

A DNA barcoding survey of *Ulva* (Chlorophyta) in Tunisia and Italy reveals the presence of the overlooked alien *U. ohnoi*

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Abstract – The cosmopolitan genus *Ulva* Linnaeus includes species of green macroalgae found in marine, brackish and some freshwater environments. Although there is a wide literature for the determination of *Ulva* taxa in Europe, they are among the most problematic algae to accurately identify, because they have few distinctive features, as well as a high intraspecific variation.

At present, the knowledge of both diversity and distribution of the genus *Ulva* in the Mediterranean Sea is almost entirely based on morphological studies and there is only a few published papers dealing with molecular data. Tunisia has a key position in the Mediterranean and constitutes a transition area with a rich habitat diversity between eastern and western basins. The latest inventory of marine macrophytes dates back to 1987, updated in 1995. The aim of the present paper is to provide a molecular-assisted alpha taxonomy survey of *Ulva* spp. along Tunisian coasts, in comparison with a few Italian sites, using the *tufA* marker. Nine genetic species groups were resolved, including the non indigenous species *Ulva ohnoi*, newly reported for Tunisia. The actual picture of the taxonomy of *Ulva* spp. in the Mediterranean as a whole is far to be clarified and the present data on Tunisian collections aim to be a step towards its clarification. This paper is the first DNA barcoding study on green macroalgae in Tunisia and it contributes to add records to the Barcode Of Life Data Systems which are publically available.

Alien species / DNA barcoding / non indigenous species / *tufA* / Tunisia / *Ulva ohnoi*

INTRODUCTION

Ulva Linnaeus includes species of cosmopolitan green macroalgae found in marine, brackish and some freshwater environments. It includes almost 600 described species of which 125 are currently accepted taxonomically (Guiry & Guiry, 2017),

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some are a major component in transitional environments and among the most common members of rocky intertidal and subtidal marine habitats worldwide.

Ulva species also include some common bloom-forming taxa (Hiraoka *et al.*, 2003; Leliaert *et al.*, 2009), and species have been recently used for bioremediation applications (El-Sikaily *et al.*, 2007), in chemical ecology (Van Alstyne *et al.*, 2007), as bioindicators of eutrophic or stressed environments (Kozhenkova *et al.*, 2006), and as food (Abbott, 1979; Xia & Abbott, 1987). However, most applicative studies on *Ulva* spp. lack reliable taxonomic identifications (Kirkendale *et al.*, 2013). This is a potential problem because it has been shown that even closely related species exhibit unique ecophysiological and chemical characteristics (Eswaran *et al.*, 2002; Michael, 2009; Paulert *et al.*, 2010; Winberg *et al.*, 2011).

Although there is a wide taxonomic literature on *Ulva* in Europe (Bliding, 1963, 1968; Koeman & van den Hoek, 1982a, b, 1984; Cormaci *et al.*, 2014), they are among the most problematic algae to accurately identify, because they have few distinctive features, as well as a high intraspecific variation. A direct consequence is the number of superfluous and synonymized species recorded (Guiry & Guiry, 2017). More difficulties are added by the knowledge that changes in morphology are induced by low salinity or salinity shock and laboratory culture experiments showed that bacteria and their exudates associated with *Ulva* may induce extensive morphological changes that cross supposedly well-defined species boundaries (Provasoli & Pintner, 1980; Blomster *et al.*, 1998; Marshall *et al.*, 2006). Species of *Ulva* are also known for their rapid proliferous growth and their capacity to tolerate a wide range of environmental conditions, and, as a consequence, are ideal candidates for human-mediated dispersal and have been detected as the most common component of the macroalgal biomass present on hulls (Mineur *et al.*, 2007; Heesch *et al.*, 2009; Couceiro *et al.*, 2011). They are among the most widely introduced species of macroalgae, even by wooden sailing vessels in previous centuries (Santelices *et al.*, 2002; Schaffelke *et al.*, 2006; Kraft *et al.*, 2010). To further complicate the issue, an understanding of native ranges is lacking for many species of *Ulva* and due to their cryptic diversity, many non-indigenous species (NIS) may go unnoticed (Melton *et al.*, 2016a). Previous unrecorded taxa could be possibly overlooked due to the variability and unreliability of accepted morphological characters, and many authors stated that, without the use of DNA sequence data, some species groups would remain with uncertain identification (Loughnane *et al.*, 2008; Coll *et al.*, 2010; Kang *et al.*, 2014). However, accurate biodiversity assessment is essential for monitoring biological introductions, and is critical for environmental management as well as to adequately evaluate temporal changes (Loughnane *et al.*, 2008; Hofmann *et al.*, 2010; Melton *et al.*, 2016a).

For *Ulva* spp. molecular identification, previous studies used the chloroplast-encoded *rbcL* and *tufA* markers and the nuclear internal transcribed spacer region 1 (ITS1) of the ribosomal cistron (Heesch *et al.*, 2009; Saunders & Kucera, 2010; Couceiro *et al.*, 2011; Kirkendale *et al.*, 2013; Melton *et al.*, 2016a). However, Kirkendale *et al.* (2013) noted that ITS1 commonly had poor amplification success producing double bands indicative of divergent copies (Saunders & Kucera, 2010), whereas *rbcL* showed low levels of genetic diversity reducing species-level resolution (Heesch *et al.*, 2009). Differently, *tufA* resulted as a viable marker with a higher resolution power at species level in comparison to the other molecular markers (Saunders & Kucera, 2010).

At present, the knowledge of both diversity and distribution of the genus *Ulva* in the Mediterranean Sea is almost entirely based on morphological studies and

there are few published papers dealing with molecular data (Wolf *et al.*, 2012; Armeli Minicante *et al.*, 2014). Checklists for Mediterranean algal taxa include a large number of species poorly known, and several taxa *inquirenda* (Coll *et al.*, 2010; Cormaci *et al.*, 2014; Gallardo *et al.*, 2016; Zenetos *et al.*, 2017) and highlight the need of an accurate revision to decipher the taxonomy of several genera, including *Ulva*.

Tunisia has a key position in the Mediterranean and constitutes a transition area with a rich habitat diversity between eastern and western basins. The Sicilian Channel is the crossroad from south to north and from east to west, and is crucial in the analysis of the marine organism distribution into the Mediterranean Sea. The presence of rocky or sandy coasts, deep bays, some protruding caps, numerous islands and islets and a shallow belt are characteristic features of the Tunisian littoral topography (Blanpied *et al.*, 1979a, b; Pergent & Kempf, 1993).

All floristic and taxonomic studies for Tunisian marine flora, have been carried out by means of a morpho-anatomic identification approach, and the latest inventory dates back to 1987, (Ben Maiz *et al.*, 1987), with an update in 1995 (Ben Maiz, 1995). Subsequently, only papers on a limited area (Ben Maiz & Shili, 2007; Shili *et al.*, 2007), dealing with NIS (Sghaier *et al.*, 2016) or with applicative purposes (Azaza *et al.*, 2008; Yaich *et al.*, 2011; Kolsi *et al.*, 2015), have been published on *Ulva* spp.; none of them used a molecular systematics approach.

The aim of the present paper is to provide a molecular-assisted alpha taxonomy (MAAT) survey of *Ulva* spp. along Tunisian coasts, as well as a few Italian sites to serve as comparison, using the *tufA* marker for the compilation of a DNA barcode inventory.

MATERIAL AND METHODS

Samples of *Ulva* were collected in 9 sites distributed along the Tunisian coastline from March 2014 to March 2016 (Fig. 1). All samples were hand collected from the intertidal zone. Samples were transported in seawater at low temperature to the laboratory, where they were washed thoroughly with seawater to eliminate debris. For each sample, a voucher specimen was prepared by pressing a single individual on an herbarium sheet with a subsample of thallus dried in silica gel for molecular analyses. Vouchers are housed in the Phycological Lab Herbarium (PhL) of the University of Messina, Italy (<http://grbio.org/institution/phyecological-lab-herbarium-university-messina>). Additional samples collected along Italian coasts included in PhL were also considered for comparison with Tunisian collections.

DNA was extracted from silica dried specimens using a standard CTAB-extraction method (Doyle & Doyle, 1987), with few modifications: 2-mercaptoethanol was excluded from the extraction buffer, while 1% PVP and 0.02% of proteinase K were added; lysis was performed at room temperature for 2 hours on a rotary shaker.

The plastidial *tufA* gene was PCR amplified as described in Saunders and Kucera (2010). Sequencing reactions were performed by an external company (Macrogen Europe, The Netherlands). Some of the Italian specimens were processed and sequenced at the University of New Brunswick, Canada (Saunders & McDevit, 2012).

Specimen data, sequences and used primers were deposited in the Barcode of Life Data Systems (BOLD, <http://www.boldsystems.org>). Forward and reverse

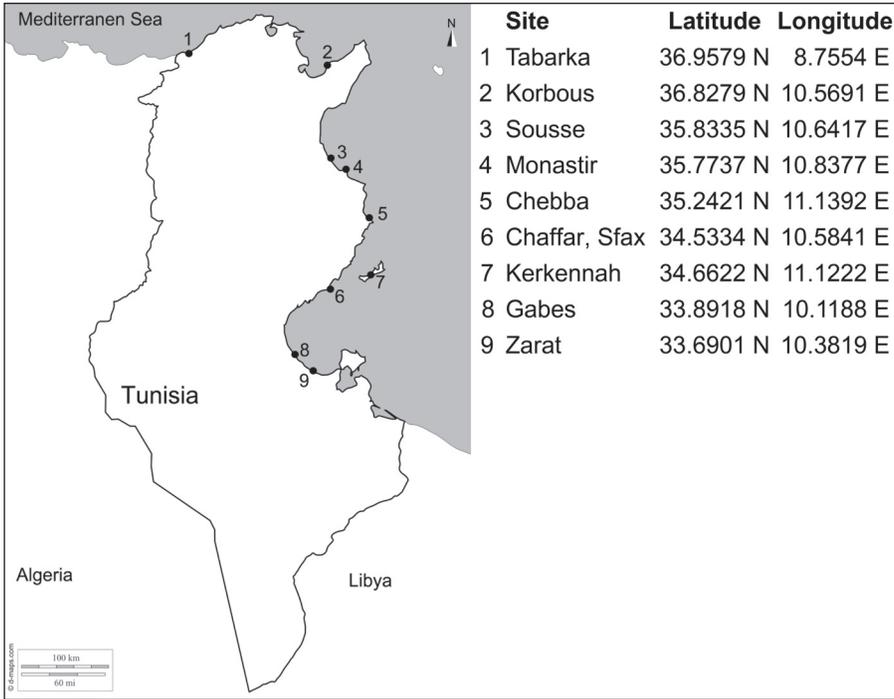


Fig. 1. Collection sites along the Tunisian coastline.

sequence reads were assembled with the software ChromasPro (v. 1.41, Technelysium Pty Ltd) and multiple sequence alignments were constructed in Seaview (v. 4.3.3, Gouy *et al.*, 2010), including sequences of the family Ulvaceae downloaded from GenBank (Benson *et al.*, 2017). Sequence alignments were subjected to distance analyses with a Neighbor-Joining algorithm under a K2P model of nucleotide substitution (Kimura, 1980) as performed in PAUP* 4b10 (Swofford, 2002) to visualize genetic clusters that may correspond to species.

