Multiple genetic marker analysis challenges the introduction history of Ulva australis (Ulvales, Chlorophyta) on French coasts

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Abstract

The green seaweeds *Ulva australis* and *U. pertusa* were described from southern Australia and Japan, respectively. They are conspecific and *U. australis*, the currently accepted taxon, is native to temperate marine waters in northeastern Asia, and known to be introduced overseas into Australasia, the Americas and Europe. Although the genetics of *U. australis* have been investigated elsewhere, along French coasts the origins and history of the introduction of this species need to be clarified. We used mitochondrial, plastid and nuclear markers to differentiate introduced populations of *U. australis* along the French Atlantic coasts. The plastid *tuf*A gene used as a barcoding marker revealed a well-defined species with a higher haplotype diversity in native *vs.* introduced areas. The ITS2 region (nuclear) and *rbc*L (plastid) were used to compare French specimens with the lectotype of *U. australis*. Putative geographic origins of the genetically determined *U. australis* were examined using genetic markers with better resolution, the plastid *atp*I-H combined with the mitochondrial *trn*A-N. Origin(s) and introduction history of French specimens were inferred from the comparison between their haplotypes and those previously described in native and non-native temperate areas worldwide. Our results indicate that the presence of *U. australis* along the French Atlantic and Mediterranean coasts is the result of multiple introductions and independent pathways, and suggest that historical oyster transfers from Japan and British Columbia can only partially explain the observed patterns in genetic markers. Alternative hypotheses for the timing and pathways of introductions are proposed in the light of the historic background of maritime transport networks and trade between north-eastern Asia and Europe.

Keywords: *atp*I-H; green macroalgae; introduced species; ITS2; *rbc*L; *trn*A-N; *tuf*A

Introduction

The green macroalgal genus *Ulva* Linnaeus, which includes species previously named under the genus *Enteromorpha* Link (Hayden *et al.*, 2003; Shimada *et al.*, 2003), is distributed worldwide in different marine, brackish and freshwater ecosystems. According to AlgaeBase, amongst the 407 historical names presently reported as *Ulva* sp. in the database, only 86 have been flagged as taxonomically accepted species (Guiry & Guiry, 2020). This spectacular number of synonyms reflects, to some extent, the notorious problems in distinguishing *Ulva* species on the basis of their habitat, morphology, anatomy, cytology and physiology (Bliding, 1969; Hoeksema & Van Den Hoek, 1983; Phillips, 1988; Fort *et al.*, 2019; 2020a). Difficulties in the delineation of *Ulva* species are linked to their high degree of phenotypic plasticity, but have also been enhanced by human-mediated biological invasions in coastal waters. *Ulva* species are common biofoulers on ship hulls (Mineur *et al.*, 2007), mariculture structures and products (Verlaque *et al.*, 2002; Boudouresque *et al.*, 2011), and hitchhikers in ballast waters (Flagella *et al.*, 2010). International trans-oceanic shipping and marine aquaculture are recognized as major vectors of introductions of non-indigenous species (NIS) in coastal waters (Carlton, 1987; Katsanevakis *et al.*, 2013). Distinguishing between native species and NIS, which often remained overlooked (Haydar & Wolff, 2011), is still a major challenge in the context of marine *Ulva* species (Baamonde López *et al.*, 2007; Hofmann *et al.*, 2010; Kraft *et al.*, 2010; Wolf *et al.*, 2012; Steinhagen *et al.*, 2019).

Molecular methods have been employed for a few decades to overcome these difficulties and new light has been shed on *Ulva* spp. taxonomy, to identify and delineate species (Coat *et al.*, 1998; Baamonde López *et al.*, 2007; Flagella *et al.*, 2010), critically re-examine inventory records (Wolf *et al.*, 2012; Steinhagen *et al.*, 2019), infer phylogenetic relationships among taxa (Hayden *et al.*, 2003; Shimada *et al.*, 2003), elucidate introduction history (Couceiro *et al.*, 2011) and characterize type materials to clarify species taxonomy and

