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The Appearance of *Ulva laetevirens* (Ulvophyceae, Chlorophyta) in the Northeast Coast of the United States of America

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Abstract Introduced species may outcompete or hybridize with native species, resulting in the loss of native biodiversity or even alteration of ecosystem processes. In this study, we reported an alien distromatic *Ulva* species, which was found in an embayment (Holly Pond) connected with Long Island Sound, USA. The morphological and anatomical observations in combination with molecular data were used for its identification to species. Anatomy of collected specimens showed that the cell shape in rhizoidal and basal regions was round and the marginal teeth along the basal and median region were not found. These characteristics were primarily identical to the diagnostic characteristics of *Ulva laetevirens* Areschoug (Chlorophyta). The plastid-encoding *tuf*A and nucleus-encoding ITS1 were used for its molecular identification. Phylogenetic analysis for the *tuf*A gene placed the specimens from Holly Pond in a well-supported clade along with published sequences of *U. laetevirens* identified early without any sequence divergence. In ITS tree, the sample also formed well-supported clades with the sequences of *U. laetevirens* with an estimated sequence divergence among the taxa in these clades as low as 1%. These findings confirmed the morpho-anatomical conclusion. Native to Australia, this species was reported in several countries along the Mediterranean coast after the late of 1990s. This is the first time that *U. laetevirens* is found in the northeast coast of United States and the second record for Atlantic North America.

Key words *Ulva laetevirens* Areschoug; introduced seaweed; Long Island Sound, USA; ITS; *tuf*A

1 Introduction

Introduced seaweeds are a growing and imminent threat throughout the world's oceans, altering ecological structure and causing economic harm (Nyberg and Wallentinus, 2005; Mathieson *et al*., 2008). Based upon floristic and molecular investigations, 23 introduced seaweeds are presently known from the Northwest Atlantic, including 3 green, 4 brown and 16 red algae (Mathieson *et al*., 2008; Thornber *et al*., 2009; Hofmann *et al*., 2010; Schneider, 2010). Many *Ulva* species are notorious biofoulers of ships' hulls and ballast waters, making them among the most commonly transported and widely introduced species of macroalgae (Nelson *et al*., 2007). *Ulva laetevirens* Areschoug, a green seaweed native to south Australia (Areschoug, 1854), was recently reported in several Mediterranean coastal countries including Italy (Furnari *et al*., 1999; Rindi *et al*., 2002; Sfiso, 2010), Slovenia (Rindi and Battelli, 2005), Israel (Einav, 2007), Greece and Cyprus (Christia *et al*., 2011). It was also recently reported in the Atlantic coast of Canada (Kirkendale *et al*., 2013). Here we reported the appearance of *Ulva laetevirens* Areschoug in the Long Island Sound, northwest Atlantic Ocean of USA.

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2 Materials and Methods

2.1 Location and Habitats

The samples were collected from two sites of Holly Pond (Stamford, Connecticut, 41˚2′57.87′′N, 73˚29′ 55.66′′W) on Jun 21, 2011. In general, temperature and salinity of site 1 are more variable than those of site 2. In site 1, plants are often exposed to air for 9–10 h during the tidal cycle. This site is rapidly influenced by fresh water runoff, with the salinity ranging from 0.0 to 26.0 (mean= 18.3). Water temperature ranges from −1.0℃ in January to 29.0℃ in August. The substratum of site 1 is dominated by mud, and *Ulva* plants attach to small pebbles or oyster shells. Site 2 is situated just above the dam. Salinity ranges from 2.0 to 28.0 (mean=23.4±5.48) and temperature ranges from −2.0℃ to 26.5℃. The bottom is characterized by shell fragments, stones and fine sediment. Currents are minimal at both sites (Mariani, 1983).

2.2 DNA Extraction and PCR

Following the collection, each specimen was cleaned

and pressed on a herbarium paper as a voucher, while a subsample was frozen for DNA extraction.