RESULTS AND DISCUSSION

It is widely acknowledged that taxonomic identification within the large genus *Ulva* Linnaeus, with 125 species and infraspecific taxa currently accepted (Guiry & Guiry, 2017), by means of dichotomous keys based on morphological characters, is a challenging task because of the paucity of available characters and their significant plasticity in response to environmental factors (Heesch *et al.*, 2009; Hofmann *et al.*, 2010; Couceiro *et al.*, 2011). As a consequence, DNA data have been progressively used as an easier alternative for species recognition.

Two categories of DNA-based species delimitation exist, namely single-locus and multi-locus methods (see Leliart *et al.*, 2014 for a review). While it is

now generally recognized that multi-locus data are essential for accurate species delimitation, single-locus approaches have proven effective for species identification (e.g. Manghisi *et al.*, 2011; Geoffroy *et al.*, 2012; Kogame *et al.*, 2016). Single-locus methods basically rely on three assumptions: a) the gene genealogy is representative of the species phylogeny; b) species are monophyletic; c) the transition between intra- and interspecific sequence variation is visible as a distinct gap in the frequency distribution, the so-called DNA barcoding gap (Hebert *et al.*, 2004). Although in several studies dealing on algal species delimitation clear discontinuities between intra- and interspecific genetic distances have been observed (e.g. Saunders, 2005; Zimmermann *et al.*, 2011), the use of distance methods may be problematic in some cases, such as among closely related species, in which intra- and interspecific genetic distances may overlap (Hamsher *et al.*, 2011; Hoef-Emden, 2012), or with increased geographic sampling resulting in a blur of barcoding gaps (Meyer & Paulay, 2005; Bittner *et al.*, 2010; Bergsten *et al.*, 2012).

Several taxonomists have claimed that adequate delimitation of species based solely on DNA sequence data is not acceptable, rather should be based on additional lines of evidence, including morphological, ultrastructural, biochemical, geographic, ecological and breeding data (e.g. Pröschold *et al.*, 2001; Vanormelingen *et al.*, 2007; Walker *et al.*, 2009; Bendif *et al.*, 2011; McManus & Lewis, 2011; Neustupa *et al.*, 2011; Ni-Ni-Win *et al.*, 2011; Lam *et al.*, 2012; Milstein & Saunders, 2012; Škaloud & Rindi, 2013). Nevertheless, molecular methods are effective tools for a first screening in order to merely identify genetic species groups in a strategy named molecular-assisted alpha taxonomy (MAAT) (Saunders, 2005). This methodology is especially useful in organisms with simple morphologies, with a high degree of phenotypic plasticity or convergence depending on environmental conditions, like the genus *Ulva*. The chloroplast-encoded *tufA* gene resulted as a feasible marker for identification of green algae, with a higher resolution power at the species level in comparison to other molecular markers commonly used in studies dealing with *Ulva* spp. molecular identification, such as the nuclear internal transcribed spacer region 1 (ITS1) and the chloroplast-encoded *rbcL* gene (Saunders & Kucera, 2010; Kirkendale *et al.*, 2013).

The main limit of MAAT, however, is that type sequences, generated from type specimens, or at least topotype material, should be used as a reference to define species or clarify species concepts (Heesch *et al.*, 2009; Hofmann *et al.*, 2010). Nevertheless, these data are rarely available, particularly when type specimens are very old, or lost, and/or type localities are unknown or vague, as is, for instance, the case with most of the *Ulva* type specimens described by Linnaeus (1753) (Womersley, 1984), and the names used in sequence data retrieved from GenBank need to be used with caution (Heesch *et al.*, 2009) as the DNA library of life is still under construction (Le Gall *et al.*, 2017). The only historical type specimen, which DNA was sequenced, is the lectotype of *Ulva lactuca* Linnaeus (selected by Papenfuss, 1960) and, according to unpublished data by C. Maggs and F. Mineur (Butler, 2007; Holden, 2007; O'Kelly *et al.*, 2010; Delnatte & Wynne, 2016), the specimen in the Linnean Herbarium matched with *Ulva fasciata* Delile. What is currently known as *U. lactuca* is in fact another species, namely *Ulva stipitata* Areschoug (presently considered as a synonym of *U. lactuca*), even if the authors stated that further work was needed to clarify the matter. However, DNA results on the lectotype of *U. lactuca* has not yet been published.

At present, published papers using MAAT approaches for Mediterranean Ulvaceae are very limited. In 2012, Wolf *et al.* presented a survey on *Ulva* spp. biodiversity in Venice lagoon, pointing the presence of the non-indigenous species

(NIS) *Ulva australis* Areschoug (as *Ulva pertusa* Kjellman, already reported by Manghisi *et al.*, 2011) and *Ulva californica* Wille (the first Mediterranean record). Later, Armeli *et al.* (2014) reported the NIS *Ulva ohnoi* Hiraoka *et Shimada* in Cape Peloro lagoon, north-eastern Sicily, for the first time in Europe, and Bertuccio *et al.* (2013/2014) updated the floristic list of the same site.

In the present work, a total of 43 *tufA* sequences of *Ulva* from Tunisia and Italy were generated. These sequences were resolved in nine genetic clusters, which are here regarded as species (Table 1, Fig. 2). Five species included specimens collected along Tunisian coasts, namely *Ulva compressa* Linnaeus, *Ulva mediterranea* Alongi, Cormaci *et G. Furnari*, *Ulva torta* (Mertens) Trevisan, *Ulva laetevirens* Areschoug and *U. ohnoi*, the latter two newly reported for Tunisia. *U. compressa*, *U. laetevirens* and *U. ohnoi* were also found along Italian coasts, together with *U. australis*, *Ulva linza* Linnaeus and *Umbrulva dangeardii* Wynne *et Furnari*, and the doubtful record of *U. fasciata*.

***Ulva compressa* Linnaeus.** Distance analysis of *tufA* sequences showed that Tunisian specimens grouped with others from various Mediterranean sites, including Venice lagoon and Torre Faro, Italy, Banyuls, France, other localities from Canada, Australia and China, as well as with an unidentified species from UK, within the type area (probably Bognor, Sussex, England, *fide* Hayden *et al.*, 2003: 289) (Fig. 2). Samples included in the present work were collected in various habitats, both undisturbed and with anthropogenic impact, and displayed a highly variable morphology (Figs 3-8), as already observed in other regions of the world (Tan *et al.*, 1999; Hofmann *et al.*, 2010; Kraft *et al.*, 2010).

Ulva compressa is regarded as a common native species in the Mediterranean Sea, thriving in non-exposed sites (Cormaci *et al.*, 2014), already reported for Tunisia (Ben Maiz *et al.*, 1987), and widely distributed in all oceans (Guiry & Guiry, 2017). It is noteworthy that Kirkendale *et al.* (2013) hypothesized that *Ulva compressa* could be introduced from Australia to the rest of the world as it display higher genetic diversity within than outside Australia. The analysis of all publicly available *tufA* sequences, including Mediterranean samples generated in the present works, showed distance values ranging from 0.00 to 1.35% (0-9 bp) within species. These values are driven by the diversity of Australian samples whereas Mediterranean isolates showed distance values ranging from 0.00 to 0.54% (0-4 bp). The large sequence divergence is comparable to that observed among species currently regarded as distinct (e.g. *U. fasciata* vs *U. ohnoi*). Therefore, it could be an evidence of cryptic diversity at the specific level, which should be investigated with supplementary sequences from other geographic sites and additional molecular markers. Meanwhile, there is no apparent reason to consider it as a NIS in the Mediterranean basin.

Ulva mediterranea* Alongi, Cormaci *et G. Furnari. The Tunisian sample collected in the present work, found in an habitat with anthropogenic impact, grouped in *tufA* analysis with isolates from Venice lagoon, Italy, and other localities from Canada, Australia, China and Korea (0.00-0.47%, 0-3 bp sequence divergence) identified as *Ulva flexuosa* Wulfen (Figs 2, 9). Hiraoka *et al.* (2017), basing on DNA data coupled with culturing and hybridisation experiments, proved that the previously listed samples do not belong to the genuine *U. flexuosa*, which had been characterized by Mareš *et al.* (2011) using molecular systematics in combination with morphological observations (including re-examination of the holotype). Hiraoka *et al.* (2017) merged the former samples with *U. mediterranea*, also including *Ulva flexuosa* Wulfen var. *linziformis* Alongi, Cormaci *et G. Furnari* and *Ulva linzoides* Alongi, Cormaci & G. Furnari among synonyms.

Table 1. Species of foliose Ulvaceae identified by DNA barcoding in the Mediterranean Sea in the present paper and in previous literature

Species	Locality	DNA Barcode	DNA Barcode reference	Global distribution	Mediterranean distribution	Notes	NIS assessment ¹
<i>Ulva australis</i> Areschoug	Venice Lagoon, Italy (as <i>U. pertusa</i> Kjellman)	BOLD# ITGRE004-11	Present study	Japan (Kjellman, 1897), Northeast Asia (Hanyuda <i>et al.</i> , 2016), New Zealand (Heesch <i>et al.</i> , 2009), Australia (Kraft <i>et al.</i> , 2010), Indonesia, Singapore and the Philippines (Silva <i>et al.</i> , 1996), The Netherlands (Stegenga <i>et al.</i> , 2007), Spain (Couceiro <i>et al.</i> , 2011), Brittany (Coat <i>et al.</i> , 1998), Canada and the USA (Saunders & Kucera, 2010), Mexico (Aguilar-Rosas <i>et al.</i> , 2008), Yemen, Kenya, Mauritius and Tanzania (Silva <i>et al.</i> , 1996)	Thau Lagoon, France (Verlaque <i>et al.</i> , 2002), Venice Lagoon, Italy (Manghisi <i>et al.</i> , 2011), Malaga (Couceiro <i>et al.</i> , 2011)	<i>U. pertusa</i> Kjellman is a synonym based on genetic data (Couceiro <i>et al.</i> , 2011; Kirkendale <i>et al.</i> , 2013)	Likely, from North-eastern Asia; genetic data compared to Kirkendale <i>et al.</i> (2013) and Hanyuda <i>et al.</i> (2016); anthropized habitat
		GB# HE600186					
		GB# HE600187					
		GB# HE600188					
		GB# HE600189					
		GB# HE600190					
		GB# HE600173					
		GB# HE600174					
		GB# HE600175					
		GB# HE600176					
<i>Ulva californica</i> Wille	Venice Lagoon, Italy	BOLD# TUGRE007-17	Present study	Indopacific, Britain, Ireland, Senegal, Guadeloupe, Antarctic and the subantarctic islands (Guiry & Guiry, 2017)	Venice Lagoon (Wolf <i>et al.</i> , 2012)		Likely; genetic sampling insufficient to assess the Mediterranean status; anthropized habitat
		BOLD# TUGRE003-17					
		BOLD# TUGRE004-17					
		BOLD# TUGRE005-17					
		BOLD# TUGRE006-17					
		BOLD# TUGRE001-17					
		BOLD# TUGRE002-17					
		BOLD# ITGRE013-11					
		BOLD# ITGRE014-11					
		BOLD# GREVE002-17					
<i>Ulva compressa</i> Linnaeus	Kerkennah, Tunisia	GB# HE600184	Present study	Cosmopolitan (Guiry & Guiry, 2017)	Common (Cormaci <i>et al.</i> , 2014)	Consistent with use in Kirkendale <i>et al.</i> (2013)	Unlikely; various habitats
		GB# HE600185					
		BOLD# GRAPP002-17					
		BOLD# GRAPP003-17					
		Lido of Venice, Italy					
		Torre Faro, Messina, Italy					
		Wolf <i>et al.</i> , 2012					
		Present study					
		Present study					
		Present study					

