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Native, invasive and cryptogenic *Ulva* species from the Israeli Mediterranean Sea: risk and potential

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Abstract

The genus *Ulva* (Chlorophyta) is ubiquitous along Israeli Mediterranean shores where it has been studied extensively due to its important ecological role and potential value in biotechnology and aquaculture. Previous identifications of *Ulva* in Israel were based only on morphology. Here, we compare species found in 2002 and in 2014-2016. Analyses of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) and elongation factor 1-alpha (*tuf*A) plastid genes (2014-2016 samples only), combined with morphological data, identified six *Ulva* species, three of which are new records for Israel and probably originate from the Indo-Pacific. *Ulva compressa*, rarely found in 2002, is now the most abundant species and exhibits two fairly distinct morphologies correlated with different haplotypes for both genes. *Ulva fasciata* was found more commonly in 2002 than in 2014-16, whereas the morphologically similar, and closely related, invasive species *U. ohnoi* seemed more frequent in recent samples. The finely branched tubular *Ulva tepida* was found in 2002 and 2015/16, and *U. chaugulii* and *Ulva aragoensis* (Bliding) Maggs, comb. nov. were discovered for the first time in 2015/16. The changing *Ulva* flora of the Israeli Mediterranean may be correlated with major environmental changes including 3°C increase in sea surface temperatures over the last two decades, as well as a generally increasing prevalence of non-native species. The local *Ulva* species now found in Israel could be of value for various industrial uses.

Keywords: Molecular taxonomy, cryptogenic species, invasive species, *rbc*L, *tuf*A, seaweeds.

Introduction

Ulva (Chlorophyta) is a common marine macroalgal genus with a worldwide distribution in marine and some freshwater environments (Mares *et al*., 2011). It is well-known for forming free-floating "green tides" such as the blooms of *U. prolifera* that appear annually in the Yellow Sea and disrupted the 2008 Beijing Olympics sailing events (Li *et al*., 2016). *Ulva* provides services for the food industry (Rouxel *et al*., 2001) and is used in aquaculture of fish (Azaza *et al*., 2008) and marine invertebrates such as shrimps and abalone (Cruz-Suarez *et al*., 2010; Brito *et al*., 2013; Viera *et al*., 2016). Due to their high photosynthetic and growth rates (Longstaff *et al*., 2002; Figueroa *et al*., 2009), *Ulva* species are used as biofilters in integrated multi-trophic aquaculture systems to reduce organic loads from fish effluents (Bartoli *et al.,* 2005; Korzen *et al*., 2016). Recently, in view of its high carbohydrate content (ca. 40% on a dry weight basis), *Ulva* biomass has been suggested as a potential source for bioethanol production (Singh & Gu, 2010; Trivedi *et al*., 2013; Korzen *et al*., 2015; Shefer *et al*., 2017).

Ulva species from Israel have been the subject of extensive physiological research (e.g. Beer *et al*., 1990; Israel & Hophy, 2002; Beer *et al*., 2008). Along Israeli Mediterranean Sea shores, *Ulva* species are mostly seasonal in the tidal zone of abrasion platforms (supplementary fig. 1). Two distinctive growth periods, one in autumn from approximately October to December and another during springtime in March to May, are related to irradiance, photoperiod and seawater temperatures (Einav & Israel, 2008). Elsewhere, the species composition of *Ulva* populations has been shown to vary temporally as well as spatially (Ogawa *et al*., 2013). Ten species of *Ulva* (including tubular forms previously classified as *Enteromorpha*; Hayden *et al.,* 2003) were included in the most recent checklist for Israeli coasts (Einav & Israel, 2008): *U. clathrata*, *U. compressa*, *U. fasciata*, *U. flexuosa*, *U. intestinalis*, *U. lactuca*, *U. laetevirens*, *U. linza*, *U. prolifera* and *U. rigida*. Studies in the Eastern Mediterranean have added *U. ohnoi* to the list of Israeli species (Awad, 2000; Zenetos *et al*., 2005; Einav, 2007; Flagella *et al*., 2009). However, despite the high recorded diversity of *Ulva* species in Israel, as yet no molecular identifications have been reported.

Ulva species exhibit wide phenotypic plasticity and rapid morphological changes in the natural environment so species identification is particularly difficult (Coat *et al*., 1998; Malta *et al*., 1999; Blomster *et al*., 2002; Kraft *et al*., 2010; Mares *et al*., 2011; Kirkendale *et al*., 2013; Pirian *et al*., 2016). A range of molecular markers has been used in the molecular systematics of this genus: initially the nuclear ribosomal spacer region ITS2 was favoured as it has appropriate levels of divergence (Blomster *et al*., 2002); addition of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbc*L) gene sequences gave a better picture of phylogeny and systematics (Hayden *et al*., 2003). More recently, other genetic markers such as elongation factor 1-alpha (*tuf*A) have been widely used for molecular-assisted identification in green algae (Guidone & Thornber, 2013). However, the identity of the species from which many published sequences were obtained is problematic (Kirkendale *et al*., 2013).

We studied the distribution of *Ulva* species on Israeli Mediterranean coasts in May and September 2002, and repeated the study in 2014-2016, to establish which species were present at those times and to determine whether changes in species composition had occurred over this time period. These decades have been a time of rapid environmental change in Israel, particularly rising sea temperatures (Gertman *et al*., 2013; Shaltout & Omstedt, 2014; Raveh *et al*., 2015; Ozer *et al*., 2016) combined with local effects of power and desalination plants (Titelboim *et al*., 2016). Also, new aliens are reported from the Mediterranean with increasing frequency (Flagella *et al.,* 2009; Wolf *et al*., 2012; Katsanevakis & Crocetta, 2014; Aragay *et al*., 2016). More than 115 introduced benthic algae and seagrass taxa have been recorded in the Mediterranean (Mineur *et al*., 2015), mainly transferred through the Suez Canal (known as "Lessepsian invaders"; Katsanevakis & Crocetta, 2014), with ballast water, water currents and other organisms identified as vectors of transportation. Lessepsian invaders have had a major impact on benthic communities in the eastern Mediterranean (e.g. Goren *et al*., 2016). There are 86 seaweeds currently regarded as alien on Israeli Mediterranean Sea shores (Israel & Einav, 2017) and new ones are detected regularly (Hoffman & Wynne, 2016). Climate change and rising water temperatures have been linked with increasing numbers of aliens in the Bay of Biscay (Díez *et al*., 2012).

Consequently, the aims of this study were to verify the taxonomic identity of *Ulva* species along the Israeli coastline in two discrete time periods, using multiple samples to investigate morphological variation. We used two molecular markers to reduce the problems in linking conspecific samples that result from non-standardization of molecular markers in green algae.

Materials and Methods

Sampling

Ulva samples were collected in the intertidal zone along the coastline of the Israeli Mediterranean Sea when they were most obvious in 2002 and again in 2014-2016 (Fig. 1). During each period, sampling was carried out when *Ulva* spp. appeared to be most abundant. In 2002, samples were collected in February, May and August–September; sampling in 2014-16 was in November to December 2014 and then in December 2015 to January 2016. Sampling was carried out on intertidal rock abrasion platforms at low tide (the tidal range is ca. 40 cm; supplementary Fig. 1). All morphological variation exhibited by *Ulva* thalli at all sites was sampled on each occasion.

In 2002, 5-10 morphologically distinct thalli were collected at each site, if available, including tubular *Enteromorpha*-type thalli. In May and August 2002 samples were obtained at Achziv (33.043111˚ N, 35.099028˚ E), and Michmoret (32.403778˚ N, 34.866306˚ E). In September 2002, sampling was at Michmoret and Tel-Baruch, north of the beach (32.1169˚ N, 34.780278˚ E). Samples were transported alive to Belfast and processed there. To establish the relationships of *Ulva* species reported from Israel (Einav & Israel, 2008) with those originally described from the British Isles (*U. compressa, U. intestinalis*, *U. linza*) or common in Atlantic Europe (*U. lactuca*) comparative field collections of these species, all of which have been extensively researched in the British Isles (Brodie *et al*., 2007), were made in Ireland and sequences were obtained from them (Table 1).