biogeography (Hanyuda & Kawai, 2018; Hughey *et al.*, 2019). Both the nuclear ribosomal internal transcribed spacer DNA (ITS nDNA) and the chloroplast-encoded *rbc*L gene have been tested alone (Coat *et al.*, 1998; Flagella *et al.*, 2010) or in tandem (e.g. Shimada *et al.*, 2003; Couceiro *et al.*, 2011). However, compared with these two molecular markers, the plastid elongation factor *tuf*A was recommended by Saunders & Kucera (2010) as a more suitable barcode marker for most marine green macroalgae including the genus *Ulva*. This was further confirmed by Kirkendale *et al.* (2013), Lee *et al.* (2019) and Fort *et al.* (2020*b*) for Australian, Korean and west European specimens, respectively, as they provided estimates of intra- and inter-specific divergence in *Ulva* species. Our current sequencing survey of hundreds of *Ulva* specimens along the French Atlantic coast found that many *tuf*A sequences fully matched GenBank sequences of *U. australis* Areschoug and *U. pertusa* Kjellman, a junior synonym (see details in Couceiro *et al.*, 2011), from Korea in the native area (Kang *et al.*, 2019), and disjunct areas where the species has been introduced e.g. Australasia (Heesch *et al.*, 2009; Kirkendale *et al.*, 2013), the Italian North Adriatic (Wolf *et al.*, 2012) and German North Sea (Steinhagen *et al.*, 2019). *U. australis*, recognized to be of north-eastern Asian origin (Hanyuda *et al.*, 2016; Hanyuda & Kawai, 2018) was thought to have been introduced into the Mediterranean Sea and along North European coasts through multiple introduction events and different vectors including maritime transport and mariculture (Baamonde López *et al.*, 2007; Couceiro *et al.*, 2011; Verlaque & Breton, 2019).

To compare our specimens unequivocally to the type material of *U. australis*, recently characterized with molecular markers (Hanyuda & Kawai, 2018), and reanalyse earlier records of the species in Brittany (Coat *et al.*, 1998), we conducted molecular analyses based on ITS2 and *rbc*L sequences. We also used both plastid and mitochondrial non-coding sequences, *atp*I-H and *trn*A-N, as suggested by Hanyuda *et al.* (2016). This latter combination of markers has allowed significant differences in genetic diversity to be resolved among

native (Japan and Korea) and non-native populations of *U. australis* (Hanyuda *et al.*, 2016; 2018) and contributed to clarifying the introduction history of *U. australis* in Australia in terms of pathways and timing (Hanyuda & Kawai, 2018). This approach is used here to provide biogeographic information about the origin of our *U. australis* specimens identified based on ITS2, *rbc*L and *tuf*A and to re-evaluate commonly accepted hypotheses concerning the vectors and timing of introductions of *U. australis* along the French coasts in view of the history of maritime transport between Europe and Northeast Asia.

Materials and methods

Sample collection

Our sampling strategy was based on collecting large sample sizes (> 100 specimens) from three sites separated by over 460 km. Green algae with large and flat rigid thalli, pale to dark green in colour and with a stiff base, were sampled from rocky shores at three sites during low spring tides from 22 January to 21 February 2019. The three sites are in Brittany at Roscoff (48.7327'N, 3.9868'W, Parmentier dock, Estacade, Ile Verte) and Concarneau (47.8601'N, 3.9148'W, pointe du Cabellou) and in Vendée at La Tranche-sur-Mer (46.3461'N, 1.4189'W, La Grière). This last site is a sand-influenced rocky shore and sampling was restricted to mid-shore pools. At each site, *c.* 120 living specimens (blade up to 7 cm^2) growing attached to rocks were collected by hand and placed individually in small plastic bags. Samples were separated by at least several metres. In the lab, each specimen was rinsed with filtered seawater to remove epiphytes and debris. Following individual photography (Olympus Stylus TG-830), specimens were preserved at -80°C in individual plastic bags numbered with letters (sampling sites) and serial numbers.