Table 1. Species of foliose Ulvaceae identified by DNA barcoding in the Mediterranean Sea in the present paper and in previous literature (*continued*)

Species	Locality	DNA Barcode	DNA Barcode reference	Global distribution	Mediterranean distribution	Notes	NIS assessment ¹
<i>Ulva fasciata</i> Delle	Venice Lagoon, Italy	BOLD# ITGRE015-11	Present study				
		BOLD# ITGRE003-11					
	Cape Peloro Lagoon, Italy	BOLD# ITGRE016-11	Bertuccio <i>et al.</i> , 2013/2014	Cosmopolitan (Guiry & Guiry, 2017)	Common (Cormaci <i>et al.</i> , 2014)	Consistent with usage in Kraft <i>et al.</i> (2010), comparison with type necessary	Doubtful, genetic data compared to Kirkendale <i>et al.</i> (2013); anthropized habitat
		BOLD# ITGRE017-11					
		BOLD# ITGRE018-11	Present study				
<i>Ulva flexuosa</i> Wulfen	Zarat, Tunisia	BOLD# TUGRE008-17	Present study				
	Venice Lagoon, Italy	GB# HE600177	Wolf <i>et al.</i> , 2012	Cosmopolitan (Guiry & Guiry, 2017)	Common (Cormaci <i>et al.</i> , 2014)	Consistent with usage in Mares <i>et al.</i> (2011); genetic data compared also to Kirkendale <i>et al.</i> (2013)	Unlikely. Anthropized habitats
<i>Ulva laetevirens</i> Areschong	Monastir, Tunisia	BOLD# TUGRE009-17					
	Sousse, Tunisia	BOLD# TUGRE011-17					
	Tabarka, Tunisia	BOLD# TUGRE010-17	Present study				
	Venice Lagoon, Italy	BOLD# ITGRE010-11					
		BOLD# ITGRE011-11					
		GB# HE600178					
	Lido of Venice, Italy	GB# HE600179	Wolf <i>et al.</i> , 2012				
		GB# HE600180					
		GB# HE600181					
		GB# HE600182					
		BOLD# ITGRE009-11		Australia, Atlantic North America, China and Japan (Kirkendal <i>et al.</i> , 2013, unpublished in Genbank)	Common (Cormaci <i>et al.</i> , 2014); reported in Tunisia as <i>Ulva rigida</i> C. Agardh, likely see Cormaci <i>et al.</i> , 2014	Consistent with use in Kraft <i>et al.</i> (2010); synonymy with <i>Ulva rigida</i> C. Agardh likely but not asserted (see text).	Doubtful, possible introduction from Australia; genetic data compared to Kirkendale <i>et al.</i> (2013); various habitats
		BOLD# GRAPP004-17					
		BOLD# GRAPP005-17					
		BOLD# GRAPP006-17					
	Torre Faro, Messina, Italy	BOLD# GRAPP007-17	Present study				
	BOLD# GRAPP008-17						
	BOLD# GRAPP009-17						
	BOLD# GRAPP010-17						
	BOLD# GRAPP011-17						
	BOLD# GRAPP012-17						
	BOLD# ITGRE009-11						
Ragusa, Italy	BOLD# ITGRE002-11	Present study					

Species	Locality	DNA Barcode	DNA Barcode reference	Global distribution	Mediterranean distribution	Notes	NIS assessment ¹
<i>Ulva linza</i> Linnéus	Venice Lagoon, Italy	GB# ITGRE005-11	Present study	Cosmopolitan (Guiry & Guiry, 2017)	Common (Cormaci <i>et al.</i> , 2014)	= <i>Ulva "procera"</i> (K. Ahlner) Hayden, Blomster, Maggs, P.C. Silva, M.J. Stanhope et J.R. Waaland <i>sensu</i> Kirkendale <i>et al.</i> (2013)	Unlikely; anthropized habitat
<i>Ulva ohnoi</i> Hiraoka et Shimada	Gabes, Tunisia Cape Peloro Lagoon, Italy	BOLD# TUGRE012-17 BOLD# ITGRE019-11 BOLD# ITGRE019-11	Present study Present study	Spain (South Atlantic) (Gallardo <i>et al.</i> , 2016), Venezuela (Melton <i>et al.</i> , 2016), India (Kazi <i>et al.</i> , 2016), Iran (Pirian <i>et al.</i> , 2016), Japan (Hiraoka <i>et al.</i> , 2004, Kirkendale <i>et al.</i> , 2013), Korea (Bae, 2010), Australia (Kirkendale <i>et al.</i> , 2013), Hawaiian Islands (O'Kelly <i>et al.</i> , 2010)	Cape Peloro Lagoon (Armeli <i>et al.</i> , 2014); not previously reported in Tunisia		Likely, from Asia, anthropized habitats
<i>Ulva tortu</i> (Mertens) Trevisan	Chaffar, Sfax, Tunisia	BOLD# TUGRE013-17	Present study	Cosmopolitan (Guiry & Guiry, 2017)	Common (Cormaci <i>et al.</i> , 2014)		Unlikely; undisturbed habitat
<i>Ulva</i> sp.	Cape Peloro Lagoon, Italy	BOLD# ITGRE020-11 BOLD# ITGRE021-11 BOLD# ITGRE023-11	Bertuccio <i>et al.</i> , 2013/2014	Australia, North America, South Korea (Guiry & Guiry, 2017)	Common (Guiry & Guiry, 2017); Italy (Furnari <i>et al.</i> , 2003)	= <i>Ulva flexuosa</i> subsp. <i>pitifera sensu</i> Bertuccio <i>et al.</i> (2013/2014); based on results of Mares <i>et al.</i> (2011)	Doubtful
<i>Umbraulva dangeardii</i> Furnari	Torre Faro, Messina, Italy	BOLD# ITGRE007-11 BOLD# ITGRE008-11	Present study	Europe, Atlantic Islands, Africa, South-west Asia, Australia and New Zealand (Guiry & Guiry, 2017)	Common (Guiry & Guiry, 2017); Italy (Furnari <i>et al.</i> , 2003)	= <i>Umbraulva olivascens</i> (P.J.L. Dangeard) G. Furnari	Unlikely; anthropized habitat

¹ Based on criteria by Heesch *et al.* (2009) and Kirkendale *et al.* (2013).