In November 2014 new exploratory collections were made at Michmoret beach, Palmachim beach (31.930111˚ N, 34.69825˚ E) and Herzliya beach (32.139612˚ N, 34.789362˚ E). General sampling of *Ulva* populations was carried out to determine the range of morphologies present and to collect material for molecular identification. In December 2015–January 2016 collections were made again at Michmoret, Palmachim, Herzliya and at four additional field sites: Rosh Hanikra (33.092278˚ N, 35.105194˚ E), Achziv beach, Shikmona marine reserve in Haifa Bay (32.826056˚ N, 34.956˚ E) and Habonim beach (32.630194˚ N, 34.920444˚ E) (Fig. 1). At each site, samples were collected approximately every 1 m along a 150 m shore transect, where all *Ulva* thalli were removed, bagged by station, and taken back to the laboratory in Haifa for sorting.

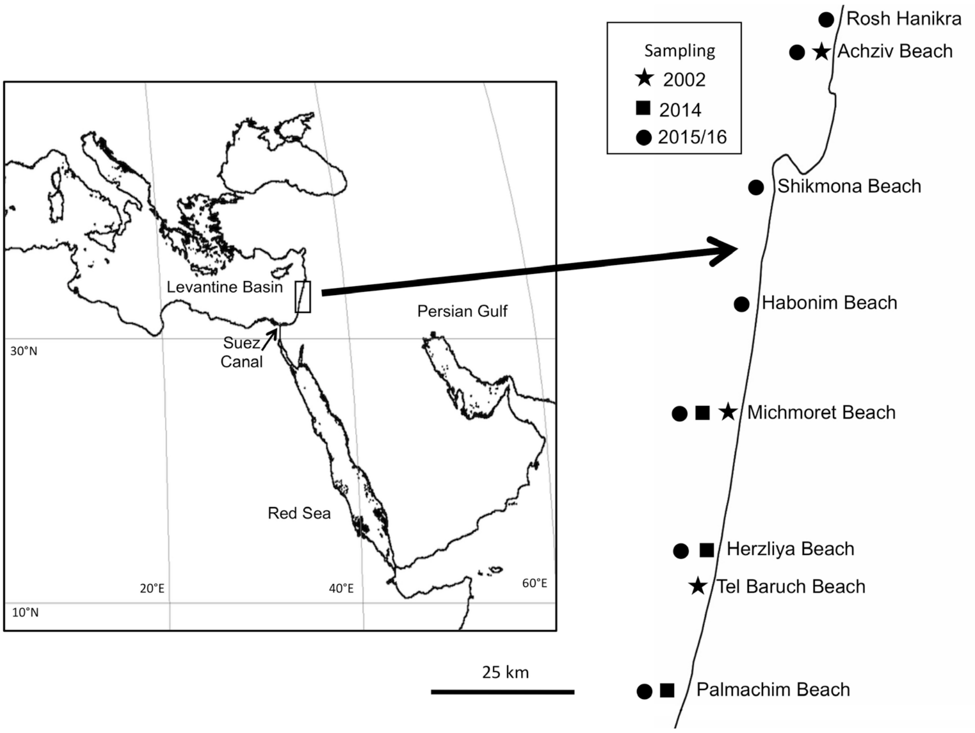

Fig. 1: Sampling sites for *Ulva* species along the Israeli Mediterranean coast in 2002, October 2014 and December 2015-January 2016.

Table 1. Collections of samples sequenced in this study (mostly representing multiple individual samples; see Table 2). Accession numbers of sequences obtained from GenBank are provided in Figs 2 and 3. No samples of *Ulva chaugulii* or *Ulva aragoensis* are available as these two species could be separated from common species only after sequencing.

Species	Sample code	Collection site	Lat/long	Collector	Date	Genbank code (*rbc*L)	Genbank code (*tuf*A)	Herbarium accession
Ulva aragoensis (Bliding) Maggs comb. nov.	HER 2 TC	Herzliya, Israel	32.139612˚*N*, 34.789362˚*E*	N. Krupnik	Dec 2015/ Jan 2016	MG704815	MG976875	No samples
Ulva chaugulii M.G.Kavale & M.A.Kazi	HER 1 TD	Herzliya, Israel		N. Krupnik	Dec 2015/ Jan 2016	MG704805	MG976863	No samples
Ulva chaugulii	HER 9 TH	Herzliya		N. Krupnik	Dec 2015/ Jan 2016	MG704804	MG976862	No samples
Ulva compressa Linnaeus	ACH 3 TC	Achziv, Israel	33.043111˚*N*, 35.099028˚*E*	N. Krupnik	Dec 2015/ Jan 2016	MG704825	MG976867	
Ulva compressa	HAB 2 TC	Habonim, Israel	32.630194˚N, 34.920444˚E	N. Krupnik	Dec 2015/ Jan 2016	MG704823	MG976868	
Ulva compressa	G	Portavogie, N. Ireland	54.4618, -5.438306	N. Carmel	14 Jun 2002	MG704814	–	IOLR-N277
Ulva compressa	16	Achziv (North)		A. Israel, N. Carmel	2 May 2002	MG704803	–	
Ulva compressa	ROS 3 TD	Rosh Hanikra, Israel	33.092278˚*N*, 35.105194˚E	N. Krupnik	Dec 2015/ Jan 2016	–	MG976869	
Ulva compressa	HER 6 TF	Herzliya		N. Krupnik	Dec 2015/ Jan 2016	MG704811	–	
Ulva compressa	HER 10 TH	Herzliya		N. Krupnik	Dec 2015/ Jan 2016	MG704824	MG976874	

(continued)