The freshly-frozen portion was ground in liquid nitrogen. DNA was extracted using a Qiagen DNeasy Plant Mini Kit (Valencia, CA, USA) following the manufacturer's protocol. The PCR mixture was prepared by mixing 25 μL of AmpliTaq Gold PCR Master Mix (Applied Biosystems, Inc., Carlsbad, CA, USA) containing AmpliTaq Gold with 0.5 μL of forward primer (10 $\mu mol\,L^{-1}$), 0.5 μL of reverse primer (10 $\mu mol\,L^{-1}$), 1 μL of genomic DNA as template (5–10 ng) and 23 μL of distilled water for a total volume of 50 μL. PCR amplification was carried out in a GeneAmp PCR System 9600 (Applied Biosystems, Inc., Carlsbad, CA, USA). Primers used to amplify and sequence ITS and *tuf*A are listed in Table 1. PCR profile for amplifying ITS was as follows: an initial denaturation at 94℃ for 5 min, followed by 1 min at 94℃ and 3 min at 60℃ for 30 cycles, and a final 10 min extension at 60℃ (Blomster *et al*., 1998). PCR condition for amplifying *tuf*A included an initial 4 min denaturation at 94℃, followed by 38 cycles of amplification including denaturing at 94℃ for 1 min, annealing at 45℃ for 30 s, extending at 72℃ for 1 min, and an extra extension at 72℃ for 7 min (Saunders and Kucera, 2010).

Amplification success was evaluated using gel electrophoresis in a 1.0% agarose gel. PCR products were purified using a Qiagen PCR product purification kit and sequenced in both directions using an ABI 3130XL Genetic Analyzer (Carlsbad, CA, USA). The DNA sequences were trimmed using Chromas 2.22 (Technelysium Pty Ltd, Tewantin, Queensland, Australia). Sequences alignment and phylogenetic analysis were conducted using *MEGA* version 5.05 (Tamura *et al*., 2011).

Table 1 Primers used in this study for PCR amplification and sequencing

Primer	Sequence	Target	Direction	Reference
18S1505	5'-TCTTTGAAACCGTATCGTGA-3'	*ITS*	Forward	Blomster *et al*. (1998)
ENT26S	5'-GCTTATTGATATGCTTAAGTTCAGCGGGT-3'	*ITS*	Reverse	Blomster *et al*. (1998)
tufGF4	5'-GGNGCNGCNCAAATGGAYGG-3'	*tuf*A	Forward	Saunders and Kucera (2010)
tufAR	5'-CCTTCNCGAATMGCRAAWCGC-3'	*tuf*A	Reverse	Fama *et al*. (2002)

3 Results

3.1 Morphology and Anatomy

Specimens of *U. laetevirens* were collected from both sites (1# and 2#) of Holly Pond (Fig.1). Thalli of *U. laetevirens* are foliose (Fig.2), which naturally grow singly or in small clumps fixed to the hard substratum with rhizoidal. The cell shape and blade thickness vary from rhizoidal parts to apical regions. In surface view, the

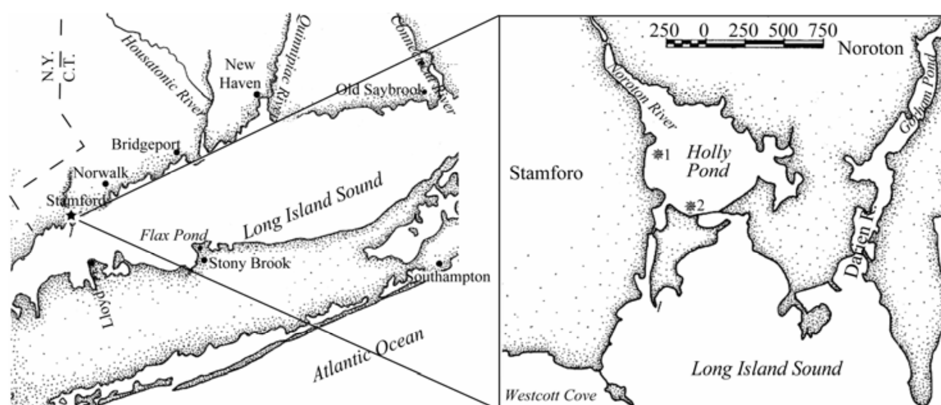

Fig.1 Map of Long Island Sound and locations where specimens of *Ulva laetevirens* were found (❋).

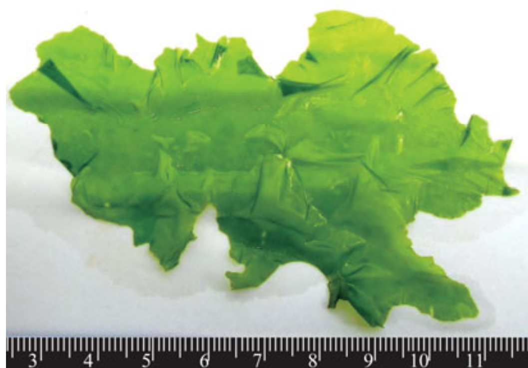

Fig.2 Blade of *U. laetevirens* from Holly Pond. Scaled to a centimeter ruler.

