suggest that several among them cannot be considered as morphologically coherent taxa.

CONCLUSIONS

A combination of morphological analyses, molecular tools, and breeding experiments is necessary for a broad reinvestigation of the genus *Ulva* in the Mediterranean Sea in order to uncover cryptic and/or cryptogenic species. The integration in these studies of genetically characterized herbarium specimens will further contribute to this effort. The analysis of herbarium specimens compared with recently collected material is needed to locate in time an introduction event and to robustly delineate taxa on the basis of the morphological descriptions of the type specimens.

ACKNOWLEDGEMENTS

This research was supported by the EU programme "Algal Introduction to European Shores" (ALIENS) (EVK3-CT2001-00062). The authors are grateful to the Port authorities and Maritime Agencies of Naples and Salerno, to Dr Beth Ballment for revision of the English text, and to the three anonymous reviewers for their useful comments on the manuscript.

REFERENCES

Aguilar-Rosas R, Aguilar-Rosas E, Pedroche FF, 2005. *Ulva fasciata* Delile (Ulvaceae, Chlorophycota): a species newly introduced into Pacific Mexico. *Botanica marina*, 48: 46-51.

Andreakis N, Procaccini G, Kooistra WHCF, 2004. *Asparagopsis taxiformis* and *Asparagopsis armata* (Bonnemaisoniales, Rhodophyta): genetic and morphological identification of Mediterranean populations. *European journal of phycology*, 39: 273-283.

Andreakis N, Procaccini G, Maggs CA, Kooistra WHCF, 2007. Phylogeography of the invasive seaweed *Asparagopsis* (Bonnemaisoniales, Rhodophyta) reveals cryptic diversity. *Molecular ecology*, 16: 2285-2289.

Ardré F, 1967. Une Ulve à thalle remarquable des côtes du Portugal. *Revue algologique nouvelle serie*, 8: 292-297.

Baamonde López S, Fernández IB, Lozano RB, Ugarte JC, 2007. Is the cryptic alien seaweed *Ulva pertusa* (Ulvales, Chlorophyta) widely distributed along European Atlantic coasts? *Botanica marina*, 50: 267-274.

Bianchi CN, Morri C, 2000. Marine biodiversity of the Mediterranean Sea: situation, problems and prospects for future research. *Marine pollution bulletin*, 5: 367-376.

Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, Winker K, Ingram KK, Das I, 2007. Cryptic species as a window on diversity and conservation. *Trends in ecology & evolution*, 22: 148-155.

Bliding C, 1963. A critical survey of European taxa in Ulvales. Part I. *Capsosiphon, Percursaria, Blidingia, Enteromorpha*. *Opera botanica*, 8: 1-160.

Bliding C, 1968. A critical survey of European taxa in Ulvales. II. *Ulva, Ulvaria, Monostroma, Kornmannia*. *Botaniska notiser*, 121: 535-629.

Boudouresque CF, 2003. The erosion of Mediterranean biodiversity. In: Rodríguez-Prieto C, Pardini G, eds. *The Mediterranean Sea: an overview of its present state and plans for future protection*. Servei de Publicacions de la Universitat de Girona: 53-112.

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

Buia MC, Gambi MC, Terlizzi A, Mazzella L, 2001. Colonization of *Caulerpa racemosa* along the southern Italian coasts: I. Distribution, phenological variability and ecological role. In: Gravez V, Ruitton S, Boudouresque CF, Le Direach L, Meinesz A, Scabbia G, Verlaque M, eds. *Fourth international workshop on Caulerpa taxifolia*. GIS Posidonie Publisher, Marseille: 352-360.

Carlton JT, 1985. Transoceanic and interoceanic dispersal of coastal marine organisms: the biology of ballast water. *Oceanography and marine biology: an annual review*, 23: 313-371.

Carlton JT, 1987. Patterns of transoceanic marine biological invasions in the Pacific Ocean. *Bulletin of marine science*, 41: 452-465.

Carlton JT, 1996. Biological invasions and cryptogenic species. *Ecology*, 77: 1653-1655.

Carlton JT, Geller R, 1993. Ecological roulette: the global transport of non-indigenous marine organisms. *Science*, 261: 78-82.