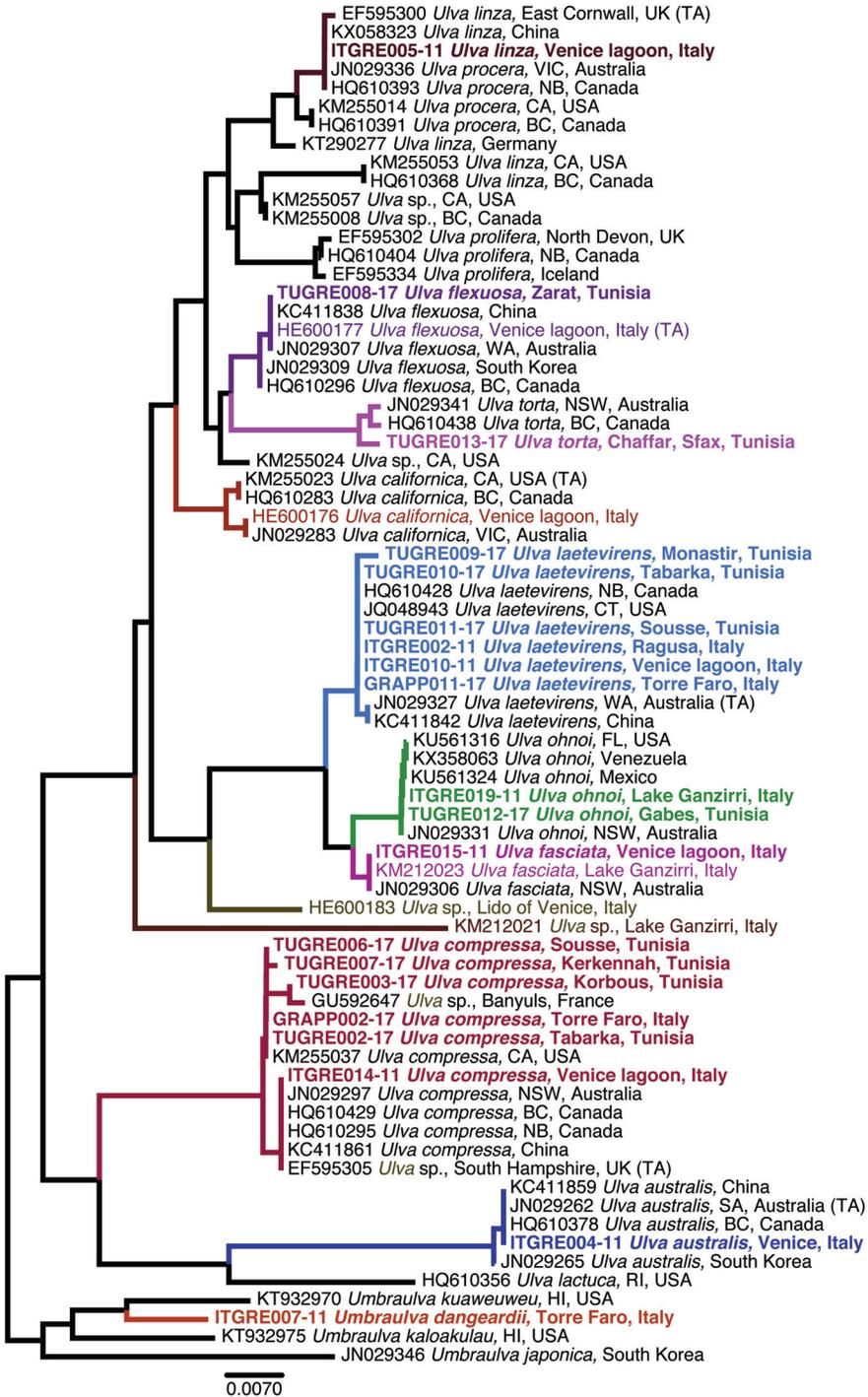
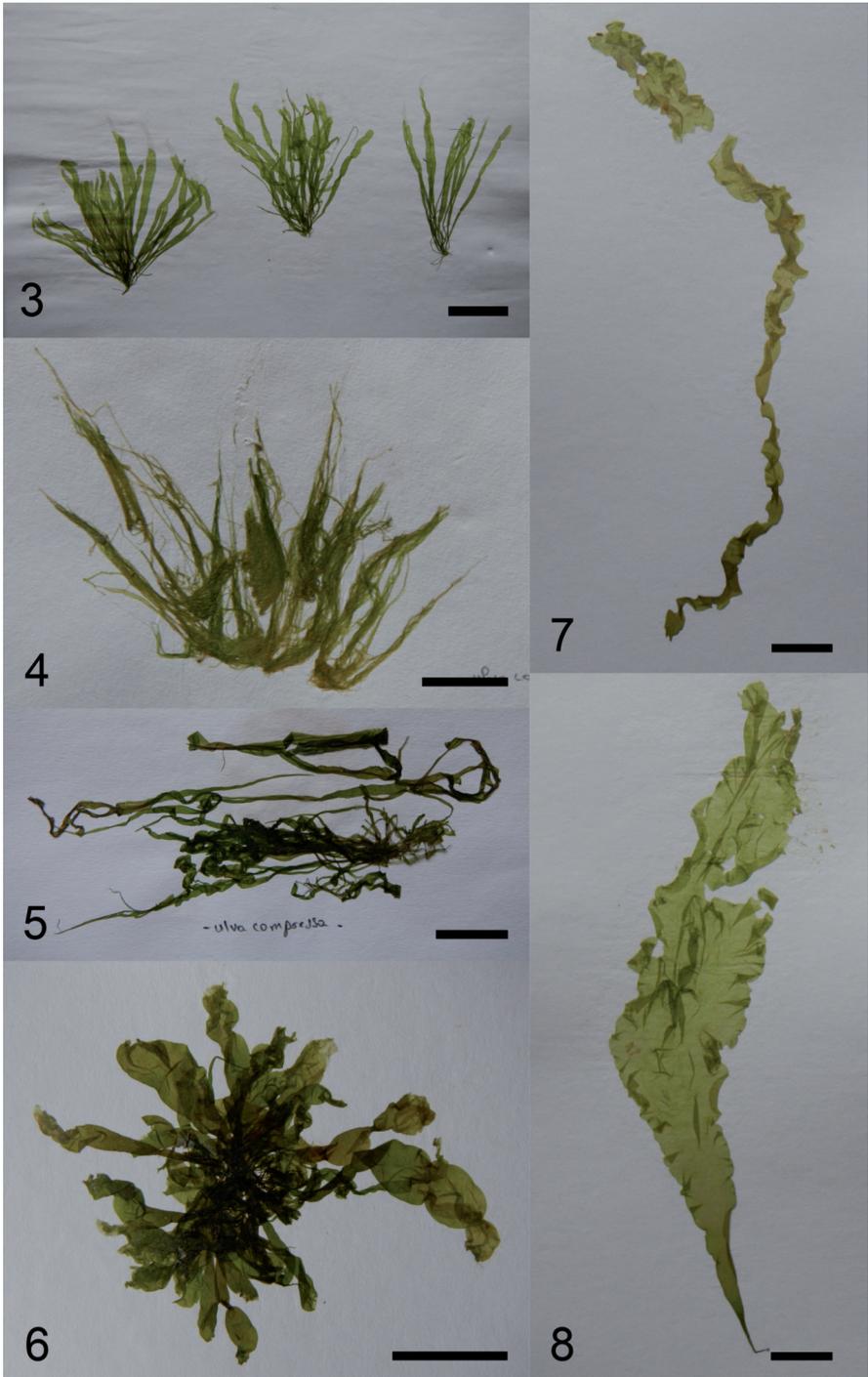


Fig. 2. Neighbor-Joining tree of *tufA* sequences generated in the present work (coloured in bold type) and of representative Ulvaceae downloaded from Genbank (TA = type area). Scale bar: substitution per site.



Figs 3-8. Specimens of *Ulva compressa*. **3.** Torre Faro, Italy (BOLD# GRAPP003-17); **4-5.** Tunisia (BOLD# TUGRE007-17, BOLD# TUGRE003-17); **6-8.** Venice Lagoon (BOLD# ITGRE013-11, BOLD# ITGRE014-11, BOLD# GREVE002-17). Scale bar: 2 cm.



Figs 9-10. **9.** *Ulva mediterranea*, Tunisia (BOLD# TUGRE008-17). **10.** *Ulva torta*, Tunisia (BOLD# TUGRE013-17). Scale bar: 2 cm.

Ulva mediterranea has been reported as a common native species in the Mediterranean Sea and it is widely distributed in all oceans (Guiry & Guiry, 2017). It is not possible to assess whether *U. mediterranea* is a new report for Tunisia or rather it was misidentified as *U. flexuosa* by Ben Maiz *et al.* (1987).

***Ulva torta* (Mertens) Trevisan.** Distance analysis showed that the Tunisian specimen grouped with *U. torta* from Pacific North America and Australia (0.00-0.40%, 0-3 sequence divergence) (Fig. 2). However, a comparison with sequences from type locality (Germany) is missing.

It has been reported as a common native species in the Mediterranean Sea (Cormaci *et al.*, 2014), already reported in Tunisia (Ben Maiz *et al.*, 1987), widely distributed in all oceans (Guiry & Guiry, 2017). Samples collected in the present work were found in an undisturbed habitat (Fig. 10).

***Ulva linza* Linnaeus.** Distance analysis showed that the specimen from Venice lagoon grouped with *U. linza* from England, in the type area (Kent, England fide Hayden *et al.*, 2003) and China, together with samples identified as *Ulva procera*

(K. Ahlner) Hayden, Blomster, Maggs, P.C. Silva, M.J. Stanhope *et al.* J.R. Waaland by Kirkendale *et al.* (2013), from Canada and Australia (0.00-1.14%, 1-8 bp sequence divergence) (Fig. 2).

Ulva linza is a common native species in the Mediterranean Sea (Cormaci *et al.*, 2014), widely distributed in all oceans (Guiry & Guiry, 2017). Several publications on *Ulva* spp. phylogenies considered *U. procera* as a separate species from *U. linza* (Heesch *et al.*, 2009; Saunders & Kucera, 2010; Kirkendale *et al.*, 2013), even if, according to Brodie *et al.* (2007), ITS sequence data had demonstrated that it is conspecific with typical *U. linza*. However, *U. linza* (including *U. procera*) has a high phenotypic plasticity induced by the environment and bloom-forming isolates in the Yellow Sea were similar in morphology to *U. prolifera* O.F. Müller and *U. procera* (Kang *et al.*, 2014). According to Kirkendale *et al.* (2013), there are actually 3-5 genetic groups in the “*linza-prolifera-procera*” cluster, with a morphology ranging from blades to tubes, which makes impossible to use morphology for identifications. A detailed study on type material of the three taxa is needed to define this cluster.

***Ulva fasciata* Delile.** Distance analysis showed that specimens from Venice lagoon and lake Ganzirri in Cape Peloro lagoon grouped with *Ulva fasciata* from Australia (Fig. 2). No sequence divergence was observed within Mediterranean isolates, while one base-pair difference (0.14%) was observed with just one Australian sample. All samples collected in the present work came from habitat with anthropogenic impact and showed a high degree of morphological variability (Figs 11-14).

Ulva fasciata has been reported as a common native species in the Mediterranean Sea (Cormaci *et al.*, 2014), widely distributed in all oceans (Guiry & Guiry, 2017). According to Kirkendale *et al.* (2013) the complete lack of sequence diversity within Australia made introduction plausible, however global genetic reference pool was lacking and habitat data did not suggest NIS. Similarly, no genetic diversity was observed among Italian isolates. Its status as a NIS or a native species is doubtful, pending a larger sampling in the Mediterranean and a comparison with type material (type locality: Alexandria, Egypt).

***Ulva laetevirens* Areschoug.** Distance analysis showed that Tunisian specimens are conspecific with *U. laetevirens* from Australia (type area, Port Phillip, South Australia) and other Mediterranean sites, including Venice lagoon, Ragusa and Torre Faro, Italy, as well as with samples from Atlantic North America and China (1-3 bp, 0.00-0.40%) (Fig. 2). In the same genetic species grouped sequences identified as *Ulva rigida* C. Agardh from Venice (data not shown, 100% sequence identity with other Italian specimens). Samples collected in the present work were found in various habitats, both undisturbed and anthropized (Figs 15-20).