rhizoidal cells are difficult to clearly distinguish from the normal cells (Fig.3) in size and color, which is different from the confusable species *U. rigida* whose rhizoidal cells appears thicker and darker (Sfriso, 2010). The cell shape in rhizoidal and basal regions is round, whereas those in median and apical regions are polygonal or quadrangular. Marginal teeth along the basal and median regions were not found in this study (Fig.3). In cross sections, the thickness reaches 100–120 μm in the rhizoidal region and decreases to 30–40 μm in the apical region (Fig.4). The rhizoidal and basal cells are distinctly cylindrical or conical, and their height is 2–3 times of their diameter, which is an important characteristic for determination of *U. laetevirens* (Sfriso, 2010). A cross-

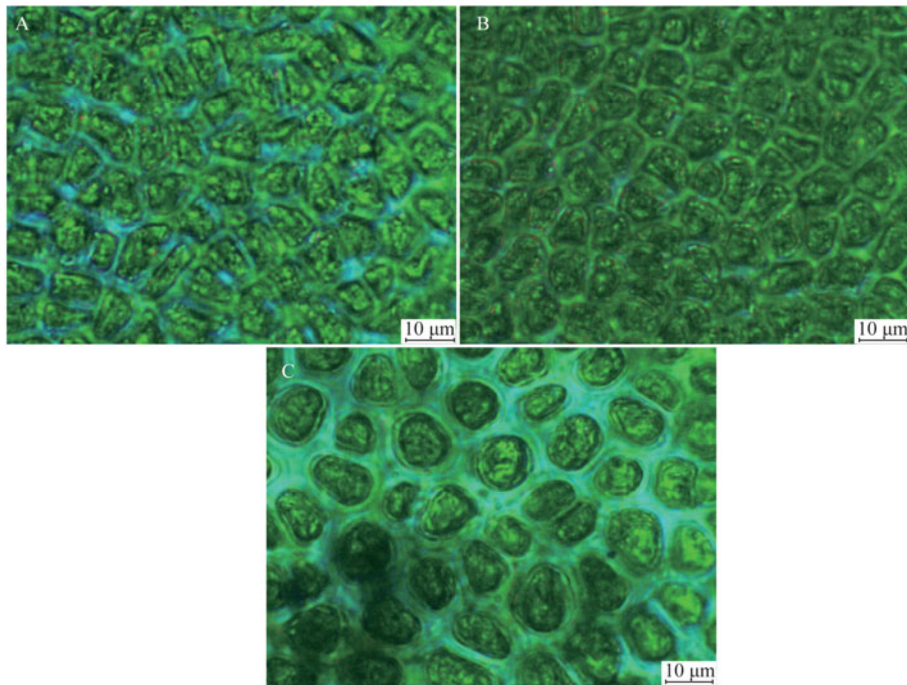

Fig.3 Microscopic surface views of *U. laetevirens* (scale bar = 10 μm). A, Apical region; B, Median region; C, Basal region.

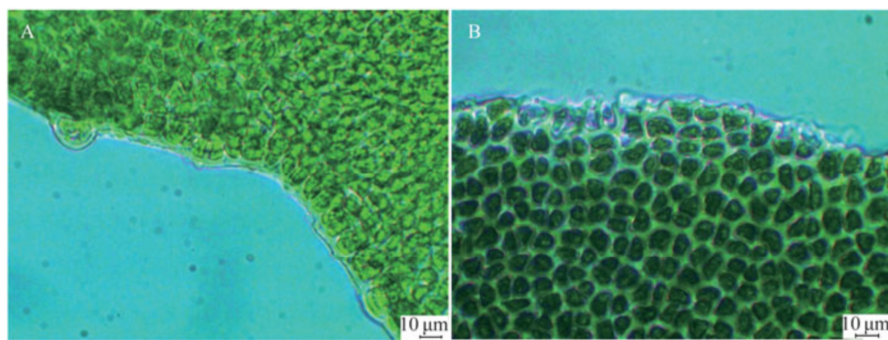

Fig.4 Marginal views of *U. laetevirens* (scale bar = 10 μm). A, Median region; B, Basal region.