DNA extraction, amplification and sequencing

Frozen tissue from each thallus was ground to a powder in liquid nitrogen. Whole genomic DNA was extracted from 0.3 mg of powder using the NucleoSpin Tissue Kit (Macherey-Nagel). The manufacturer's standard protocol for tissues was followed, except for the following steps: (1) an overnight tissue digestion in proteinase K and (2) DNA was eluted in two steps, each consisting of a 3 min incubation with 25 µl of dH_2O pre-heated at 70°C, for a final volume of 50 µl. DNA quality and quantity were assessed using a Nanodrop ND-2000 spectrophotometer (Thermo Scientific), a Qubit 1.0 (Thermo Scientific) fluorometer (dsDNA HS Assay Kit), and 1x agarose gel electrophoresis. Primers for *tuf*A were designed based on Saunders & Kucera (2010) but modified to reduce the number of degenerate bases using the complete *Ulva* chloroplast genomes available on Genbank on 16 March 2019 (Supplementary table S1). The nuclear internal transcribed spacer 2 (ITS2) together with the plastid ribulose 1, 5-bisphosphate carboxylase large chain (*rbc*L) gene were used to compare our sequences with the type material of *U. australis* housed in the Swedish Museum of Natural History (http://herbarium.nrm.se/specimens/A2025). The lectotype was characterized by Hanyuda & Kawai (2018) based on partial sequences of ITS2 (LC331301, 242 bp) and *rbc*L (LC331300, 197 bp). Following ITS2 and *rbc*L validations, further analyses of genetic variability used the intergenic regions of chloroplast *atp*I and *atp*H (*atp*I-H) and mitochondrial *trn*A and *trn*N (*trn*A-N) genes amplified with primers from Hanyuda *et al.* (2016). Primer sequences and PCR amplification cycles are presented in Supplementary table S1 and fig. S1. PCRs were carried out with a Sensoquest labcycler using the TaKara ExTaq reaction kit (Takara Bio). PCR amplicons were checked on a 1x agarose gel electrophoresis prior to purification and Sanger sequencing in both forward and reverse directions by Eurofins Genomics (Ebersberg-Germany: www.eurofinsgenomics.eu/).

Data analysis

For each DNA sequence, chromatograms were cleaned manually with Geneious Prime 2019 (www.geneious.com); primer sequences were trimmed and sequences were checked for the presence of ambiguities and stop codons. Forward and reverse chromatograms were then assembled and consensus sequences were submitted to Genbank (Accession Numbers in Supplementary table S2). Newly generated consensus sequences were compared with sequences available on Genbank and aligned using Muscle 3.8.425 (Edgar, 2004). Our last search on GenBank (17 October 2020) returned 181 *tuf*A sequences identified as *U. australis* (174 sequences) or *U. pertusa* (7 sequences) including the two recent releases of complete chloroplast genomes of *U. pertusa* LC507117 (Mitsuhashi *et al.*, 2020) and MN853875 (Han, direct submission 9 December 2019). Fifty-five *tuf*A sequences not yet available on Genbank were also utilized including our sequences (MT078952 from Roscoff and MT078953 from Concarneau), and 10 sequences manually retrieved from the doctoral dissertation of Melton (2017). Our final data set contained 219 sequences since 2 and 15 sequences were excluded due to inconsistencies and mismatches in sequence length, respectively (see details in Supplementary table S3). DnaSP v6 (Rozas *et al.*, 2017) was used to identify haplotypes from the 659 bp-long *tuf*A alignment performed on our data set. Uncorrected p distances were calculated from the *tuf*A alignment matrix using PAUP* v.4.0 (Swofford, 2002) and are presented as percentages. A median-joining network (Bandelt *et al.*, 1999) was constructed to visualize relationships among *tuf*A haplotypes using PopART with default settings (http://popart.otago.ac.nz). Two *tuf*A sequences of *U. fenestrata* were used as an outgroup as there is consistent support for a close taxon relationship of *tuf*A between *U. australis* and *U. fenestrata* (Kirkendale *et al.*, 2013; Hughey *et al.*, 2019; Kang *et al.*, 2019): HQ610325 from Saunders & Kucera (2010) and MK456404 as the holotype of *U. fenestrata* (Hughey *et al.*, 2019). Sequence similarity between the type material and our specimens for ITS2 and *rbc*L was calculated using BLASTn (Zhang *et al.*, 2000) with default parameters