Table 1. continued

Ulva compressa	PAL 2 TA	Palmachim, Israel	31.930111˚*N*, 34.69825˚*E*	N. Krupnik	Dec 2015/ Jan 2016	–	MG976866	
Ulva compressa	ACH 4 TA	Achziv	33.043111˚*N*, 35.099028˚*E*	N. Krupnik	Dec 2015/ Jan 2016	–	MG976855	
Ulva compressa morph 1 (*rbc*L haplotype 1 and *tuf*A haplotype 1)	SHI 1 TA	Shikmona	32.826056˚*N*, 34.956˚*E*	N. Krupnik	Dec 2015/ Jan 2016	MG704813	BoLD BIM489-16	IOLR-GA00653
Ulva compressa morph 2 (*rbc*L haplotype 2 and tufA haplotype 2)	HER 1 TH	Herzliya		N. Krupnik	Dec 2015/ Jan 2016	MG704812	BoLD BIM490-16	IOLR-GA00660
Ulva fasciata Delile	Sample D	Aquaculture facility, Haifa		Y. Bron-fman	Jun 2015	MG704807	–	
Ulva fasciata	Sample E	Aquaculture facility, Haifa		Y. Bron-fman	Jun 2015	MG704808		
Ulva fasciata	MIC 5 TF	Michmoret, Israel	32.403778˚*N*, 34.866306˚*E*	N. Krupnik	Dec 2015/ Jan 2016	MG704806	MG976861	
Ulva fasciata	10	Achziv	33.043111˚*N*, 35.099028˚*E*	A. Israel, N. Carmel	02 05 2002	MG704801	–	
Ulva fasciata	11	Achziv		A. Israel, N. Carmel	02 05 2002	MG704802	–	BM001216503
Ulva fasciata	12	Achziv		A. Israel, N. Carmel	02 05 2002	MG704803	–	BM001216504
Ulva fasciata	Mik2	Michmoret		A. Israel, N. Carmel	09/09/2002	MG704829	–	BM001216501
Ulva lactuca Linnaeus	F	Portavogie, N. Ireland		N. Carmel	30 Jun 2002	MG704797	–	IOLR-N276
Ulva linza Linnaeus	CAM1057	Portaferry, Co. Down, N. Ireland	54.381958˚*N*, 5.550219˚*W*	F. Mineur	2 May 2002	MG704800	–	IOLR-N278
Ulva ohnoi Hiraoka & Shimada	C	Aquaculture facility, Haifa		Y. Bron-fman	Jun 2015	MG704809	–	
Ulva ohnoi	HER 2 TG	Herzliya		N. Krupnik	Dec 2015/ Jan 2016	MG704817	MG976876	
Ulva ohnoi	MIC 2 TF	Michmoret		N. Krupnik	Dec 2015/ Jan 2016	MG704826	MG976877	
Ulva ohnoi	PAL 5 TF	Palmachim, Israel		N. Krupnik	Dec 2015/ Jan 2016	–	MG976878	
Ulva ohnoi	PAL 4 TF	Palmachim		N. Krupnik	Dec 2015/ Jan 2016	MG704810	MG976879	
Ulva ohnoi	27	Achziv		A. Israel	1 Aug 2002	MG704795	–	BM001216505
Ulva ohnoi	28	Achziv		A. Israel	1 Aug 2002	MG704831	–	BM001216506
Ulva ohnoi	9	Achziv North		A. Israel, N. Carmel	2 May 2002	MG704833	–	
Ulva ohnoi	Mik1	Michmoret		A. Israel, N. Carmel	09/09/2002	MG704835		BM001216507
Ulva ohnoi	Mik3	Michmoret		A. Israel, N. Carmel	09/09/2002	MG704827		BM001216508
Ulva ohnoi?	Mik4	Michmoret		A. Israel, N. Carmel	09/09/2002	MG704828		BM001216502
Ulva ohnoi	Mik5	Michmoret		A. Israel, N. Carmel	09/09/2002	MG704830		BM001216509
Ulva ohnoi	MIC 4 TF	Michmoret		N. Krupnik	Dec 2015/ Jan 2016	MG704818	MG976858	

(continued)

Table 1. continued

Ulva ohnoi	HER 1 TG	Herzliya		N. Krupnik	Dec 2015/ Jan 2016	–	MG976860	
Ulva ohnoi	HER 4 TF	Herzliya		N. Krupnik	Dec 2015/ Jan 2016	–	MG976859	
Ulva ohnoi	MIC 3 TF	Michmoret		N. Krupnik	Dec 2015/ Jan 2016	MG704816	MG976880	
Ulva ohnoi *rbc*L haplotype A	HER 8 TG*	Herzliya		N. Krupnik	Dec 2015/ Jan 2016	MG704819	BIM488-16	IOLR-GA00610
Ulva ohnoi *rbc*L haplotype B	HER 3 TF	Herzliya		N. Krupnik	Dec 2015/ Jan 2016	MG704821	MG976881	
*Ulva ohnoi tuf*A haplotype 1	HER 5 TG*	Herzliya		N. Krupnik	Dec 2015/ Jan 2016	–	MG976857	
Ulva tepida Masakiyo & S.Shimada	MIC 3 TA	Michmoret		N. Krupnik	Dec 2015/ Jan 2016	MG704820	MG976864	
Ulva tepida	MIC 4 TA	Michmoret		N. Krupnik	Dec 2015/ Jan 2016	MG704822	MG976872	
Ulva tepida	15	Achziv North		A. Israel, N. Carmel	2 May 2002	MG704799	–	IOLR-N280
Ulva tepida	Mik6	Michmoret		A. Israel, N. Carmel	9 Sep 2002	MG704834		BM001216500
Umbraulva dangeardii M.J.Wynne & G.Furnari	CAM1062	Hayling Is., England	50.799067° *N*, 0.938272° *W*	C. Maggs	5 May 2002	MG704796	–	IOLR-N279

Morphological description

Field-collected samples were initially sorted based on morphological parameters such as thallus shape, margin and colour (Coat *et al*., 1998; Shimada *et al*., 2003; Leskinen *et al*., 2004; Loughnane *et al*., 2008; Wolf *et al*., 2012; Guidone & Thornber, 2013). Herbarium voucher specimens were prepared from specimens sorted in the laboratory. In Belfast, whole tissue mounts were made displaying cells in surface view, and sections made with a cryostat microtome (Leica Microsystems, UK) were stained with 10% aniline blue, post-fixed in 1% HCl and mounted in 10% Karo high fructose corn syrup (ACH Food Co. Inc., USA). Cells were measured with a graticule, and drawn using a camera lucida. In Israel in 2014-2016, whole thalli were photographed (Leica FX300, Wetzlar, Germany) and cross and longitudinal sections prepared (Cryostat CM1850 microtome, Leica, Wetzlar, Germany) for light microscopy with an Olympus BX50 upright microscope (Olympus, Hamburg, Germany) equipped with a XC30 Color View camera (Hamburg, Germany) and a Soft Imaging System program (Munster, Germany)

DNA extraction

In Belfast, DNA extraction used the DNeasy Plant Mini Kit (Qiagen UK). In Haifa, DNA extractions followed Coat *et al*.'s (1998) CTAB protocols. About 1 cm^2 fresh *Ulva* thallus was placed in an Eppendorf tube containing 500 µl of a CTAB solution (0.1M Tris HCl, 0.05 M EDTA, 1.5 M NaCl, 0.05 M DTT and 2% CTAB) and milled using a Tissuelyzer '2' (Retsch, Haan, Germany) at 30 Hertz for 30 s. Then, 500 µl of chloroform:isoamyl alcohol 24:1 (V/V) were added and centrifuged at 7000 *g* for 30 min at room temperature. The aqueous upper phase was transferred into a new vial, and 2 volumes of 100% cold ethanol were added and incubated overnight at -20° C. The samples were centrifuged at 21,000 *g* for 30 minutes at 4° C, and washed with 500 µl 70% (V/V) cold ethanol and centrifuged again at 21,000 *g* for 30 min at 4° C. Pellets were dried overnight in a hood and resuspended in 50 µl DDW.

DNA amplification and sequencing

In Belfast, protocols for PCR amplification of the plastid-encoded *rbc*L (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) gene followed Hayden *et al.* (2003), using oligonucleotide primers RH1 (5'-ATGTCACCACAAACAGAAACTAAAGC-3') and 1385r (5'- AATTCAAATTTAATTTCTTTCC-3'). PCR products were sequenced commercially (Fusion Antibodies, Belfast or Macrogen, Seoul, Korea). In Haifa, *rbc*L genes were amplified using the same primers RH1 and 1385R, following Hayden & Waaland (2002). The plastid gene for elongation factor 1-alpha (*tuf*A) was amplified using forward and reverse primers tufAF4 5'-GGNGCNGCNCAAATGGAYGG-3' and tufAR 5' CCTTCNCGAATMGCRAAWCGC-3' following Saunders & Kucera (2010). Amplification was carried out using a QSR PCR machine (QSR Technology, Gyeonggi, Korea) programmed as follow: 95°C, 5 min; (95°C, 1 min; 45°C, 1 min; 72°C, 1 min) x 35 cycles; 72°C 10 min. The *rbc*L product was sequenced by Stab Vida (Stab Vida, Caparica, Portugal) and the *tuf*A product by Macrogen (Macrogen, Seoul, Korea).