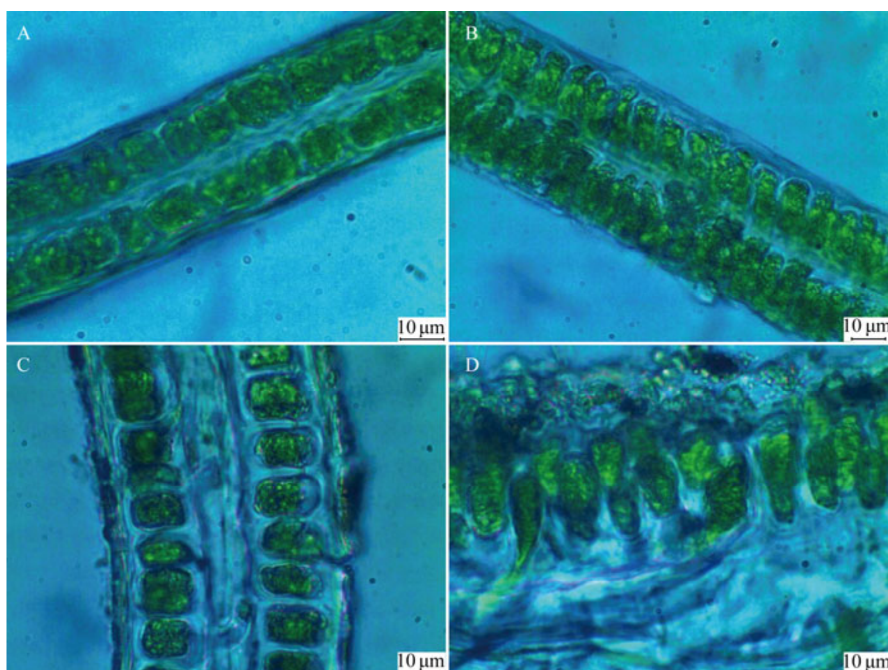

Fig.5 Microscopic cross sections of *U. laetevirens* (scale bar = 10 μm). A, Apical region; B, Median region; C, Basal region; D, Rhizoidal region.

section from the rhizoidal region reveals a thick band of secondary rhizoids. In the median and apical areas, the cell length is not distinctive, but they have a conical shape as they taper towards the thallus surface.

3.2 Molecular Evidences

We sequenced partial plastid elongation factor gene, *tuf*A, which has recently been developed as a routine barcode of green algae (Saunders *et al*., 2010). After trimming the ambiguous nucleotides, the sequence length of *tuf*A from voucher UCCY20110621P03 was 815 bp (JQ048942.1) and that was 780 bp for voucher UCCY 20110621P05 (JQ048943.1). The alignment showed that the two sequences were identical at 780 aligned sites. All available accessions of *Ulva tuf*A genes in GenBank were collected with duplicates removed. In total, 746 sites among 35 *tuf*A sequences were aligned, which represented 15 *Ulva* species, and used for reconstructing the phylogenetic tree. Molecular phylogenetic data for *tuf*A gene placed these two specimens from Holly Pond in a well-supported clade along with published sequences of *U. laetevirens* (HQ610428.1) identified early without any divergence (Fig.6).

JQ048946.1 and JQ048947.1 were assigned to the ITS sequence of voucher UCCY20110621P03 and UCCY 20110621P05, respectively. The two ITS sequences are the same in length (516 bp). Alignment shows that the two ITS sequences are identical each other. There are quite a number of ITS sequences for *Ulva* in Genbank. The following criteria were used for picking up the representatives from GenBank for constructing ML tree: i) higher value of query cover and similarity value (>90%) against JQ048946.1; ii) with foliose thalli; iii) naturally distributing along the coast of nothern Atlantic. In ITS ML trees (Fig.7), voucher UCCY20110621P03 and UCCY20110621P05 form well-supported clades with sequences of *U. laetevirens*, as well as a mixture of GenBank accessions given as *U. amoricana*, *U. rigida* and *U. scandinavica*. The estimated sequence divergence between the taxa in these clades was as low as 1%.