(http://blast.ncbi.nlm.nih.gov/Blast.cgi). BLASTn was also used for Coat *et al.* (1998)'s molecular data for testing the two ITS sequences U.rot.4 (MT078968) and U.rot.5 (MT078969) erroneously labelled as *U. rotundata*. These two sequences with corrected labels were submitted to Genbank on behalf of B. de Reviers (Supplementary table S2). Correspondence between our specimens and the type material of *U. australis* using ITS2 and *rbc*L allowed the *atp*I-H and *trn*A-N intergenic sequences of our specimens to be aligned with, respectively, the 15 (haplotypes cH1 to cH15) and 27 (haplotypes mH1 to mH27) sequences defined by Hanyuda *et al.* (2016) as descriptive of the genetic diversity of native *U. australis* populations. All sequences were concatenated and analysed following Hudson *et al.* (1992) using DnaSP v6 (Rozas *et al.*, 2017) in order to detect haplotypes identical to any of the 50 combined haplotypes H1-H48 and H49-H50 described by Hanyuda *et al.* (2016) and Hanyuda *et al.* (2018), respectively. The metadata associated with *Ulva* sequences deposited in GenBank, including geographic location, were retrieved from flatfiles. Geographic information, when absent, was manually compiled from published references (Supplementary table S3). Maps were edited with ArcGIS ArcMap 10.6 (ESRI).

Results

Sequencing success across markers

A total of 339 *Ulva* samples were sequenced for *tuf*A. Forty-one samples matched the *U. australis* (or *U. pertusa*) *tuf*A sequences, and 298 represented other *Ulva* species (Table 1). Amplification and/or sequencing of ITS2 and *rbc*L were less successful. Of the 41 samples putatively assigned to *U. australis*, only 32 and 28 were successfully sequenced at ITS2 and *rbc*L, respectively. However, sequencing of the *atp*H-I and *trn*A-N intergenic regions was successful for all of the same 41 samples (Table 1). This last dataset was used to identify

which combined haplotypes among those previously described from Japan were also present in France.

tuf*A sequences*

The 41 specimens identified as *U. australis* were unevenly distributed among localities: 31 samples were from Roscoff and 10 from Concarneau; no *U. australis* specimens were detected from La Tranche in Vendée (Table 1). All 41 *tuf*A sequences (659 bp) are identical to 106 Genbank sequences, and differ by 1, 2 and 3 substitutions from 105, 6 and 1 sequences, respectively. Uncorrected p distances calculated between our sequences (from Roscoff and Concarneau) and *U. australis* sequences produced by others ranged from 0-0.46%.

The haplotype network revealed two well-separated groups, the *U. fenestrata* outgroup, and the other incorporating all *U. australis* haplotypes. Thirty-eight substitutions separated the *U. fenestrata* outgroup from its closest relative *U. australis*. Within the alignments for *U. australis*, there were 8 variable sites (Supplementary fig. S2) but only up to 3 substitutions per sequence (Fig. 1). The network of *U. australis* is star-like and included two very common haplogroups here named tuH0 and tuH1, which are associated with six rare haplotypes (Fig. 1). Our *tuf*A sequences from Brittany matched tuH0, the commonest (163 samples), recorded from Northern Asia (27%), Australasia (18%), North and South America (9%) and Western Europe (46%) including the Mediterranean Sea (Figs 1, 2). *Tuf*A sequences differing from tuH0 by one transition constitute the second haplogroup tuH1 (103 samples), from Northern Asia (91%), British Columbia (BC), California (5%) and Western Europe (4%). All other *tuf*A sequences (7 samples) with at least 2 mutations from tuH0 were from north-eastern Asia (Fig. 2) within the Yellow Sea from Donggang, China (Du *et al.*, 2014) to Jeju Island, Korea (Kang *et al.*, 2019; Lee *et al.*, 2019). For simplicity, these *tuf*A sequences were labelled tuH2 and tuH3, as they had 2 and 3 substitutions, respectively. Substitutions in

tuH2a and tuH2b haplotypes were at the 5' end of the *tuf*A gene and tuH2c, tuH2d, tuH2e and tuH3 haplotypes substitutions were at the 3' end (see Supplementary fig. S2). The two commonest haplogroups and five of the six rare haplotypes were all recorded in Asia (Fig. 1), from Jeju Island (Fig. 2, Yellow Sea map).