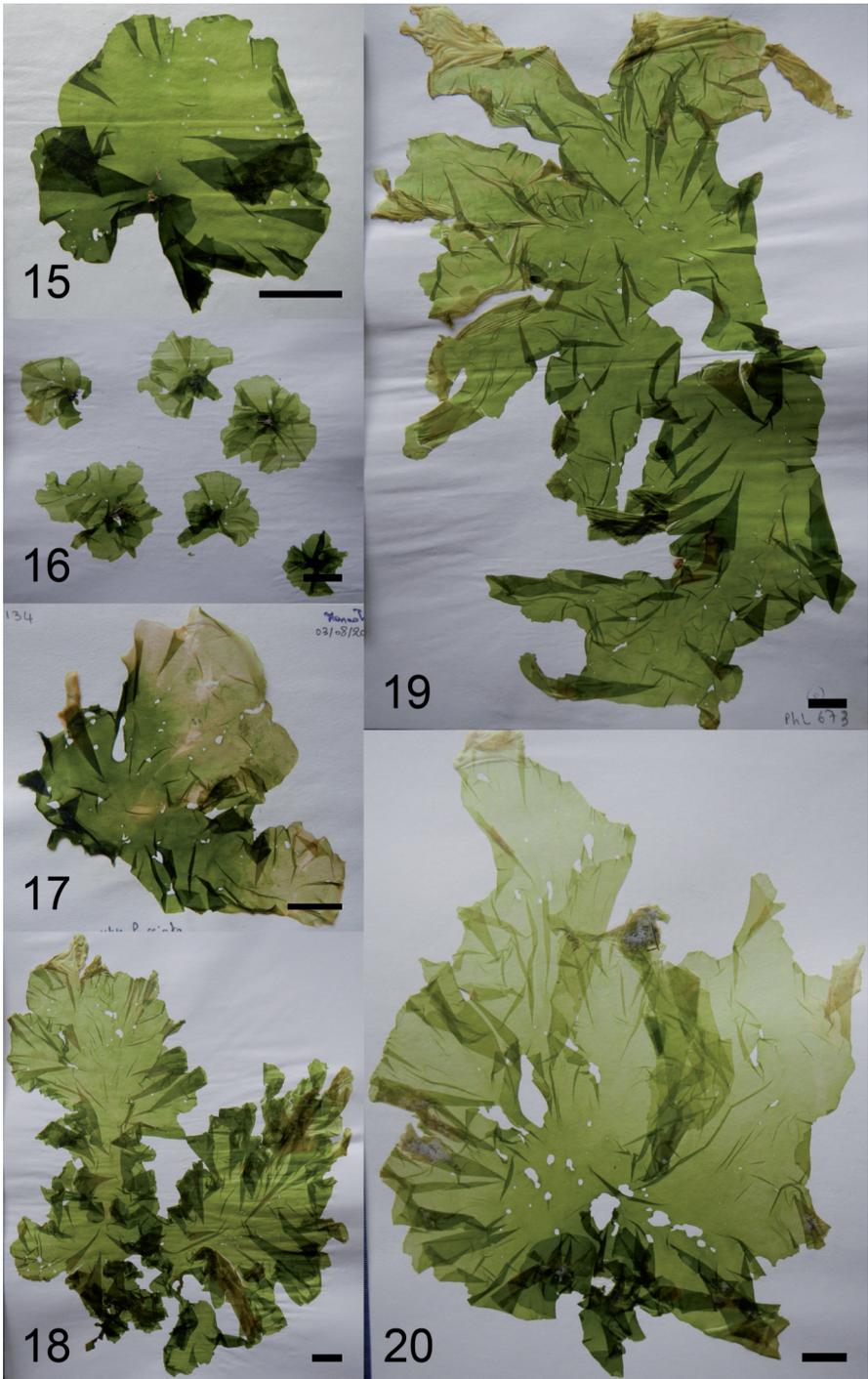
U. laetevirens is commonly reported in the Mediterranean Sea, in a variety of sites both exposed and sheltered, mainly oligotrophic (Cormaci *et al.*, 2014), and also found in Atlantic North America, China and Japan (Kirkendale *et al.*, 2013; unpublished in Genbank). Synonymy of *U. laetevirens* with *U. rigida* was discussed by Phillips (1988), which noted that specimens described by Bliding (1968) were misidentified, and she concluded that *U. rigida sensu* Bliding *non* C. Agardh should be referred to *U. laetevirens*. However, Phillips (1988) included erroneously *U. australis* among synonyms of *U. rigida* (Kraft *et al.*, 2010) and, as a consequence, it is possible that she did not analyze the genuine *U. rigida* C. Agardh. Since Bliding's study, many investigations conducted in Europe have recorded the presence of *U. rigida sensu* Bliding, presumably also molecular articles (Hayden *et al.*, 2003; Loughnane *et al.*, 2008), therefore, accessions in GenBank bearing the name



Figs 11-14. Specimens of *Ulva fasciata*. 11-14. Cape Peloro lagoon, Italy (BOLD# ITGRE018-11, BOLD# ITGRE016-11, BOLD# ITGRE003-11, BOLD# GRAPP013-17). Scale bar: 2 cm.

U. rigida are likely to actually represent *U. laetevirens*. Molecular sequences for *U. laetevirens* from the type region (Port Phillip Bay, Australia) was reported in Kraft *et al.* (2010), while a morphological and molecular study of *U. rigida sensu* C. Agardh from the type region (Cadiz, Spain) is not available. At present, the synonymy between *U. rigida* and *U. laetevirens*, is most likely but cannot be asserted.

In Venice lagoon, Sfriso (2010) reported the coexistence of *U. rigida* and *U. laetevirens* as two distinct species on the basis of subtle anatomical differences. The present finding of *U. laetevirens* in Tunisia is unlikely to be a new report, as it most likely corresponds to *Ulva rigida* already recorded by Ben Maiz *et al.* (1987). Based on genetic data compared to those of Kirkendale *et al.* (2013), it might be



Figs 15-20. Specimens of *Ulva laetevirens*. **15-16, 18-19**. Torre Faro, Italy (BOLD# GRAPP014-17, BOLD# GRAPP006-17, BOLD# GRAPP008-17, BOLD# GRAPP012-17); **17**. Tunisia (BOLD# TUGRE009-17); **20**. Venice Lagoon, Italy (BOLD# ITGRE010-11). Scale bar: 2 cm.

considered as a NIS introduced from Australia to the Mediterranean, but it is preferable to consider it as a cryptogenic species *sensu* Carlton (1996), pending upon clarification of its relationship with *U. rigida* and the identification of its native region.

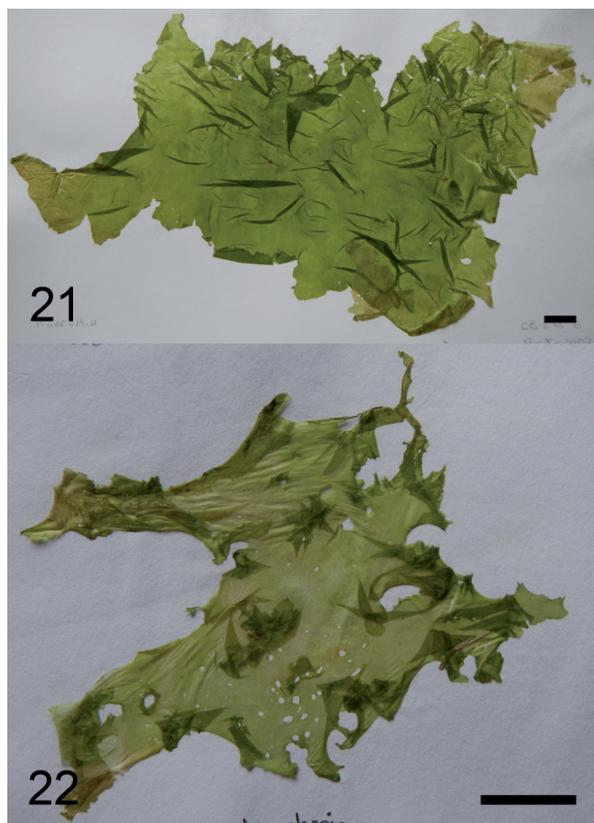
***Ulva australis* Areschoug.** Distance analysis showed that Venetian specimens grouped (0 bp sequence divergence) with other isolates from Venice lagoon and from Australia (type area, Port Adelaide, South Australia), Canada, Korea and China (0.00-0.29%, 1-2 bp) (Fig. 2), confirming the presence of the NIS *Ulva australis* in Venice lagoon, already reported by Manghisi *et al.* (2011).

Its native range includes Northeast Asia, Australia and New Zealand (Heesch *et al.*, 2009; Kraft *et al.*, 2010; Hanyuda *et al.*, 2016) and it is also reported as a NIS in most oceans, introduced by aquaculture or maritime activities, as a fouling species or in ballast waters of trans-ocean shipping (as *U. pertusa*, Hanyuda *et al.*, 2016). In the Mediterranean Sea, *Ulva australis* has been recorded also in Thau lagoon, France (as *U. pertusa*, Verlaque *et al.*, 2002), and Malaga, Spain (Couceiro *et al.*, 2011). In most instances, molecular data were decisive in appropriate taxonomic identification of this species, which was often previously misidentified by morphological data alone (Aguilar-Rosas *et al.*, 2008; Couceiro *et al.*, 2011; Hanyuda *et al.*, 2016). Therefore, its presence in the Mediterranean might even be considerably wider than presently known.

***Ulva ohnoi* Hiraoka & Shimada.** Distance analysis showed that the Tunisian specimen grouped with *U. ohnoi* from Cape Peloro lagoon, Italy (0 bp sequence divergence), and from Australia, China, North and South America (0.00-0.54%, 0-4 bp) (Fig. 2).

The new record of the Japanese bloom-forming *U. ohnoi* in Gabes, Tunisia, in a highly impacted area, close to commercial and fishing ports, chemical and oil industries, and tourist pathways (Figs 21-22) is noteworthy. The species was previously recorded in lake Ganzirri, Cape Peloro lagoon, northeastern Sicily, Italy, by Armeli *et al.* (2014), first report in the field in the Mediterranean and in Europe. The species was also found in Atlantic Spain (Gallardo *et al.* 2016), Gulf of Mexico and Florida (Melton *et al.*, 2016a), Venezuela (Melton *et al.*, 2016b), Iran (Piriani *et al.* 2016), India (Kazi *et al.* 2016), Japan (Hiraoka *et al.* 2004, Kirkendale *et al.* 2013), Korea (Bae 2010), Australia (Kirkendale *et al.* 2013), and Hawaiian Islands (O'Kelly *et al.* 2010).

In the Mediterranean, *U. ohnoi* had been previously identified in the algal fouling biomass on ships arriving at the commercial harbour at Sète, Mediterranean coast of France, a medium size port (Mineur *et al.*, 2007) and, similarly, in ballast water from ships arriving to Naples harbour, Italy, coming from Singapore, which had loaded water in Egypt and Lebanon (Flagella *et al.*, 2010). However, as stated by the latter authors themselves, colonization by this species had not been detected in the area (Flagella *et al.*, 2010). The Tunisian sample was found in impacted habitats, as well as those found in Cape Peloro lagoon, and the most likely mean of introduction is the maritime traffic considering that it is relevant both in Gabes harbour and in the Straits of Messina. Further studies are needed to investigate the actual distribution of this species in the Mediterranean, especially considering that all reports worldwide have been made by the use of DNA tools and that the species has been often misidentified under a mix of taxon names (Mineur *et al.*, 2007; O'Kelly *et al.*, 2010; Melton *et al.*, 2016a). Basing on present knowledge, this taxon is commonly regarded as a NIS, having a disjoint distribution and being recorded in highly disturbed sites. However, its presence in the Mediterranean, as well as in other sites, might be underestimated and its actual distribution, as well as its native range, be far different from what presently known.



Figs 21-22. Specimens of *Ulva ohnoi*. **21.** Cape Peloro lagoon, Italy (BOLD# ITGRE019-11); **22.** Tunisia (BOLD# TUGRE012-17). Scale bar: 2 cm.