Data analysis

In order to identify our samples by molecular identification, *rbc*L sequences for representatives of other *Ulva* clades reported by Hayden *et al*. (2003) were obtained from Genbank for comparison, including named samples from the Mediterranean. Some unidentified Mediterranean samples were also included as there are comparatively few sequences available for named specimens. Sequences were aligned manually initially using MacClade 3 software (Maddison & Maddison, 1992), SeaView version 4 (Gouy *et al*., 2010), Bioedit v7.2.5 (Hall, 1999) and Clustal X v2.0 (Higgins & Sharp, 1989). Multiple sequence alignments were constructed, and some identical sequences were removed prior to analysis. In 2014, preliminary analyses of *rbc*L and *tuf*A sequences were used to divide the recently collected material into provisional morphological groups, and *rbc*L and *tuf*A sequences were compared to those in databases (NCBI using BLAST and BoLD, Barcode of Life Data Systems, http://www.boldsystems.org/views/idrequest.php). Relevant sequences in GenBank were included in the alignment. Trees for 2014 and 2015/16 data were initially constructed using MEGA v.6 (Tamura *et al*., 2013) and BAPS (Tang *et al*., 2007). Final phylogenetic trees including sequences from representative of all Israeli taxa sampled were computed in SeaView (Gouy *et al*., 2010), with *Umbraulva* species and *Ulvaria obscura* as outgroups, using maximum likelihood (PhyML; Guindon *et al.,* 2010; GTR, 4 rate categories, 1000 replicates), parsimony (PHYLIP, Felsenstein, 2013; sequence order randomized 5 times, treating gaps as unknown, more thorough tree search, 100 replicates), and distance (Neighbor Joining; Jukes-Cantor; treating gaps as unknown states; 1000 bootstrap replicates).

Herbarium vouchers for the *Ulva* sequences were deposited in the Israel Oceanographic & Limnological Research (IOLR) Herbarium or Natural History Museum London (BM) and sequences were uploaded to BoLD or GenBank (Table 1).

Results

Morphological and molecular identification of Ulva *species*

In 2002 the majority of samples collected were of the blade-like *Ulva* morphology, with very few tubular "*Enteromorpha*"-type samples observed. By contrast, in 2014-2016, tubular forms were clearly dominant at some sites. Morphological data were combined with our molecular analyses (Figs 2-3) to identify the *Ulva* species present in our samples from Israel from both collecting periods. Phylogenetic analyses of *rbc*L sequences from samples collected in Israel in 2014-2016 plus selected GenBank sequences (Fig. 2) were congruent with those of our *tuf*A alignments (Fig. 3). Although fewer *tuf*A sequences than *rbc*L sequences are available in GenBank for *Ulva* species, they included close matches (>99% similarity) for each of our species identified by comparison with *rbc*L sequences of types or other authentic sequences.

Comparison of 2002 samples and 2014-2016 samples, based on morphology and the *rbc*L gene, revealed some differences, which are described below. *Ulva compressa* samples, collected at one site in 2002 and at all sites in 2014-2016, formed a robust clade (Figs 2, 3). Israeli samples were represented in three other major clades in both *rbc*L and *tuf*A analyses, with relationships differing somewhat probably due to sequence availability. *U. chaugulii* (not found in 2002) and *U. tepida* were both well-resolved within a robust clade in *rbc*L and *tuf*A trees (Figs 2, 3). The *U. californica/aragoensis* clade included one Israeli sample collected in 2015-2016. A large clade consisting of several closely related species including *U. rigida*, *U. fasciata* and *U. ohnoi* was strongly supported in both trees (Figs 2, 3). These analyses indicate that in total six *Ulva* species were collected on Israeli coasts, as follows.

Ulva compressa ***Linnaeus***

Sequences of Israeli *Ulva compressa* samples, collected at one site in 2002 and at all sites in 2014-2016, formed a robust clade with other representative *U. compressa* sequences including those from near the type locality. This clade was sister to *U. intestinalis* (Figs 2, 3). Two common Israeli haplotypes of *U. compressa* differed by 2 bp in both the 1300 bp *rbc*L alignment and the 739 bp *tuf*A alignment. Several other haplotypes diverged by 1 bp. The common haplotypes for both *rbc*L and both *tuf*A were represented worldwide in GenBank; our sequence from Northern Ireland (sample G), close to the type locality for this species (England: Brodie *et al*., 2007), was Israeli *rbc*L haplotype 1. The common haplotypes were morphologically distinguishable to some extent: in 2014-2016, two common morphs (Figs 4, 5) were broadly congruent with *rbc*L haplotypes 1 and 2 and *tuf*A haplotypes 1 and 2, which were found at all sampling sites.

Morph 1 consisted of tubular thalli with narrow axes (Fig. 4A) or narrow tubular axes bearing flattened tubes with smooth edges (Fig. 4B). Cells were in straight rows in narrow axes (Fig. 4C), or in a less regular arrangement in larger blades (Fig. 4D), rounded in surface view, ca. 10 x 6 µm, with hood-shaped chloroplasts with 1(-4) large pyrenoids (Fig. 4E, F).

Morph 2 exhibited flat, narrow, elongate thalli with ruffled edges (Fig. 5A, B), sometimes emerging singly from the base (Fig. 5A). Cells were in short rows, smaller than in morph 1 (average 6.7 x 4.9 µm), sometimes

Fig. 2: Phylogenetic relationships based on *rbc*L gene sequences from *Ulva* samples collected in Israel with selected sequences from collections in Ireland and GenBank (new sequences indicated in bold; see Table 1 for sample details). The tree shown is ML with bootstrap values as follows: Maximum Likelihood (ML) representing PhyML (1000 replicates); Maximum Parsimony (MP; 100 replicates after removal of identical sequences); and Neighbor Joining (NJ), 1000 replicates. Branches with < 60% support are unlabelled.

with hood-shaped chloroplasts (Fig. 5C-E) and with 2-3 pyrenoids (Fig. 5E).

Ulva chaugulii M.G.Kavale & M.A.Kazi and *Ulva tepida* Masakiyo & S.Shimada

Ulva chaugulii and *U. tepida* were both well-resolved within a robust clade in *rbc*L and *tuf*A trees (Figs 2, 3). Two samples of *U. chaugulii* collected at Herzliya in 2015/16 (Fig. 6) closely resembled *U. compressa* morph 2 (Fig. 5B), and could not be distinguished from *U. compressa* when collected.

Ulva tepida was collected in 2002 at Achziv and Michmoret (one sample from each site). In 2015/16 it was again collected at Michmoret (two samples), but not observed at Achziv. Branched tubular thalli were long, narrow, hair-like (< 1 mm wide), and light yellow-green in colour (Fig. 7), similar to some forms of *U. compressa* (Fig. 4A). Cells were arranged in long curved rows, polygonal to oval (Fig. 7C), with plastids located near the cell wall, sometimes hood-like (Fig. 7C) with 2-5 (usually 3) pyrenoids (not shown).

Ulva aragoensis (Bliding) Maggs comb. nov.