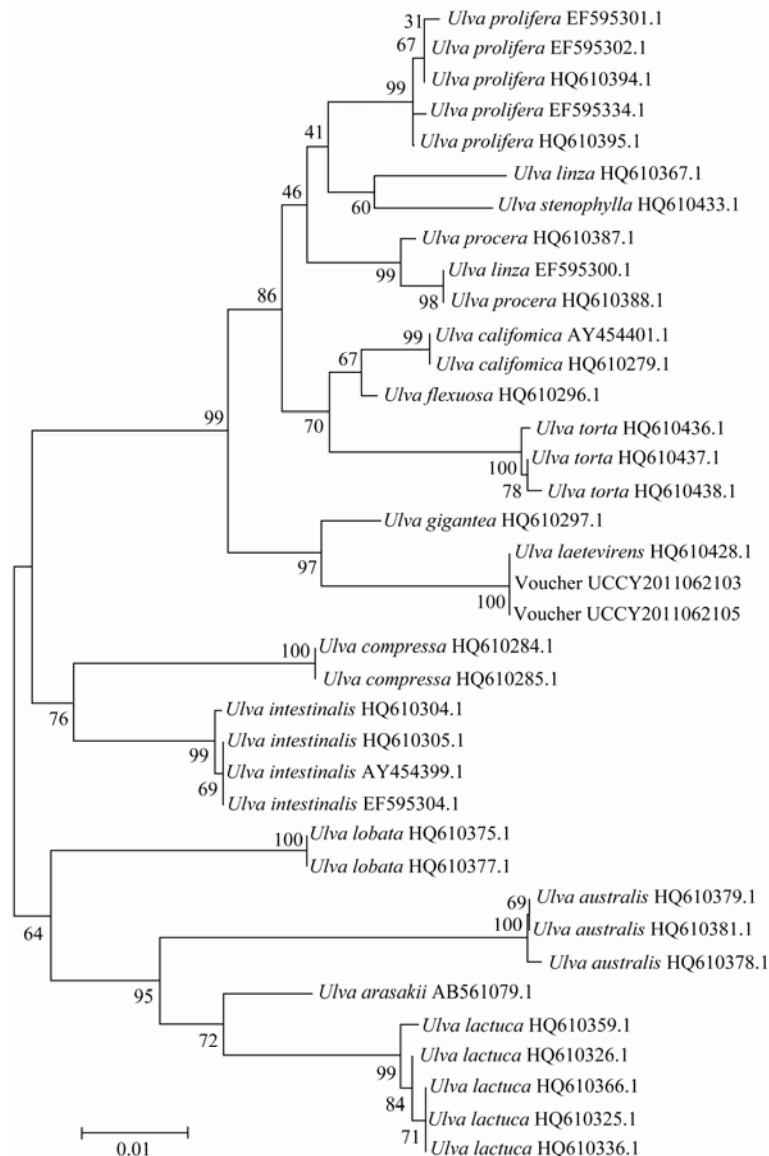

Fig.6 Unrooted maximum-likelihood (ML) tree for plastid-coding elongation factor gene (*tuf*A) sequence data with ML bootstrap support values.

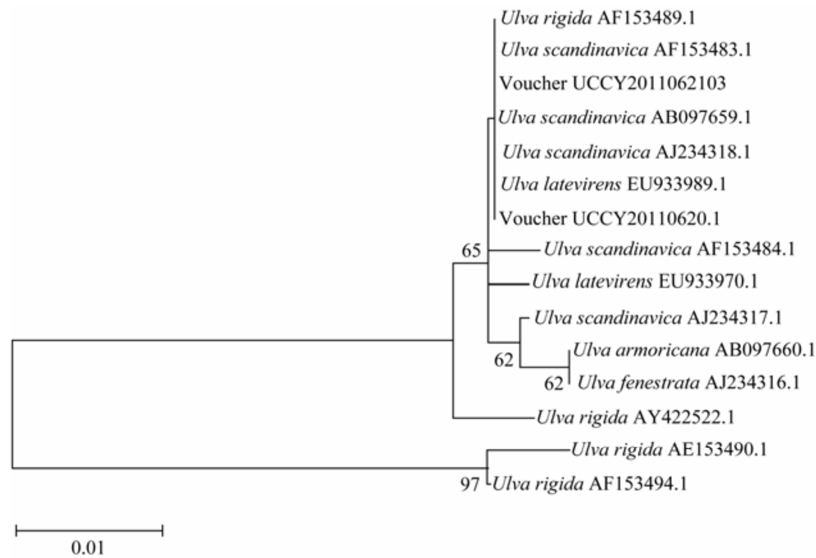

Fig.7 Unrooted phylogram generated with maxima-likelihood analysis from the *ITS* for samples included in this study.