CONCLUSIONS

The Mediterranean Sea has a long history of bioinvasions with a number of records higher than other European seas (Galil *et al.*, 2016). Many species of *Ulva* are often considered as widespread due to natural dispersal (cosmopolitan species), anthropogenic transport (NIS), or both (Kirkendale *et al.*, 2013). *Ulva* species have high dispersal potential due to their peculiar features, including good fouling capacity, dispersal efficiency and fast growth. It is recognized that species of *Ulva* can metabolize different environmental sources of nitrogen producing large biomass and, consequently, green tides (Melton *et al.*, 2016a). Therefore, they have a high potential for introduction and invasiveness if they arrive to altered environments with high availability of nutrients.

Criteria specific to distinguish between NIS and cosmopolitan species have been proposed by Heesch *et al.* (2009) and Kirkendale *et al.* (2013). A taxon is likely to be a NIS if it is found in highly modified environments and/or in close vicinity to potential vectors, such as frequent maritime traffic, aquaculture sites and artificial modifications of natural barriers (i.e. Suez Canal). However, this is not strict and native species might also thrive in sites with high anthropogenic impacts, as well as introduced species can occur widely, including in undisturbed sites. In addition, NIS

usually exhibit higher genetic similarity in comparison to samples from native area. Furthermore, it should be noted that most of the macroalgal species found on hulls are considered cosmopolitan and, consequently, at low risk of introduction outside their present range (Mineur *et al.*, 2007). This assumption may be misleading, however, as little information is available about their original native ranges. Marine floristic studies were uncommon for most sites before the twentieth century and an unknown amount of supposed cosmopolitan species may have had restricted ranges prior to their anthropogenic dispersal (Carlton 1996). It is likely that the distribution pattern of at least some *Ulva* species has been confused by repetitive human-mediated introductions over centuries of ship travelling.

The actual picture of the taxonomy of *Ulva* spp. in the Mediterranean as a whole is far to be clarified and the present data on Tunisian collections aim to be a step towards its clarification. At the best of our knowledge, this is the first study on the Tunisian green algal flora using DNA barcoding methods. The current taxonomic, biogeographical and ecological data gaps can be filled only by cooperative work and standardized methodologies (Galil *et al.*, 2016; Zenetos *et al.*, 2017). DNA barcoding demonstrated as a fast and effective tool for the genetic labelling of challenging taxa, such as Ulvacean species, especially in those areas that are subject to anthropogenic disturbances and quick changes of biodiversity. Data collected in such perspective are also valuable to increase the BOLD system catalogue, amplifying the biodiversity knowledge linked to geographical information, which becomes freely available for the scientific community. DNA barcodes are permanent labels assigned to specimens regardless any subsequent taxonomic or nomenclature variation. An effective monitoring of the biodiversity changes by means of a quick and accurate protocol, such as DNA barcoding, is essential to provide the basis for a correct environmental management.

Acknowledgements. Dr. Gary Saunders and his colleagues at the Center for Environmental & Molecular Algal Research, University of New Brunswick, are kindly acknowledged for processing specimens within the ALGA (Algal Life Global Audit) project under the iBOL initiative WG1.8 Marine Bio-surveillance. Two anonymous referees are kindly acknowledged for their valuable contributions to improve the manuscript. This study was supported by grants to R.M. (Emmag Erasmus Mundus fellowship) and to G.G. and M.M. (INNOVAQUA PON02_00451_3362185).

REFERENCES

- ABBOTT I.A., 1979 — The importance of taxonomy to the utilization of marine algae. *Actas symposium sobre algas marinas Chilenas*: 51-58.
- 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 (3): 201-207.
- ARMELI MINICANTE S., GENOVESE G. & MORABITO M., 2014 — Two new alien macroalgae identified by the DNA barcoding. Due nuove macroalghe aliene identificate mediante il dna barcoding. *Biologia marina Mediterranea* 21 (1): 70-72.
- AZAZA M., MENSÍ F., KSOURI J., DHRAIEF M., BRINI B., ABDELMOULEH A. & KRAIEM M., 2008 — Growth of Nile tilapia (*Oreochromis niloticus* L.) fed with diets containing graded levels of green algae ulva meal (*Ulva rigida*) reared in geothermal waters of southern Tunisia. *Journal of applied ichthyology* 24 (2): 202-207.
- BAE H.B., 2010 — Ulotrichales, Ulvales. In: Bae E.H., Kim H.-S., Kwon C.-J., Hwang I.-K., Kim G.H. & Klochkova T.A. (eds), *Algal flora of Korea*. Volume 1, Number 1. Chlorophyta: Ulvophyceae: Ulotrichales, Ulvales, Cladophorales, Bryopsidales. Marine green algae. Incheon, National Institute of Biological Resources, pp. 7-52.

- BEN MAIZ N., 1995 — Étude nationale sur la diversité biologique de la flore marine et aquatique en Tunisie (Monographie). Projet MEAT/PNUE/GEF, Ministère de l'Environnement, Tunisie. 78 p.
- BEN MAIZ N., BOUDOURESQUE C.-F. & OUAHCHI F., 1987 — Inventaire des algues et des phanérogames marines benthiques de la Tunisie. *Giornale botanico Italiano* 121: 259-304.
- BEN MAIZ N. & SHILI A., 2007 — Les peuplements phytobenthiques du Lac Nord de Tunis. In *Proceedings of the 3rd Mediterranean Symposium on Marine Vegetation, Marseille, 27-29 March 2007*, 247-249.
- BENDIF E.M., PROBERT I., HERVE A., BILLARD C., GOUX D., LELONG C., CADORET J.-P. & VERON B., 2011 — Integrative taxonomy of the Pavlovophyceae (Haptophyta): a reassessment. *Protist* 162: 738-761.
- BENSON D.A., CAVANAUGH M., CLARK K., KARSCH-MIZRACHI I., LIPMAN D.J., OSTELL J. & SAYERS E.W., 2017 — GenBank. *Nucleic acids research* 45 (Database issue): D37-D42.
- BERGSTEN J., BILTON D.T., FUJISAWA T., ELLIOTT M., MONAGHAN M.T., BALKE M., HENDRICH L., GEIJER J., HERRMANN J., FOSTER G.N., RIBERA I., NILSSON A.N., BARRACLOUGH T.G. & VOGLER A.P., 2012 — The effect of geographical scale of sampling on DNA barcoding. *Systematic biology* 61: 851-869.
- BERTUCCIO C., GENOVESE G., MANGHISI A., CRUAUD C., COULOUX A., LE GALL L. & MORABITO M., 2013/2014 — Changes in the benthic algal flora of Lake Ganzirri, North-Eastern Sicily (Italy). Cambiamenti della flora bentonica del Lago di Ganzirri, Sicilia nord orientale (Italia). *Natura rerum* 3: 79-91.
- BITTNER L., HALARY S.B., PAYRI C., CRUAUD C., DE REVIERS B., LOPEZ P. & BAPTESTE E., 2010 — Some considerations for analyzing biodiversity using integrative metagenomics and gene networks. *Biology direct* 5 (1): 1-47.
- BLANPIED C., BUROLLET P.F., CLAIREFOND P. & SHIMI M.D., 1979a — Sédiments actuels et holocènes. *Annales de l'université de Provence, France* 6: 61-82.
- BLANPIED C., BURROLET P.F., CLAIREFOND P. & SECHIMI M., 1979b — Cadre géographique et géologique du plateau continental de la Tunisie. In *Géologie Méditerranéenne, la Mer Pélagienne. Annales de l'université de Provence, France* 6 (1): 19-22.
- BLIDING C., 1963 — *A critical survey of European taxa in Ulvales. I. Capsosiphon, Percursaria, Blidingia, Enteromorpha*. Stockholm, Almqvist & Wiksell, 160 p.
- BLIDING C., 1968 — *A critical survey of European taxa in Ulvales. II. Ulva, Ulvaria, Monostroma, Kornmannia*. *Botaniska notiser* 121: 535-629.
- BLOMSTER J., MAGGS C.A. & STANHOPE M.J., 1998 — Molecular and morphological analysis of *Enteromorpha intestinalis* and *Enteromorpha compressa* (Chlorophyta) in the British Isles. *Journal of phycology* 34: 319-340.
- BOOTH D., PROVAN J. & MAGGS C.A., 2007 — Molecular approaches to the study of invasive seaweeds. *Botanica marina* 50 385-396.
- BOUDOURESQUE C.F. & VERLAQUE M., 2002 — Biological pollution in the Mediterranean Sea: invasive versus introduced macrophytes. *Marine pollution bulletin* 44 (1): 32-38.
- BRODIE J., MAGGS C.A. & JOHN D.M. (eds.), 2007 — *Green seaweeds of Britain and Ireland*. London, British Phycological Society, [v], vi-xii, 242 p, 101 figs.
- BUTLER D., 2007 — Ancient algal mixup sorted. *Nature News* doi:10.1038/news.2007.396.
- CARLTON J.T., 1996 — Biological Invasions and Cryptogenic Species. *Ecology* 77 (6): 1653-1655.
- COAT G., DION P., NOAILLES M.-C., DE REVIERS B., FONTAINE J.-M., BERGER-PERROT Y. & LOISEAUX-DE GOËR S., 1998 — *Ulva armoricana* (Ulvales, Chlorophyta) from the coast of Brittany (France). II. Nuclear rDNA ITS sequence analysis. *European journal of phycology* 33: 81-86.
- COLL M., PIRODDI C., STEENBEEK J., KASCHNER K., BEN RAIS LASRAM F., AGUZZI J., BALLESTEROS E., BIANCHI C.N., CORBERA J., DAILIANIS T., DANOVARO R., ESTRADA M., FROGLIA C., GALIL B.S., GASOL J.M., GERTWAGEN R., GIL J., GUILHAUMON F., KESNER-REYES K., KITSOS M.-S., KOUKOURAS A., LAMPADARIOU N., LAXAMANA E., LÓPEZ-FÉ DE LA CUADRA C.M., LOTZE H.K., MARTIN D., MOUILLOT D., ORO D., RAICEVICH S., RIUS-BARILE J., SAIZ-SALINAS J.I., SAN VICENTE C., SOMOT S., TEMPLADO J., TURON X., VAFIDIS D., VILLANUEVA R. & VOULTSIADOU E., 2010 — The Biodiversity of the Mediterranean Sea: Estimates, Patterns, and Threats. *PLoS ONE* 5 (8): e11842.
- CORMACI M., FURNARI G. & ALONGI G., 2014 — Flora marina bentonica del Mediterraneo: Chlorophyta. *Bollettino accademia Gioenia di scienze naturali* 47 (377): 11-436.
- COUCEIRO L., CREMADES J. & BARREIRO R., 2011 — Evidence for multiple introductions of the Pacific green alga *Ulva australis* Areschoug (Ulvales, Chlorophyta) to the Iberian Peninsula. *Botanica marina* 54 (4): 391.