In the *rbc*L tree (Fig. 2), sequences of *U. californica* constituted one clade of a robust lineage consisting of *U. californica* and sequences reported as *U. flexuosa*. Pacific "*U. flexuosa*" samples are not morphologically similar to nor closely related to authentic (European) *U. flexuosa* (Fig. 2; see Mares *et al*., 2011), but instead have been identified as *Ulva mediterranea* Alongi, Cormaci & G.Furnari, which is closely related to *U. californica* (Hiraoka *et al*., 2017). In both *rbc*L and *tuf*A analyses the clade included one Israeli sample (HER-2-TC) collected at Herzliya in 2015/16 (Fig. 8). The correct name

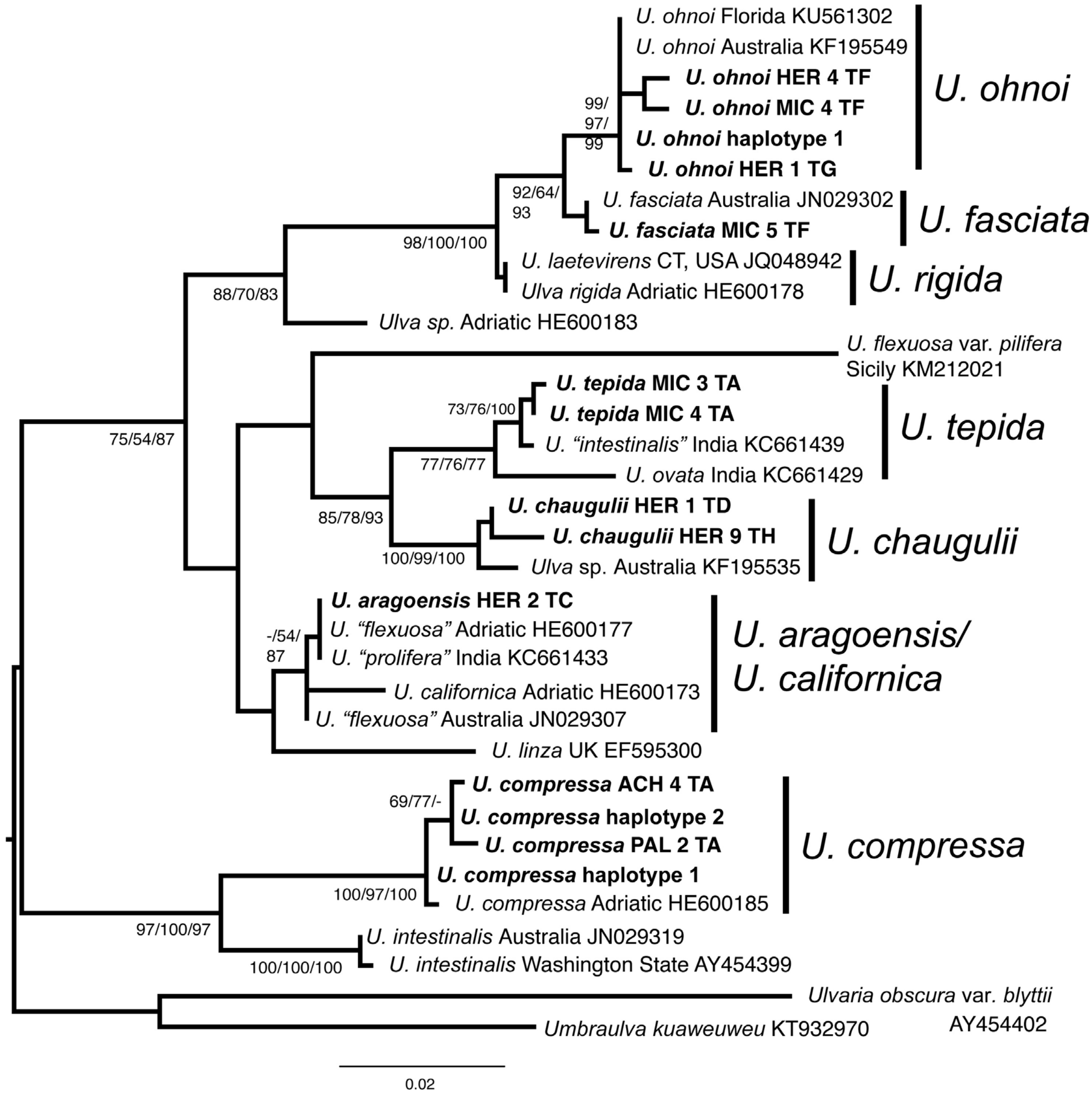

Fig. 3: Phylogenetic relationships based on tufA gene sequences from *Ulva* samples collected in Israel with selected sequences from GenBank (new sequences indicated in bold; see Table 1 for sample details). See legend to Figre 2 for other information.

for this entity is *U. aragoensis*, as explained in the Discussion. The *U. californica/U. aragoensis* clade is sister to *Ulva linza* and *Ulva prolifera* in *rbc*L analyses (Fig. 2), although it was rather poorly resolved in the *tuf*A tree (Fig. 3).

Ulva fasciata Delile and *Ulva ohnoi* Hiraoka & Shimada

A large clade consisting of several closely related species including *U. rigida*, *U. fasciata* and *U. ohnoi* was strongly supported in both trees (Figs 2, 3) although sequence divergences are low and the component taxa are weakly resolved. Samples from Israel were resolved as members of two currently recognized species. In 2002 larger blade-like *Ulva* specimens could be separated into two groups, identifiable as *U. fasciata* and *U. ohnoi*. *U. fasciata* (Fig. 9) had long parallel-sided strips, which were very obvious in tank-grown specimens but sometimes poorly developed in field-collected specimens (Fig. 9A). However, small blade-like specimens lacked distinctive habit features and were very fragile with no definite shape (Fig. 9B). *U. fasciata* thalli were dark to light green in colour, with ruffled or frilly margins, had small holes (1-5 mm diameter) and cells were arranged in short to long curved rows (Fig. 9D), with (rarely 1) 2-5 pyrenoids (not shown). In 2002, *U. fasciata* was collected at all sites except the southernmost Tel-Baruch (Fig. 1; Table 2), but it was not observed or collected at all in 2014 and only one sample was sequenced in 2015/16, from Michmoret. Material sampled in 2016 from aquaculture tanks at Haifa consisted of a mixture of *U. fasciata* and *U. ohnoi* (Fig. 2). Cultivation tanks include 40 and 600 L fiberglass units equipped with aeration and running seawater pumped

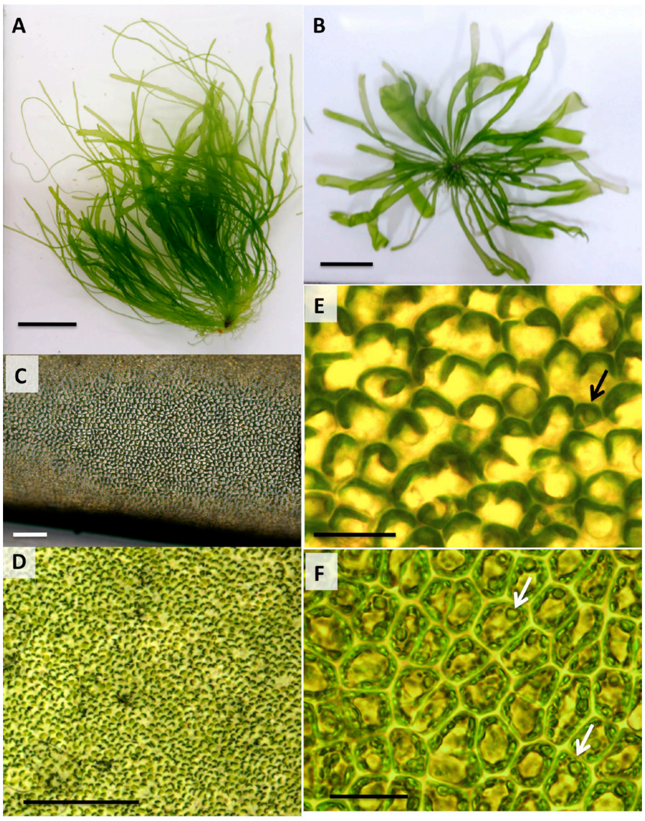

Fig. 4: *Ulva compressa* morph 1, all samples collected in Israel in Nov 2015/Jan 2016. A) Thallus with narrow branches, Palmachim (PAL-TA; scale = 10 mm); B) Thallus with wider branches, Habonim (HAB-TC; scale = 10 mm); C) Cells of narrow axis, in straight or curved rows, Achziv (scale bar = 50 µm); D) Cells of wider thallus, less obviously in rows, chloroplasts hood-shaped, Rosh Anikra (ROS-TC; scale = 200 µm); E) Cells showing hood-shaped chloroplasts and visible pyrenoids (arrow; scale = 20 µm). F) Cells showing chloroplasts throughout the cell and visible pyrenoids (arrows; scale = 20 µm).