Visual inspection of 14 aligned sequences shorter than 659 bp (excluded from our data set) and two sequences from BC positioned in 349-1122 bp of the complete *tuf*A sequence (see GenBank numbers in Supplementary table S3) concurred with the assumptions that (1) both tuH0 and tuH1 haplogroups are present within the Bohai Sea, (2) tuH0 is recorded in Chile and (3) tuH1 occurred along both sides of the North Atlantic (Germany and Virginia, USA). However, one sequence (MH538644) collected in Schleswig-Holstein, Germany, near Cuxhaven at the mouth of the Elbe River (Steinhagen *et al.*, 2019) and two identical sequences (HQ610379 and HQ610380) from BC (Saunders & Kucera, 2010) differed from tuH0 by one and two transitions, respectively. They would represent two new additional haplotypes not shown here (Fig. 1) as the nucleotide substitutions are out of the 659 bp range of our analysis.

ITS2 and rbc*L regions*

Thirty-one of the 35 ITS2 sequences, 25 from Roscoff (MT078941) and 6 from Concarneau (MT078940), were identical to the ITS2 sequence from the lectotype of *U. australis* (Supplementary table S4). Two other sequences from Roscoff and one from Concarneau were poorer quality (50–92% of untrimmed based with phred score > 40). Two were < 184 bp (134 and 164 bp, respectively) and one Roscoff sequence had three ambiguities (S instead of C or G). Another sequence, from Concarneau, had one substitution (T instead of G at position 84). These variants could be due to the co-amplification of multiple alleles/copies of ITS2. The ITS1-5.8S-ITS2 sequences MT078968 (U.rot.4) and MT078969 (U.rot.5) originated from

Coat *et al.* (1998) and incorporated ITS2 sequences identical to that of the lectotype *U. australis*.

Among all the ITS2 sequences related to *Ulva australis* and/or *U. pertusa* available in GenBank (Supplementary table S4), 172 sequences from the literature (80 *U. australis* and 92 *U. pertusa*) fully matched the ITS2 of lectotype material. Only six additional ITS2 sequences revealed intra- and inter-individual variation with one (5 specimens) to three (1 specimen) substitutions or indels. AJ234321, initially labelled *U. pertusa* but misidentified as *U. fasciata* (Hayden *et al.*, 2003), differed from the lectotype of *U. australis* at 25 positions, and has the lowest percentage identity with the lectotype ITS2.

In contrast to ITS2, the 28 *rbc*L sequences from France (195 bp) displayed no variation, were identical between Roscoff (MT078958) and Concarneau (MT078959), and showed 100% similarity with the lectotype *rbc*L gene sequence of *U. australis*.

atp*I-H and* trn*A-N marker regions*

For each marker, three haplotypes were detected, resulting in four combined haplotypes, three identified as H5, H42, H44 by Hanyuda *et al.* (2016), and a new combination H51 (Table 1). Two combined haplotypes, H42 and H44, were recorded at Roscoff, the former with 90% relative abundance. At Concarneau, H5, H42 and H51 were recorded. Both H42 and H51 are rare (10% relative abundance; Table 1, Fig. 3). H42, shared by Roscoff and Concarneau, was previously reported from the Thau Lagoon (Mediterranean Sea) by Hanyuda *et al.* (2016); haplotype H5 was previously observed in the Netherlands (Fig. 3).

Discussion

Conspecificity of Ulva australis *and* Ulva pertusa

Since its first morphological description by Kjellman from Japan in 1897, and until the recent work of Couceiro *et al.* (2011), the specific status of *Ulva pertusa* has not been seriously challenged (see discussion in Tanner, 1979). Very common in Japan, *U. pertusa* is polymorphic (Okamura, 1921) even including 'stalked-*Ulva*' forms (Hiraoka *et al.*, 2003) and floating green tides (Yoshida *et al.*, 2015). In contrast, the status of the South Australian *U. australis*, which was described before *U. pertusa*, has long been controversial. Several nomenclatural synonyms have been proposed, including *U. rigida* C. Agardh (Agardh (1883) and *U. lactuca* Linnaeus by Womersley (1956, p. 354) following the view that *U. lactuca* is a cosmopolitan species with multiple varieties. Contradictory opinions on *U. australis* were finally clarified using ITS1 and *rbc*L analyses on Spanish, Japanese and Australian specimens (Couceiro *et al.*, 2011). Molecular evidence of the identity of *U. australis* and *U. pertusa* presented by Couceiro *et al.* (2011) corroborates previous results of Kraft *et al.* (2010), who distinguished a clade of *U. australis* and *U. pertusa* from a more speciose clade with *U. laetevirens*, *U. rigida* and *U. scandinavica* using ITS and *rbc*L markers. They reinstated *U. australis* as a valid species and suggested *U. pertusa* as its junior synonym (see details in the Appendix S1 of Kraft *et al.*, 2010). Kirkendale *et al.* (2013) confirmed this view using *rbc*L and *tuf*A markers.