- DELNATTE C.S. & WYNNE M.J., 2016 — A revised checklist of marine algae and seagrasses of Martinique, French West Indies. *Nova Hedwigia* 103 (3-4): 415-440.
- DOYLE J.J. & DOYLE J.L., 1987 — A rapid isolation procedure for small quantities of fresh leaf tissue. *Phytochemical bulletin* 19 (1): 11-15.
- EL-SIKAILY A., NEMR A.E., KHALED A. & ABDELWEHAB O., 2007 — Removal of toxic chromium from wastewater using green alga *Ulva lactuca* and its activated carbon. *Journal of hazardous materials* 148: 216-228.
- ESWARAN K., GANESAN M., PERIYASAMY C. & RAO P.V.S., 2002 — Effect of ultraviolet-B radiation on biochemical composition of three *Ulva* species (Chlorophyta) from southeast coast of India. *Indian journal of marine sciences* 31: 334-336.
- FLAGELLA M.M., ANDREAKIS N., HIRAOKA M., VERLAQUE M. & BUIA M.C., 2010 — Identification of cryptic *Ulva* species (Chlorophyta, Ulvales) transported by ballast water. *Journal of biological research-Thessaloniki* 13: 47-57.
- GALIL B.S., MARCHINI A. & OCCHIPINTI-AMBROGI A., 2016 — East is east and West is west? Management of marine bioinvasions in the Mediterranean Sea. *Estuarine, Coastal and Shelf Science*. doi.org/10.1016/j.ecss.2015.12.021.
- GALLARDO T., BÁRBARA I., AFONSO-CARRILLO J., BERMEJO R., ALTAMIRANO M., GÓMEZ GARRETA A., BARCELÓ MARTÍ M.C., RULL LLUCH J., BALLESTEROS E. & DE LA ROSA J., 2016 — Nueva lista crítica de las algas bentónicas marinas de España – A new checklist of benthic marine algae of Spain. *Algas* 51: 7-52.
- GELLER J.B., DARLING J.A. & CARLTON J.T., 2010 — Genetic perspectives on marine biological invasions. *Annual review of marine science* 2: 367-393.
- GEOFFROY A., LE GALL L. & DESTOMBE C., 2012 — Cryptic introduction of the red alga *Polysiphonia morrowii* Harvey (Rhodomelaceae, Rhodophyta) in the North Atlantic Ocean highlighted by a DNA barcoding approach. *Aquatic botany* 100: 67-71.
- GOUY M., GUINDON S. & GASCUEL O., 2010 — SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular biology and evolution* 27 (2): 221-224.
- GUIRY M.D. & GUIRY G.M., 2017 — *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. <http://www.algaebase.org/>; searched on 16 May 2017.
- HAMSHER S.E., EVANS K.M., MANN D.G., POULICKOVA A. & SAUNDERS G.W., 2011 — Barcoding Diatoms: Exploring Alternatives to COI-5P. *Protist* 162 (3): 405-422.
- HANYUDA T., HEESCH S., NELSON W., SUTHERLAND J., ARAI S., BOO S.M. & KAWAI H., 2016 — Genetic diversity and biogeography of native and introduced populations of *Ulva pertusa* (Ulvales, Chlorophyta). *Phycological research* 64 (2): 102-109.
- 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. *European Journal of phycology* 38 (3): 277-294.
- HEBERT P.D.N., STOECKLE M.Y., ZEMLAK T.S. & FRANCIS C.M., 2004 — Identification of Birds through DNA Barcodes. *PLOS Biology* 2 (10): e312.
- HEESCH S., BROOM J.E.S., NEILL K.F., FARR T.J., DALEN J.L. & NELSON W.A., 2009 — *Ulva*, *Umbraulva* and *Gemina*: genetic survey of New Zealand taxa reveals diversity and introduced species. *European Journal of Phycology* 44 (2): 143-154.
- HIRAOKA M., ICHIHARA K., ZHU W., SHIMADA S., OKA N., CUI J., TSUBAKI S. & HE P., 2017 — Examination of species delimitation of ambiguous DNA-based *Ulva* (Ulvophyceae, Chlorophyta) clades by culturing and hybridisation. *Phycologia* 56 (5): 517-532.
- HIRAOKA M., SHIMADA S., UENOSONO M. & MASUDA M., 2003 — A new green-tide-forming alga, *Ulva ohnoi* Hiraoka et Shimada sp. nov. (Ulvales, Ulvophyceae) from Japan. *Phycological research* 51 (1): 17-29.
- HOEF-EMDEN K., 2007 — Revision of the genus *Cryptomonas* (Cryptophyceae) II: incongruences between the classical morpho-species concept and molecular phylogeny in smaller pyrenoidless cells. *Phycologia* 46: 402-428.
- HOFMANN L.C., NETTLETON J.C., NEEFUS C.D. & MATHIESON A.C., 2010 — Cryptic diversity of *Ulva* (Ulvales, Chlorophyta) in the Great Bay Estuarine System (Atlantic USA): introduced and indigenous distromatic species. *European journal of phycology* 45 (3): 230-239.
- HOLDEN C., 2007 — Sea scum rebranded. *Science* 318: 1839.
- KANG E.J., KIM J.-H., KIM K., CHOI H.-G. & KIM K.Y., 2014 — Re-evaluation of green tide-forming species in the Yellow Sea. *Algae* 29 (4): 267.
- KAZI M.A., KAVALE M.G. & SINGH V.V., 2016 — Morphological and molecular characterization of *Ulva chaugulii* sp. nov., *U. lactuca* and *U. ohnoi* (Ulvophyceae, Chlorophyta) from India. *Phycologia* 55 (1): 45-54.

- KIMURA M., 1980 — A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of molecular evolution* 16: 111-120.
- KIRKENDALE L., SAUNDERS G.W. & WINBERG P., 2013 — A Molecular Survey of *Ulva* (Chlorophyta) in Temperate Australia Reveals Enhanced Levels of Cosmopolitanism. *Journal of phycology* 49 (1): 69-81.
- KJELLMAN F.R., 1897 — Marina chlorophyceer från Japan. *Bihang til Kongliga Svenska Vetenskaps-Akademiens Handlingar, Afd. III* 23(11): 1-44, 7 figs, 7 plates.
- KOEMAN R.P.T. & VAN DEN HOEK C., 1982a — The taxonomy of *Enteromorpha* Link, 1820, (Chlorophyceae) in the Netherlands. I. The section *Enteromorpha*. *Archiv für hydrobiologie (Algological studies* 32) 63: 279-330.
- KOEMAN R.P.T. & VAN DEN HOEK C., 1982b — The taxonomy of *Enteromorpha* Link, 1820, (Chlorophyceae) in the Netherlands. II. The section *Proliferae*. *Cryptogamie, Algologie* 3: 37-70.
- KOEMAN R.P.T. & VAN DEN HOEK C., 1984 — The taxonomy of *Enteromorpha* Link, 1820, (Chlorophyceae) in the Netherlands. III. The sections *Flexuosae* and *Clathratae* and an addition to the section *Proliferae*. *Cryptogamie, Algologie* 5 (1): 21-61.
- KOGAME K., UWAI S., ANDERSON R.J., CHOI H.G. & BOLTON J.J., 2016 — DNA barcoding of South African geniculate coralline red algae (Corallinales, Rhodophyta). *South African journal of botany*.
- KOLSI R., FRIKHA D., JRIBI I., HAMZA A., FEKIH L. & BELGITH K., 2015 — Screening of antibacterial and antifungal activity in marine macroalgae and magnoliophyta from the coast of Tunisia. *International journal of pharmacy and pharmaceutical sciences* 7 (3): 47-51.
- KOZHENKOVA S., CHERNOVA E. & SHULKIN V., 2006 — Microelement composition of the green alga *Ulva fenestrata* from Peter the Great Bay, Sea of Japan. *Russian journal of marine biology* 32: 289-296.
- 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. *Journal of phycology* 46 (6): 1257-1277.
- LAM D.W., ENTWISLE T.J., ELORANTA P., KWANDRANS J. & VIS M.L., 2012 — Circumscription of species in the genus *Sirodotia* (Batrachospermales, Rhodophyta) based on molecular and morphological data. *European journal of phycology* 47 (1): 42-50.
- LE GALL L., DELSUC F., HOURDEZ S., LECOINTRE G. & RASPLUS J.-Y., 2017 — Toward the DNA Library of Life. *European Journal of Taxonomy*. doi: 10.5852/ejt.2017.266
- LELIAERT F., VERBRUGGEN H., VANORMELINGEN P., STEEN F., LÓPEZ-BAUTISTA J.M., ZUCCARELLO G.C. & DE CLERCK O., 2014 — DNA-based species delimitation in algae. *European journal of phycology* 49 (2): 179-196.
- LELIAERT F., ZHANG X., YE N., MALTA E.Á., ENGELEN A.H., MINEUR F.D.R., VERBRUGGEN H. & DE CLERCK O., 2009 — Research note: identity of the Qingdao algal bloom. *Phycological research* 57 (2): 147-151.
- LINNAEUS C., 1753 — *Species plantarum, exhibentes plantas rite cognitatas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas. Vol. 1* Holmiae [Stockholm], Impensis Laurentii Salvii, [12], 560 p.
- LOUGHNANE C.J., MCIVOR L.M., RINDI F., STENGEL D.B. & GUIRY M.D., 2008 — Morphology, *rbcL* phylogeny and distribution of distromatic *Ulva* (Ulvophyceae, Chlorophyta) in Ireland and southern Britain. *Phycologia* 47 (4): 416-429.
- MANGHISI A., ARMELI MINICANTE S., BERTUCCIO C., MORABITO M., TORRICELLI P. & GENOVESE G., 2011 — A cryptic alien seaweed spreading in Mediterranean coastal lagoons. *Transitional waters bulletin* 5 (1): 1-7.
- MANGHISI A., BERTUCCIO C., ARMELI MINICANTE S., FIORE V., LE GALL L., GENOVESE G. & MORABITO M., 2011 — Identifying alien macroalgae through DNA barcoding: the case of *Hypnea cornuta* (Cystocloniaceae, Rhodophyta). *Transitional waters bulletin* 5 (1): 42-49.
- MAREŠ J., LESKINEN E., SITKOWSKA M., SKÁČELOVÁ O. & BLOMSTER J., 2011 — True identity of the European freshwater *Ulva* (Chlorophyta, Ulvophyceae) revealed by a combined molecular and morphological approach. *Journal of phycology* 47 (5): 1177-1192.
- MARSHALL K., JOINT I., CALLOW M.E. & CALLOW J.A., 2006 — Effect of marine bacterial isolates on the growth and morphology of axenic plantlets of the green alga *Ulva linza*. *Microbial ecology* 52 (2): 302-310.
- MELTON J.T.I., COLLADO-VIDES L. & LOPEZ-BAUTISTA J.M., 2016a — Molecular identification and nutrient analysis of the green tide species *Ulva ohnoi* M. Hiraoka & S. Shimada, 2004 (Ulvophyceae, Chlorophyta), a new report and likely nonnative species in the Gulf of Mexico and Atlantic Florida, USA. *Aquatic invasions* 11 (3): 225-237.