4 Discussion

Holly Pond locates approximately 50 km east of New York Harbor. Noroton River flows into Holly Pond and empties into Long Island Sound (LIS) (Fig.1). It has been estimated that the pond refreshes only 30% of its total volume daily because of a dam constructed in 1960, which is about 80 cm higher than mean sea level (Harris, 1973). Reduced flushing of the pond and accumulation of pollutants associated with the sediments result in eutrophic waters. From the late of 1970s, *Ulva* species inhabiting Holly Pond have been intensively and systematically investigated. Mariani (1983) described four 'types' of *Ulva* species in Holly Pond. Type 1 appears morphologically similar to *U. lactuca*; type 2, type 3 and type 4 conform to the description of *U curvata*, *U. rotundata*, *U. laetevirens*, respectively. *Ulva rigida* and *U. laetevirens* are difficult to be accurately identified, as they display few distinctive diagnostic features, as well as large degrees of morphological plasticity within these characters. The cell features in the cross section of the rhizoidal and basal regions are considered to be a diagnostic character (Sfriso, 2010). In the rhizoidal, and sometimes in the basal region, *U. rigida* has large, rectangular cells. These cells are taller and narrower in other regions. In contrast, in the rhizoidal and basal regions of *U. laetevirens*, the cells are tall and narrow, with a conical shape (Phillips, 1988). In the specimens collected from Holly Pond, the cell shapes in the rhizoidal and basal regions are identical with the diagnostic characters of *U. laetevirens* described by Sfriso (2010). Marginal teeth along the basal and median region were not found in this study, which has been used as a morphological character of *U. rigida* (Innes *et al.*, 1981; Hoeksema and van den Hoek, 1983) to distinguish these two species, even though it may not be adequate (Philips, 1988).

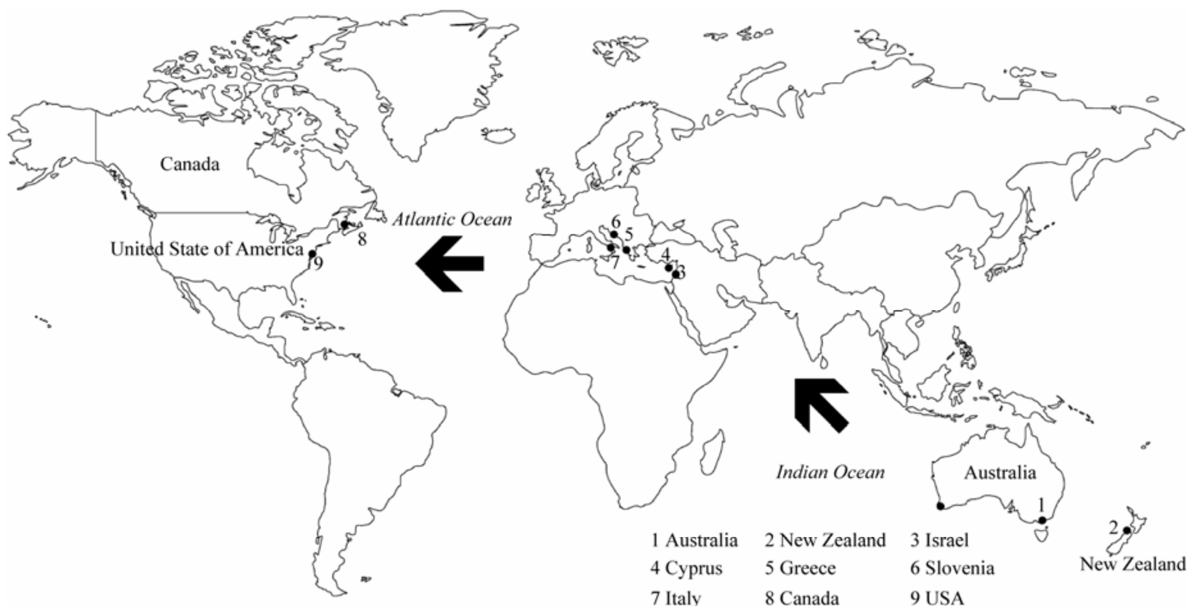

Fig.8 Distribution of *Ulva laetevirens* in the world drawn by referring to the documented early (Furnari *et al.*, 1999; Rindi *et al.*, 2002; Sfiso, 2010; Rindi and Battelli, 2005; Einav, 2007; Christia *et al.*, 2011; Kirkendale *et al.*, 2013).

Ulva laetevirens was originally described by J. E. Areschoug in 1854 based on the specimen collected from the Port Phillip, Victoria, Australia. From the late 1990s, this species has been reported in several Mediterranean coastal countries. Recently, this species was found in New Brunswick, Canada (Kirkendale *et al*., 2013). Based on the information available, we could infer the expanding tendency of this species (Fig.8). But for fully understanding its migration pattern, more specimens from North America, Mediterranean Seas and Australia should be collected to conduct the molecular genetic diversity analysis.

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