Despite known limitations associated with the use of ITS (Saunders & Kucera, 2010), more recent advances in secondary structural analysis of ITS2 have advocated the use of ITS2 as a potential DNA barcode for the class Chlorophyceae (Buchheim *et al.*, 2011). Species identification of *Ulva* specimens based on ITS2 (Hanyuda *et al.*, 2016) also supports the conspecificity of *U. australis* and *U. pertusa*. All available ITS2 sequences labelled as *U. australis* or *U. pertusa* in GenBank (Supplementary table S4) are identical or nearly identical to the historic lectotype material of *U. australis* characterized with ITS2 and *rbc*L by Hanyuda & Kawai (2018). The inter-specimen variability *i.e.* 1 to 6 substitution(s) here

reported from this large data set is consistent with previous analyses made on a more restricted data set (Melton, 2017, fig. 3.11, p. 98). Our synopsis of ITS2 data suggests misidentification of two specimens previously labelled *U. fasciata* (AF099728) and *U. lactuca* (AF099729), and their molecular identification as *U. australis* (100% match with the lectotype ITS2 sequence). Both were collected in Victoria, Australia (Woolcott & King, 1999). Until the recent study of Hughey *et al.* (2021), the type material of *U. pertusa* was thought to be unavailable (Tanner, 1979, p. 219). However, both ITS and *rbc*L sequences are available from specimens collected at Yokohama (Shimada *et al.*, 2003), one of three syntype Japanese localities, together with Hakodate and Yenoshima (Kjellman, 1897): ITS2 (AB097657) and *rbc*L (AB097627) sequences from the specimen voucher SAP:095069 were identical to ITS2 (LC331301) and *rbc*L (LC331300) sequences from the lectotype of *U. australis*, respectively. Most recently, Hughey *et al.* (2021) retrieved the type material of *U. pertusa* from the Museum of Evolution (UPS) at Uppsala University, and confirmed that *U. pertusa* is conspecific with *U. australis* on the basis of *rbc*L gene sequences from one newly designated lectotype (MT815855) from Yenoshima and two syntype specimens (MT815856, MT815857).

A large body of literature shows the high level of genetic similarity in *rbc*L gene sequences between populations of *U. australis* and *U. pertusa* from Australia (Kraft *et al.*, 2010; Kirkendale *et al.*, 2013), BC-Canada (Saunders & Kucera, 2010), California (Hayden *et al.*, 2003; Hayden & Waaland, 2004), Chile (Oróstica *et al.*, 2017), China (Du *et al.*, 2014; Kang *et al.*, 2014), Japan (Matsumoto & Shimada, 2015), Korea (Kang *et al.*, 2014), Italy (Wolf *et al.*, 2012) and Spain (Baamonde López *et al.*, 2007; Couceiro *et al.*, 2011).