Coat G, Dion P, Noailles MC, De Reviers B, Fontaine JM, Berger-Perrot Y, Loiseaux-de Goer S, 1998. *Ulva armoricana* (Ulvales, Chlorophyta) from the coasts of Brittany (France). II. Nuclear rDNA ITS sequence analysis. *European journal of phycology*, 33: 81-86.

Coutts ADM, Moore KM, Hewitt CL, 2003. Ship's sea-chests: an overlooked transfer mechanism for non-indigenous marine species? *Marine pollution bulletin*, 46: 1504-1515.

Davis MH, 2004. The role of man-aided dispersal in the spread of the immigrant *Styela clava* Herdman, 1882. *Journal of marine science and environment*, 1: 18-24.

Dobler JP, 2002. Analysis of shipping patterns in the Mediterranean and Black Seas. In: Briand F, ed. *CIESM*

2002. Alien marine organisms introduced by ships in the Mediterranean and Black seas. Workshop Monographs 20, Monaco: 19-28.

Felsenstein J, 1985. Confidence intervals on phylogenies: an approach using the bootstrap. *Evolution*, 39: 783-791.

Flagella MM, Lorenti M, Buia MC, 2005. Assessment of the potential success of *Asparagopsis taxiformis*. In: Özhan E, ed. *Proceeding of the seventh international conference on the Mediterranean Coastal Environment MEDCOAST*. Middle East Technical University, Ankara: 611-620.

Flagella MM, Soria A, Buia MC, 2006. Shipping traffic and introduction of non-indigenous species: Study case in two Italian harbours. *Ocean & coastal management*, 49: 947-960.

Flagella MM, Verlaque M, Soria A, Buia MC, 2007. Macroalgal survival in ballast water tanks. *Marine pollution bulletin*, 54: 1395-1401.

Funk G, 1955. Meeresalgen von Neapel. *Pubblicazioni stazione zoologica Napoli*, 25: 1-178.

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.

Furnari G, Giaccone G, Cormaci M, Alongi G, Serio D, 2003. Biodiversità marina delle coste italiane: catalogo del macrofitobenthos. *Biologia marina mediterranea*, 10: 350-352.

Gabrielson TM, Brochmann C, Rueness J, 2003. Phylogeny and interfertility of North Atlantic populations of "*Ceramium strictum*" (Ceramiales, Rhodophyta): how many species? *European journal of phycology*, 38: 1-13.

Galil B, Zenetos A, 2002. A sea change – exotics in the eastern Mediterranean. In: Leppäkoski E, Gollasch S, Olenin S, eds. *Invasive aquatic species of Europe. Distribution, impacts and management*. Kluwer Academic Publishers, Dordrecht, The Netherlands: 325-336.

Gallardo T, Gómez Garreta A, Ribera MA, Cormaci M, Furnari G, Giaccone G, Boudouresque CF, 1993. Check-list of Mediterranean seaweeds. II. Chlorophyceae Wille s.l. *Botanica marina*, 36: 399-421.

Gambi MC, Terlizzi A, 1998. Record of a large population of *Caulerpa racemosa* (Forsskål) J. Agardh (Chlorophyceae) in the Gulf of Salerno (Southern Tyrrhenian Sea, Italy). *Biologia marina mediterranea*, 5: 553-556.

Gollasch S, Macdonald E, Belson S, Botnen H, Christensen JT, Halmer JP, Houvenaghel G, Jelmer A, Lucas I, Masson D, Mccollin T, Olenin S, Persson A, Wallentinus I, Wetsteyn LPM, Wittling T, 2002. Life in ballast tanks. In: Leppäkoski E, Gollasch S, Olenin S, eds. *Invasive aquatic species of Europe. Distribution, impacts and management*. Kluwer Academic Publishers, Dordrecht, The Netherlands: 217-231.

Guiry MD, Guiry GM, 2008. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. (http://www.algaebase.org).

Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic acids symposium series*, 41: 95-98.

Hayden HS, Waaland JR, 2004. A molecular systematic study of *Ulva* (Ulvaceae, Ulvales) from the northern Pacific. *Phycologia*, 43: 364-382.

Hayden SH, Blomster J, Maggs CA, Silva PC, Stanhope MJ, Waaland JR, 2003. Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. *European journal of phycology*, 38: 277-294.