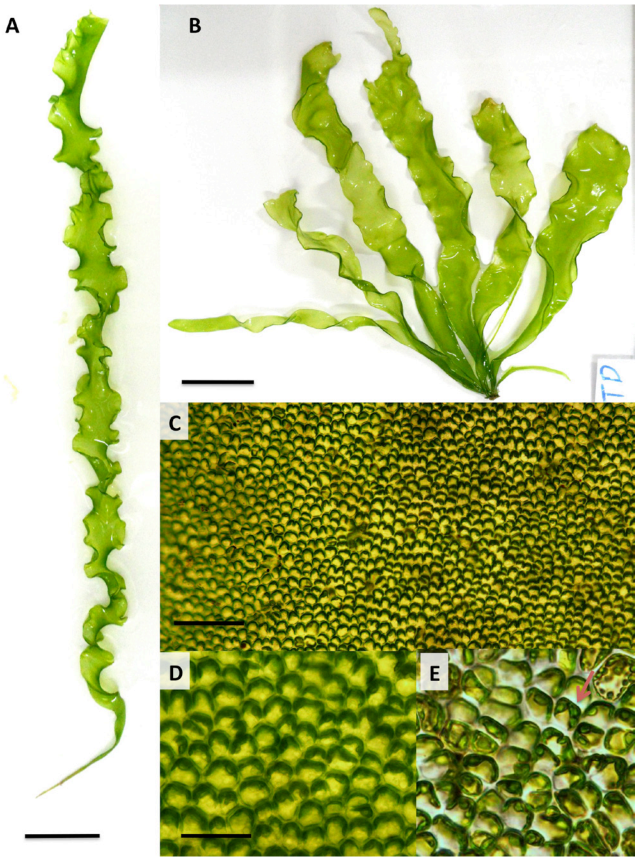

Fig. 5: *Ulva compressa* morph 2, all samples collected in Israel in Nov 2015/Jan 2016.
A) Single elongate ruffled blade from Herzliya (HER-TH; scale = 20 mm); B) Several flattened blades, Herzliya (HER-2-TD; scale = 10 mm); C) Cells in short rows with hood-shaped chloroplasts, Habonim (HAB-TD; scale = 100 µm); D) Cells with hood-shaped chloroplasts (HAB-TD; scale = 50 µm); E) Cells with up to 3 pyrenoids visible (arrow; scale as D).

Fig. 6: *Ulva chaugulii* from Herzliya, Israel, collected in Nov 2015/Jan 2016, showing pale green elongate, ruffled blades (sample HER-1-TD; scale = 1 cm).

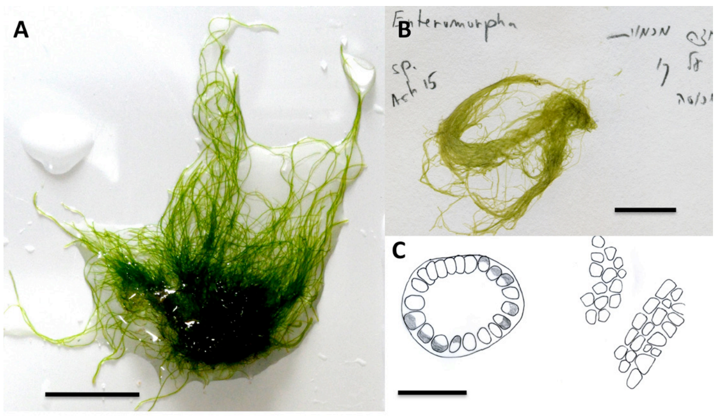

Fig. 7: *Ulva tepida*. A) Habit of finely branched live sample collected at Michmoret in Nov 2015/Jan 2016 (sample MIC-4-TA; scale = 10 mm); B) Herbarium specimen (Achziv 15) collected at Achziv North, 2 May 2002 (scale = 10 mm); C) Transverse section of tubular axis, showing chloroplasts on outer walls, and surface view of cells in rows (Mik6 from Michmoret, 9 Sept 2002; scale = 100 µm).

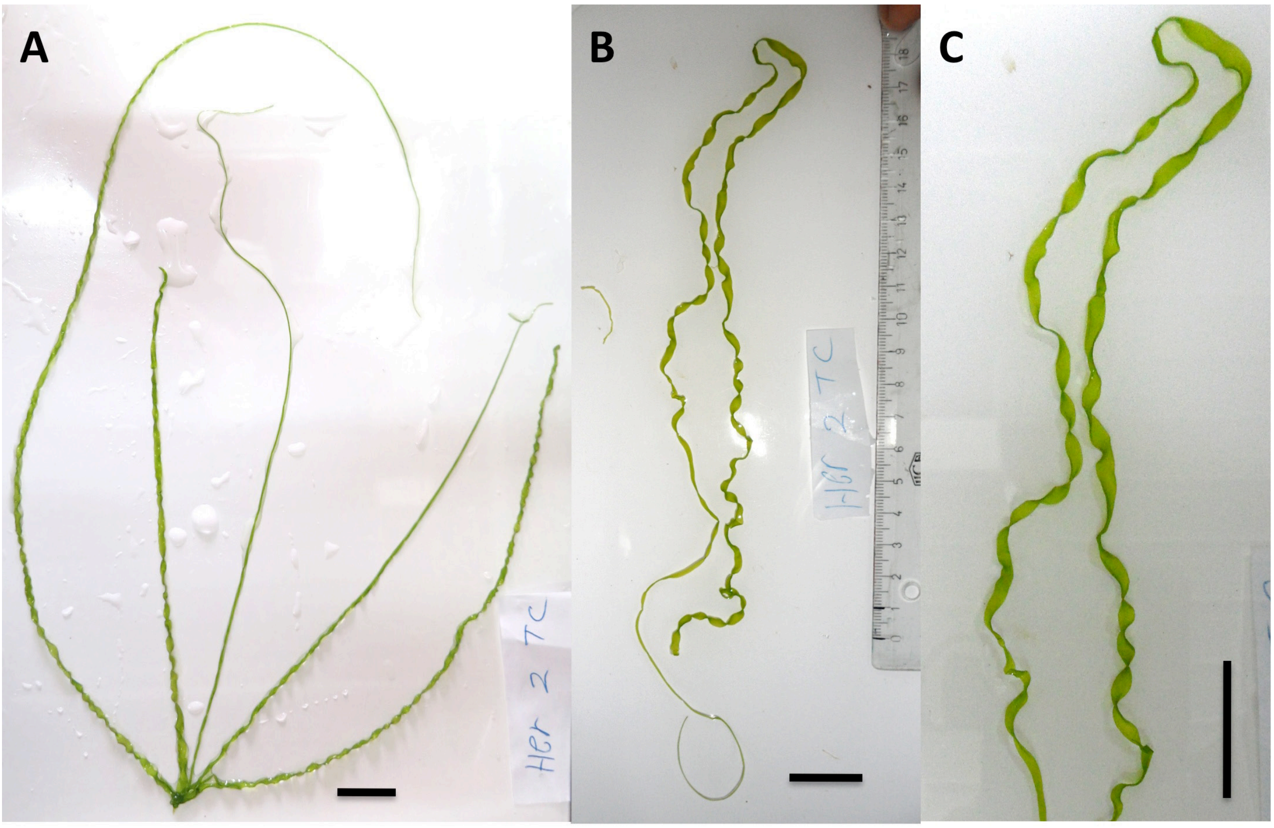

Fig. 8: *Ulva aragoensis* from Herzliya (sample HER-2-TC). A) Thallus showing several narrow elongate twisted axes; B) Single axis; C) Enlargement of part of axes (scales = 20 mm).