Synopsis of French records

Ulva australis is now widespread within the Thau lagoon (Mediterranean Sea), particularly within oyster farming zones (Verlaque *et al.*, 2002), and is one of the commonest macroalgal NIS species, as *U. pertusa*, in the lagoon (Boudouresque *et al.*, 2011). The first record is thought to be the herbarium specimen labelled *Ulva* sp. from Thau in 1984 collected by Ben Maiz (see details and references in Verlaque *et al.*, 2002). Molecular identification of *U. australis* at Thau was recently confirmed (Hanyuda *et al.*, 2016). Along the French Atlantic coast, *U. australis* was first reported as *U. pertusa* from Arcachon Bay in 2005–2006 (Verlaque *et al.*, 2008), and as *U. australis* from Port of Le Havre in 2012 (Verlaque & Breton, 2019). All these records were based on morphological diagnoses (Okamura, 1921; Verlaque *et al.*, 2002), and as pointed out by Verlaque & Breton (2019), still required molecular confirmation. To the best of our knowledge, the first molecular sequences came from Brittany (Coat *et al.*, 1998), including two ITS1-5.8S-ITS2 sequences misidentified as *U. rotundata* Bliding. They are representative of at least six specimens collected from Roscoff (attached thalli from Île Verte, Embarcadère and Pointe du Bloscon) plus one specimen from Lannion Bay (floating thalli during a green tide event at Plestin-les-Grèves) in 1994–1995. Woolcott & King (1999) clearly suspected the *U. rotundata* specimens to be misidentified as they wrote 'There are again strong molecular similarities between Australian isolates here' i.e. *U. australis* 'and European *Ulva* species in Coat *et al.* (1998)'. This taxonomic inconsistency was also highlighted by Malta *et al.* (1999) following a comparison of ITS between *U. rotundata* from Coat *et al.* (1998) and specimens labelled *U. rigida* from Flinders, Australia (UrigAus: AF153494, collected by van Oppen) and Oosterschelde, The Netherlands (UrigOS: AF153490). Blastn comparisons among these sequences confirmed their similarity (Supplementary table S4). On the other hand, Shimada *et al.* (2003) noted that *U. rotundata* from Brittany clustered with *U. pertusa* from Japan in phylogenetic ITS1-5.8S-ITS2 trees. Hayden & Waaland (2004) highlighted a similar grouping in ITS and *rbc*L trees of *U.*

rotundata from Britanny and *U. pertusa* specimens from Japan and California. Baamonde López *et al.* (2007) first recognized the presence of *U. pertusa* in Europe based on the molecular data of Coat *et al.* (1998) following Shimada *et al.* (2003) and Hayden & Waaland (2004). This conclusion was also accepted by Flagella *et al.* (2010) before the final suggestion that *U. pertusa* is a synonym of *U. australis* (Couceiro *et al.*, 2011). Molecular identification of *U. australis* at Roscoff was also confirmed by Hanyuda *et al.* (2016).

Our observations of *U. australis* at Roscoff from the same sampling sites as those selected by Coat *et al.* (1998) suggest that the species has been present at Roscoff over the past 25 years. Concarneau (Pointe du Cabellou) is a new locality for *U. australis* in Southern Brittany (Fig. 3, Table 1). Observations here supplement previous reports of *U. australis* in Brittany (Fort *et al.*, 2020a, 2020b) during spring and summer 2018 green tides at BegMeil (near Concarneau), Bay of Brest (Moulin Blanc), Roscoff and Lannion Bay (Plestin les Grèves) (Fig. 3), demonstrating the relative dominance of *U. australis* in green tides in Brittany. These observations are scattered both temporally and spatially (Fig. 3) suggesting that the distribution of *U. australis* along the French Channel, Bay of Biscay (from Brest to Biarritz) and Mediterranean coasts might be more continuous than presently reported.

Current distribution of **Ulva australis** *in Europe*

Ulva australis has been reported from Germany, the Netherlands, Ireland, Spain and Italy incorporating the oldest records and corrected misidentifications as *U. pertusa* (Baamonde López *et al.*, 2007; Couceiro *et al.*, 2011). The first records in the German Wadden Sea were in Helgoland and Schleswig-Holstein in 2014 (Steinhagen *et al.*, 2019). Records from the Netherlands came from the Oosterschelde at least from 1995 (Stegenga *et al.*, 2006), the Dutch Delta area (Fort *et al.*, 2018, 2019, 2020a), where *U. pertusa* was recognized in herbarium specimens collected in the area as early as 1993 (Wolff, 2005 and references