Hiraoka M, Enomoto S, 1998. The induction of reproductive cell formation of *Ulva pertusa* Kjellman (Ulvales, Ulvophyceae). *Phycological research*, 46: 199-203.

Hiraoka M, Shimada S, Uenosono M, Masuda M, 2004. A new green-tide forming alga, *Ulva ohnoi* Hiraoka et Shimada sp. nov. (Ulvales, Ulvophyceae) from Japan. *Phycological research*, 52: 17-29.

Leppäkoski E, Gollasch S, Olenin S, 2002. Alien species in European waters. In: Leppäkoski E, Gollasch S, Olenin S, eds. *Invasive aquatic species of Europe. Distribution, impacts and management*. Kluwer Academic Publishers, Dordrecht, The Netherlands: 1-6.

Lewis P, Hewitt CL, Riddle M, McMinn A, 2003. Marine introduction in the Southern Ocean: an unrecognised hazard to biodiversity. *Marine pollution bulletin*, 46: 213-223.

Malta EJ, Draisma SGA, Kamermans P, 1999. Free-floating *Ulva* in the southwest Netherlands: species or morphotypes? A morphological, molecular and ecological comparison. *European journal of phycology*, 34: 443-454.

Mineur F, Johnson MP, Maggs CA, Stegenga H, 2007. Hull fouling on commercial ships as a vector of macroalgal introduction. *Marine biology*, 151: 1299-1307.

Mooney HA, Cleland EE, 2001. The evolutionary impact of invasive species. *Proceedings of the national academy of sciences USA*, 98: 5446-5451.

Ohno M, 1988. Seasonal changes of the growth of green algae, *Ulva* sp. in Tosa Bay, southern Japan. *Marine fouling*, 7: 13-17.

Posada D, Crandall KA, 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics*, 14: 817-818.

Provasoli L, 1968. Media and prospects for the cultivation of marine algae. In: Watanabe A, Hattori A, eds. *Cultures and collections of algae*. Proceedings of US-Japan conference, the Japanese society of plant physiologists, Hakone, Japan: 63-75.

Ribera Siguan MA, 2003. Pathways of biological invasions of marine plants. In: Ruiz G, Carlton JT, eds. *Invasive species: vectors and management strategies*. Island Press, London: 183-226.

Santelices B, Aedo D, Hoffman A, 2002. Banks of microscopic forms and survival to darkness of propagules and microscopic stages of macroalgae. *Revista Chilena de historia natural*, 75: 547-555.

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

Streftaris N, Zenetos A, Papathanassiou E, 2005. Globalisation in marine ecosystems: the story of non-indigenous marine species across European Seas. *Oceanography and marine biology*, 43: 419-453.

Swofford DL, 2002. PAUP*. *Phylogenetic Analysis Using Parsimony* (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tan HI, Blomster J, Hansen G, Leskinen E, Maggs CA, Mann DG, Sluiman HJ, Stanhope MJ, 1999. Molecular phylogenetic evidence for a reversible morphogenetic switch controlling the gross morphology of two common genera of green seaweeds, *Ulva* and *Enteromorpha*. *Molecular biology and evolution*, 16: 1011-1018.

Tanner C, 1986. Investigations of the taxonomy and morphological variation of *Ulva* (Chlorophyta): *Ulva californica* Wille. *Phycologia*, 25: 510-520.

Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: 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.

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 CA, 2001. Intraspecific variation in seaweeds: The application of new tools and approaches. *Advances in botanical research*, 35: 171-212.

White TJ, Bruns T, Lee S, Taylor JW, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols: A guide to methods and applications*. Academic Press, New York: 315-322.

Worm B, Lotze HK, Sommer U, 2001. Algal propagule banks modify competition, consumer and resource control on Baltic rocky shores. *Oecologia*, 128: 281-293.

Zenetos A, Cinar ME, Pancucci-Papadopoulou MA, Harmelin JG, Furnari G, Andaloro F, Bellou N, Streftetis N, Zibrowius H, 2005. Annotated list of marine alien species in the Mediterranean with records of the worst invasive species. *The mediterranean marine science journal*, 6: 63-118.