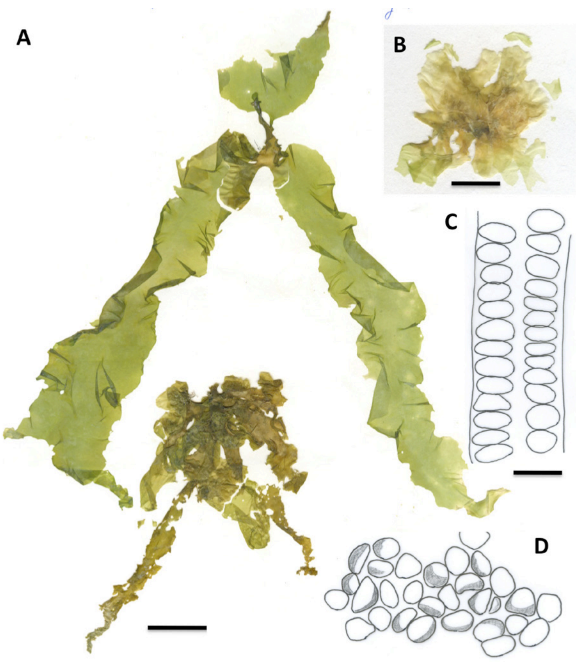

Fig. 9: Ulva fasciata. A) Thalli collected from rock platform at Achziv on 2 May 2002 (lower specimen) and after growth in culture tank at Shikmona (sample 11; scale = 20 mm); B) Sample from Michmoret, 9 Sept 2002 (Mik2; scale = 10 mm); C) Transverse section of blade and D) cells in surface view showing plastids (Achziv 14; scales = 50 µm).

from a nearby site. Stocks of *Ulva* species kept in these tanks were originally collected from the Haifa area.

Multiple collections of *U. ohnoi* (Fig. 10) were made at all sites sampled in 2002, but in 2014 and 2015/16 this species was found only on the southern part of the coast, at Michmoret, Herzliya and Palmachim (Fig. 1), where it made up the majority of samples collected. Flat, rounded leaf-like thalli lacked parallel-sided divisions; the shape and texture of specimens varied from rounded and entire (Fig. 10A) to divided and ruffled (Fig. 10D), often rigid near the base but sometimes fragile throughout. Thalli were light green, generally lacked holes, and cells were in short to long curved rows of thick-walled polygonal cells, usually with 2-5 (rarely up to 10) pyrenoids (Fig. 10B,C). The same common *rbc*L haplotype was found in 2002 and in multiple samples in 2015/16. Haplotypes differing by 1-2 bp, one of which was identical to GenBank sequences from India and Japan, were found in the later sampling period (Fig. 2). Due to the very low sequence divergences, analyses are susceptible to minor sequence ambiguities. A common *tuf*A haplotype was observed in 2014 and 2015/16, and two relatively unusual haplotypes differed by 1 bp.

Table 2. Comparison of *Ulva* species and morphs/haplotypes 1 and 2 of *U. compressa* identified by *rbc*L sequences (in 2002 and 2014-2016) and *tuf*A sequences (in 2014-2016). Bars (–) indicate that material was not sampled.

	2002		2014		2015/16	
Sites (North to South)	**Species/haplotype**	**Number of samples**	**Species/haplotype**	**Number of samples**	**Species/haplotype**	**Number of samples**
Rosh Anikra	–		–		*U. compressa* 1	4
					U. compressa 2	2
Achziv	*U. compressa* 2	1	–		*U. compressa* 1	2
	U. fasciata	6			*U. compressa* 2	1
	U. ohnoi	4				
	U. tepida	1				
Shikmona	–		–		*U. compressa* 1	4
					U. compressa 2	2
Habonim	–		–		*U. compressa* 1	0
					U. compressa 2	4
Michmoret	*U. fasciata*	1	*U. compressa* 1	1	*U. compressa* 1	2
	U. ohnoi	3	*U. compressa* 2	5	*U. ohnoi*	4
	U. tepida	1	*U. ohnoi*	30	*U. tepida*	2
Herzliya	–		*U. compressa* 1	1	*U. compressa* 1	4
			U. compressa 2	27	*U. compressa* 2	7
					U. chaugulii	2
					U. aragoensis	1
					U. ohnoi	8
Palmachim/ Tel-Baruch	*U. ohnoi*	6	*U. compressa* 1	11	*U. compressa* 1	4
			U. compressa 2	7	*U. compressa* 2	2
					U. ohnoi	7

Discussion

Previous morphological studies and surveys on Israeli coasts reported more than ten *Ulva* species but identification has been inconsistent (Einav, 2007) as a result of high phenotypic plasticity (Blomster *et al*., 2002), and ours is the first attempt to use molecular identification methods for *Ulva* species from Israel. Here, we demonstrated the presence of six *Ulva* species along the Israeli Mediterranean Sea coastline, only three of which have previously been reported.

Ulva compressa was recorded from Israel earlier and we found two haplotypes differing by 2 bp in both *rbc*L and *tuf*A markers. Surprisingly, this relatively small sequence divergence was associated with recognizable gross morphological differences. Israeli populations were distinct from those found in Britain and Ireland, from where this species was originally described (Hayden *et al*., 2003; Brodie *et al*., 2007). Although *U. compressa* is notoriously morphologically variable in its habit (Blomster *et al.,* 1998), Israeli material differed significantly from type material in cellular features, having multiple pyrenoids whereas European samples consistently have only one pyrenoid per cell (Blomster *et al*., 1998; Brodie *et al*., 2007). Nevertheless, the distinctive hood-shaped chloroplasts were visible in many specimens (Figs 4-5). The variation in pyrenoid number despite its diagnostic value in Britain and Ireland emphasizes the difficulty of identifying *Ulva* species globally without molecular markers (Pirian *et al*., 2016; Wolf *et al*., 2012). It is possible that we were dealing with populations differing in reproductive phase and/or ploidy (e.g. asexual, sexual) as reported for *U. chrismaggs* (as *U. mediterranea*) and *U. californica* by Hiraoka *et al.* (2017).

U. chaugulii M.G.Kavale & M.A.Kazi (type locality: Vayangani Maharashtra, near Goa, India) is a new record for Israel and the Mediterranean, and it was not observed in 2002. Pirian *et al*. (2016) discussed the close relationships and morphological overlap between *U. chaugulii* and *U. tepida* (as *U. paschima*).

Ulva paschima F.Bast was described recently from India (Bast *et al*., 2014) and then reported from the Persian Gulf (Pirian *et al*., 2016). Bast *et al.* (2014) based their new species on analyses of ITS sequences. Subsequently Phillips *et al*. (2016) described *Ulva sapora* J.A.Phillips, R.J.Lawton & C.Carl from Japan and Australia with ITS