- MELTON J.T.I., GARCÍA-SOTO G.C. & LÓPEZ-BAUTISTA J.M., 2016b — A new record of the bloom-forming green algal species *Ulva ohnoi* (Ulvales, Chlorophyta) in the Caribbean Sea. *Algas* 51: 62-64.
- MEYER C.P. & PAULAY G., 2005 — DNA barcoding: error rates based on comprehensive sampling. *PLOS Biology* 3 (12): e422.
- MICHAEL T.S., 2009 — Glycoconjugate organization of *Enteromorpha* (= *Ulva*) *flexuosa* and *Ulva fasciata* (Chlorophyta) zoospores. *Journal of phycology* 45: 660-677.
- MILSTEIN D. & SAUNDERS G.W., 2012 — DNA barcoding of Canadian Ahnfeltiales (Rhodophyta) reveals a new species – *Ahnfeltia borealis* sp. nov. *Phycologia* 51: 247-259.
- MINEUR F., JOHNSON M.P., MAGGS C.A. & STEGENGA H., 2007 — Hull fouling on commercial ships as a vector of macroalgal introduction. *Marine biology* 151 (4): 1299-1307.
- MCMANUS H.A. & LEWIS L.A., 2011 — Molecular phylogenetic relationships in the freshwater family Hydrodictyaceae (Sphaeropleales, Chlorophyceae), with an emphasis on *Pediastrum duplex*. *Journal of phycology* 47: 152-163.
- NEUSTUPA J., ŠTASTNÝ J., NEMJOVÁ K., MAZALOVÁ P., GOODYER E., POULÍČKOVÁ A. & ŠKALOUD P., 2011 — A novel, combined approach to assessing species delimitation and biogeography within the well-known desmid species *Microsterias fimbriata* and *M. rotata* (Desmidiales, Steptophyta). *Hydrobiologia*, 667: 223-239.
- NI-NI-WIN, HANYUDA, T., DRAISMA, S.G.A., FURNARI, G., MEINESZ, A. & KAWAI, H., 2011 — *Padina ditristomatica* sp. nov. and *Padina pavonicoides* sp. nov. (Dictyotales, Phaeophyceae), two new species from the Mediterranean Sea based on morphological and molecular markers. *European Journal of phycology* 46: 327-341.
- O'KELLY C.J., KURIHARA A., SHIPLEY T.C. & SHERWOOD A.R., 2010 — Molecular assessment of *Ulva* spp. (Ulvophyceae, Chlorophyta) in the Hawaiian Islands. *Journal of phycology* 46: 728-735.
- PAPENFUSS G.F., 1960 — On the genera of the Ulvales and the status of the order. *Journal of the Linnean society. Botany* 56: 303-318.
- PAULERT R., EBBINGHAUS D., URLASS C. & MOERSCHBACHER B.M., 2010 — Priming of the oxidative burst in rice and wheat cell cultures by ulvan, a polysaccharide from green macroalgae, and enhanced resistance against powdery mildew in wheat and barley plants. *Plant pathology* 59: 634-642.
- PERGENT G. & KEMPF M., 1993 — *L'environnement marin côtier en Tunisie. (1) Rapport de synthèse*. Brest, France, Rapport IFREMER DEL Brest 92.06, 55 p.
- PHILLIPS J.A., 1988 — Field, anatomical and developmental studies on southern australian species of *Ulva* (Ulvaceae, Chlorophyta). *Australian systematic botany* 1: 411-456.
- PIRIAN K., PIRI K., SOHRABIPOUR J., TAMADONI JAHROMI S. & BLOMSTER J., 2016 — Molecular and morphological characterisation of *Ulva chaugulii*, *U. paschima* and *U. ohnoi* (Ulvophyceae) from the Persian Gulf, Iran. *Botanica marina* 59 (2-3): 147-158.
- PRÖSCHOLD T., MARIN B., SCHLOSSER U.G. & MELKONIAN M., 2001 — Molecular phylogeny and taxonomic revision of *Chlamydomonas* (Chlorophyta). I. Emendation of *Chlamydomonas* Ehrenberg and *Chloromonas* Gobi, and description of *Oogamochlamys* gen. nov. and *Lobochlamys* gen. nov. *Protist* 152: 265-300.
- PROVASOLI L. & PINTNER I.J., 1980 — Bacteria induced polymorphism in an axenic laboratory strain of *Ulva lactuca* (Chlorophyceae). *Journal of phycology* 16 (2): 196-201.
- 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.
- SAUNDERS G.W., 2005 — Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. *Philosophical transactions of the Royal Society of London, Series B: Biological sciences* 360 (1462): 1879-1888.
- SAUNDERS G.W. & KUCERA H., 2010 — An evaluation of *rbcL*, *tufA*, *UPA*, *LSU* and *ITS* as DNA barcode markers for the marine green macroalgae. *Cryptogamie, Algologie* 31 (4): 487-528.
- SAUNDERS G.W. & MCDEVIT D.C., 2012 — Methods for DNA Barcoding Photosynthetic Protists Emphasizing the Macroalgae and Diatoms DNA Barcodes. In: Kress W.J. & Erickson D.L. (eds.), *DNA Barcodes: Methods and protocols*, Humana Press, pp. 207-222.
- SCHAFFELKE B., SMITH J.E. & HEWITT C.L., 2006 — Introduced macroalgae — A growing concern. *Journal of applied phycology* 18: 529-541.
- SFRISO A., 2010 — Coexistence of *Ulva rigida* and *Ulva laetevirens* (Ulvales, Chlorophyta) in Venice Lagoon and other Italian transitional and marine environments. *Botanica marina* 53: 9-18 (erratum: 193).

- SGHAIER Y.R., ZAKHAMA-SRAIEB R., MOUELI S., VAZQUEZ M., VALLE C., RAMOS-ESPLA A.A., ASTIER J.M., M. V. & CHARFI-CHEIKHROUHA F., 2016 — Review of alien marine macrophytes in Tunisia. *Mediterranean marine science* 171 (1): 109-123.
- SHILI A., MAIZ N.B. & BOUDOURESQUE C., 2007 — Diversité des peuplements phytobenthiques dans les lagunes du Cap Bon (Nord-Est de la Tunisie). In *Proceedings of the 3rd Mediterranean Symposium on Marine Vegetation, Marseille, 27-29 March 2007*, 79-184.
- SILVA P.C., BASSON P.W. & MOE R.L., 1996 — *Catalogue of the benthic marine algae of the Indian Ocean*. Berkeley, University of California Press, XIV+1259 p.
- ŠKALOUD P. & RINDI S., 2013 — Ecological differentiation of cryptic species within an asexual protist morphospecies: a case study of filamentous green alga *Klebsormidium* (Streptophyta). *Journal of eukaryotic microbiology* 60: 350-362.
- STEGENGA H., KARREMANS M. & SIMONS J., 2007 — Zeewieren van de voormalige oesterputten bij Yerseke. *Gorteria* 32: 125-143.
- SWOFFORD D.L., 2002 — PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). In Sinauer Associates, Sunderland, Massachusetts.
- TAN I.H., BLOOMSTER J., HANSEN G., LESKINEN E., MAGGS C.A., MANN D.G., SLUIMAN H.J. & STANHOPE M.J., 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 (8): 1011-1018.
- VAN ALSTYNE K., KOELLERMEIER L. & NELSON T., 2007 — Spatial variation in dimethylsulfoniopropionate (DMSP) production in *Ulva lactuca* (Chlorophyta) from the Northeast Pacific. *Marine biology* 150: 1127-1135.
- VANORMELINGEN P., HEGEWALD E., BRABAND A., KITSCHKE M., FRIEDL T., SABBE K. & VYVERMAN W., 2007 — The systematics of a small spineless *Desmodesmus* species, *D. constato-granulatus* (Sphaeropleales, Chlorophyceae), based on ITS2 rDNA sequence analyses and cell wall morphology. *Journal of phycology* 43: 378-396.
- VERLAQUE M., BELSHER T. & DESLOUS-PAOLI J.M., 2002 — Morphology and reproduction of Asiatic *Ulva pertusa* (Ulvales, Chlorophyta) in Thau Lagoon (France, Mediterranean Sea). *Cryptogamie, Algologie* 23 (4): 301-310.
- WALKER R.H., BRODIE J., RUSSELL S., IRVINE L.M. & ORFANIDIS S., 2009 — Biodiversity of coralline algae in the northeastern Atlantic including *Corallina caespitosa* sp. nov. (Corallinoideae, Rhodophyta). *Journal of phycology* 45: 287-297.
- WINBERG P., SKROPETA D. & ULLRICH A., 2011 — Seaweed cultivation pilot trials: towards culture systems and marketable products. Rural Industries Research & Development Corporation Technical Report. Canberra. PRJ-000162.
- WOLF M.A., SCIUTO K., ANDREOLI C. & MORO I., 2012 — *Ulva* (Chlorophyta, Ulvales) Biodiversity in the North Adriatic Sea (Mediterranean, Italy): Cryptic Species and New Introductions. *Journal of Phycology* 48(6): 1510-1521.
- WOMERSLEY H.B.S., 1984 — *The marine benthic flora of southern Australia. Part I*. Canberra, Australia, D.J. Woolman, 329 p.
- XIA B.M. & ABBOTT I.A., 1987 — Edible seaweeds of China and their place in the Chinese diet. *Economic botany* 41 (3): 341-353.
- YAICH H., GARNA H., BESBES S., PAQUOT M., BLECKER C. & ATTIA H., 2011 — Chemical composition and functional properties of *Ulva lactuca* seaweed collected in Tunisia. *Food chemistry* 128 (4): 895-901.
- ZENETOS A., ÇINAR M.E., CROCETTA F., GOLANI D., ROSSO A., SERVELLO G., SHENKAR N., TURON X. & VERLAQUE M., 2017 — Uncertainties and validation of alien species catalogues: The Mediterranean as an example. *Estuarine, coastal and shelf science*. 191: 171-187.
- ZIMMERMANN J., JAHN R. & GEMEINHOLZER B., 2011 — Barcoding diatoms: evaluation of the V4 subregion on the 18S rRNA gene, including new primers and protocols. *Organisms diversity and evolution* 11: 173-192.