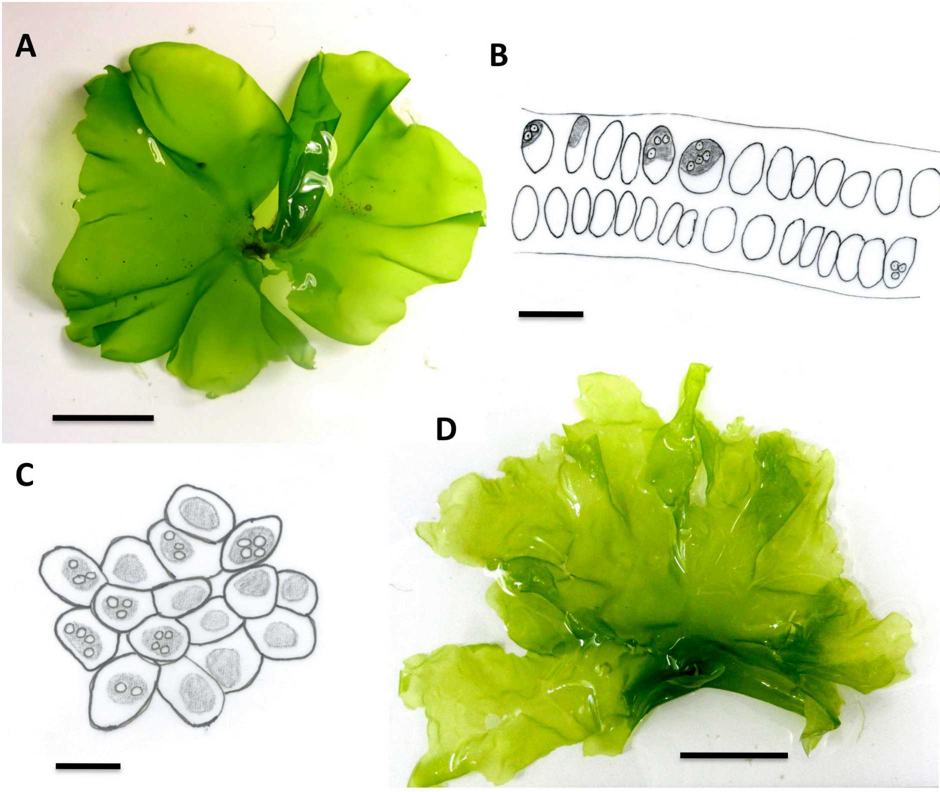

Fig. 10: *Ulva chrismaggs*. A) Thallus with smooth margins (Pal TF; scale = 10 mm); B) Transverse section of blade and C) cells in surface view showing plastids and multiple pyrenoids (sample Achziv 28, 1 Aug 2002; scales = 30 µm (B) and 40 µm (C)); D) Delicate thallus with ragged margins from Herzliya, December 2015 (sample Her 1 TG; scale = 10 mm).

sequences 99% similar to those of *U. paschima*. However, *U. sapora* is a later synonym of *U. tepida* Masakiyo & S.Shimada, published in February 2014, and the sequences are 100% identical. *Ulva tepida* thus has priority over *U. paschima*, which was published in October 2014. Pirian *et al*. (2016) used both ITS and *rbc*L markers in their investigation of *U. paschima* from the Persian Gulf, which allowed us to identify our samples as *U. tepida*, the correct name for the species reported by Pirian *et al*. (2016) as *U. paschima. U. tepida* in Japan is characterized by bright green or yellowish green tubular thalli up to 11 cm long and 8 mm in diameter, with cells in rows, chloroplasts covering the outer cell walls, and 1-5 (mostly 2-3) pyrenoids per cell (Masakiyo & Shimada, 2014) and our four specimens of *U. tepida* (2002: Achziv North 15, Michmoret 6; 2015/16: MIC 3 TA, MIC 4 TA) were morphologically similar. It should be noted that the *rbc*L GenBank HM572265 sequence of "*Ulva ovata*" (deposited by V. Gupta and others) from Gopnath is very similar to *U. tepida* (as *U. paschima*) sequences from Iran. Gopnath in the Gulf of Khambhat, India, is the type locality of *Enteromorpha ovata* F. Thivy & V. Visalakshmi ex H.V. Joshi & V. Krishnamurthy (1972). The relationship between *Ulva tepida* and *E. ovata* requires further investigation, as *E. ovata* may in turn be an older name for this species. To our knowledge, this is the first report of *U. tepida* from the Mediterranean, but we have shown that it has been present in Israel, though rare, since at least 2002. Both *U. chaugulii* and *U. tepida* are potential new introductions to the Mediterranean, but it is also possible that earlier records of *U. linza* (Einav & Israel, 2008) represent one or both of these species.

Another species found in Israel for the first time in 2015-2016 has been reported widely from the Pacific Ocean as *U. flexuosa*, and recently in the Adriatic, from molecular identifications (Wolf *et al*., 2012) based on Shimada *et al*. (2003) sequences, but it is not closely related to *U. flexuosa sensu stricto* (Hiraoka *et al*., 2017). It was linked by Hiraoka *et al*. (2017) to *U. mediterranea* Alongi, Cormaci & G.Furnari (Alongi *et al*., 2014). *Ulva mediterranea* is based on the type specimen of *Enteromorpha aragoensis* Bliding (1960, p. 174, *'aragoënsis'*) from Banyuls, Mediterranean France, described because the authors considered that *E. aragoensis* was originally named invalidly, in accordance with Hayden *et al.* (2003). However, Bliding (1963, p. 113) indicated that the holotype of *E. aragoensis* was in Lund. The relevant specimen capsule in Lund (https://lu.app.box.com/s/psphzgf3lt739ihlfksm6a9yj6qrym8c/file/239146265958) is clearly labeled "Typus" and "*Enteromorpha aragoënsis* Bliding *Pyr. Orient., Banyuls, Lab. Arago 6/6 1958* Leg. *Carl Bliding*", and therefore seems to meet the requirements of Article 40.1 of the Melbourne code (McNeill *et al*., 2012): "Publication on or after 1 January 1958 of the name of a new taxon of the rank of genus or below is valid only when the type of the name is indicated." Hence the name *Ulva mediterranea* is a superfluous name change, and we consider the correct name to be *Ulva aragoensis* (Bliding) Maggs, comb. nov. [basionym: *Enteromorpha aragoensis* Bliding 1960, Bot. Not. 113, p. 174, fig. 2a-f].

U. fasciata was originally described from Alexandria (Delile, 1813) and it has been widely recorded in Israel (Einav & Israel, 2008). However, although we found it commonly at three sites in 2002, in both spring and autumn, it was not observed in 2014 and was rare in 2015/16.

U. ohnoi Hiraoka & Shimada is an invasive species originally described from the warm temperate regions of southern and western Japan where it forms green tides (Hiraoka *et al*., 2004), and our 2002 collections were the first from natural habitats in the Mediterranean. *U. ohnoi* was first reported in the Mediterranean during a survey of ballast water in the harbour of Naples (Flagella *et al*., 2007). The ballast water had come from Port Said (Egypt), very close to the Suez Canal. It could be inferred that the native area of this species is the Indo-Pacific region and it is spreading currently by the way of anthropogenic vectors (i.e. hull fouling and ballast water). However, it is possible that earlier records from Israel of *U. rigida* and *U. lactuca* (Einav & Israel, 2008) were misidentifications of this species. *Ulva ohnoi* is very closely related to and can interbreed with *U. fasciata* (Hiraoka *et al*., 2004), which has often been found in Israel (Beer *et al*., 1990; Einav & Israel, 2013). *Ulva fasciata* grows well in aquaculture tanks, and was present in our 2002 collections, but now seems to be rare in natural habitats. As suggested by Flagella (2007), it is possible that *U. ohnoi* has invaded the area with ballast water or another vector – it can produce green tides, causing environmental and economic damage. Massive green tides and algal blooms are known worldwide for their harmful effects (Hiraoka *et al*., 2004; Guidone *et al.,* 2013).

There is a marked pattern of introductions into the Mediterranean Sea via the Suez Canal, usually with shipping (Israel & Einav, 2017) and Lessepsian species are often reported first in Israel (Nunes *et al*., 2014). A positive aspect of detecting new invasives is the opportunity to incorporate them into local industries and aquaculture. *Ulva* species, including new arrivals, may be valuable for the local bioeconomy (Chemodanov *et al*., 2017). The results are especially important given the growing interest in using *Ulva* biomass for various food industries, in bioremediation, or as a source for bioethanol production. Any future industrial-scale cultivation of *Ulva* will rely initially on collections of material from the wild. Given that sustainable food supplies, renewable energy and water treatment are major challenges for the near future, *Ulva* species could be the answer to many of these challenges.

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