therein), the entrance of Rotterdam port (Gittenberger *et al.*, 2014) and the Dutch Wadden Sea including major ports (Gittenberger *et al.*, 2015). Stegenga *et al.* (1997, p. 42 note n° 8) mentioned the occurrence of an aberrant *Ulva* species with a thicker thallus than *U. rigida* in Zeeland, referencing a 1984 collection, but it may not be represented in the material housed at the Leiden Herbarium (Stegenga, pers. comm. 19 Feb. 2020). *Ulva australis* has also been reported from Galway, Ireland since 2018 (Fort *et al.*, 2020*a*). In Spain, the oldest record was morphologically recognized from a herbarium specimen collected from La Coruña (Galicia) in 1990 (Baamonde López *et al.*, 2007). Spanish records of live specimens came from both the Atlantic (Fort *et al.*, 2020*b*) and Mediterranean coasts (Couceiro *et al.*, 2011). The first Italian records were from Venice in 2010 (Manghisi *et al.*, 2011) and *U. australis* was widespread within the lagoon in 2011 (Wolf *et al.*, 2012). *Ulva pertusa* at Naples was mentioned by Wolf *et al.* (2012) and Cormaci *et al.* (2014) according to Flagella *et al.* (2010). A closer examination of the Flagella *et al.* (2010) data suggests an erroneous interpretation of their ITS1 phylogenetic tree. Only *U. ohnoi* Hiraoka and Shimada and *U. fasciata* Delile, today known as *U. lactuca* Linnaeus (Hughey *et al.*, 2019), were reported from ballast waters in the Neapolitan harbour.

Worldwide genetic variability in U. australis tuf*A*

Among the eight *tuf*A haplotypes (659 bp) found in this study (Fig. 1), seven matched those reported from Jeju Island (Kang *et al.*, 2019; Lee *et al.*, 2019). The eighth was found only at Donggang Rizhao, China, some 700 km northeast in the Yellow Sea (Du *et al.*, 2014). At Jeju Island, Lee *et al.* (2019) reported seven haplotypes for *tuf*A including one haplotype with three substitutions from tuH0 not included in our analysis. All these results support a scenario of a drastic population bottleneck following the introduction of *U. australis* outside its native range: (1) a high level of genetic diversity for *tuf*A within the native area (Kang *et al.*, 2019;

Lee *et al.*, 2019), (2) the predominance of a unique haplogroup (tuH0) worldwide and (3) the occurrence of a second one (tuH1) in a few European and North American countries (Figs 1, 2). According to Rius *et al.* (2015), a founder effect is not the most common scenario in marine invasions. Introduced populations exhibiting similar or even higher genetic diversity than native populations have been described, as multiple introductions from large native regions with highly genetically structured populations can lead to increased diversity at introduction sites (e.g. Simon-Bouhet *et al.*, 2006). Nonetheless, all results for *tuf*A from Australia (Kirkendale *et al.*, 2013) and New Zealand (Lawton *et al.*, 2013, 2020) showed a lack of genetic diversity (only tuH0, Figs 1, 2). This supports previous findings that *U. australis*, despite its abundance and spread along these temperate coasts, is a NIS in Australia (Kirkendale *et al.*, 2013) and New Zealand (Heesch *et al.*, 2009). A similar conclusion was reported for combined genetic markers *atp*I-H and *trn*A-N in *U. australis* from Australasia compared with Japan and Korea (Hanyuda *et al.*, 2016, 2018) : 1 and 5 combinations in Australia and New Zealand, respectively *vs.* 48 in both Japan and Korea.

The first records of *U. australis* as *U. pertusa* in North America are recent and were validated by molecular analyses in BC in 2005-2006 (Saunders & Kucera, 2010) and California in 1999 (Hayden & Waaland, 2004). Aguilar-Rosas *et al.* (2008) also recorded *U. australis* (determined by ITS2 sequences) from Baja California, Mexico in 2006-2007 and suggested the species to be a NIS in range expansion *via* transoceanic shipping. Along the Eastern Pacific coast, two *tuf*A haplotypes (tuH0 and tuH1) were observed (Figs 1, 2), in Canada (Saunders & Kucera, 2010) and Chile (Melton, 2017; Oróstica *et al.*, 2017), but only tuH1 in California (Saunders, 2014). However, visual checks of Saunders & Kucera's (2010) *tuf*A sequences (774 bp) suggested the occurrence of a third haplotype exclusive to BC, Canada, and not recorded from Korea (Kang *et al.*, 2019; Lee *et al.*, 2019). This may support the view of repeated introductions into BC) waters from different Asian populations. Scarcity

of full-length *tuf*A data from Japan (Mitsuhashi *et al.*, 2020) hinders the discussion of this hypothesis for now. The status of *U. australis* along the North-eastern Pacific coast as a NIS or a native (Amphi-Pacific with disjunct temperate areas) was already questioned by Miller *et al.* (2011). Among vectors involving transoceanic transport of NIS from the western to the eastern North Pacific ocean, the oyster trade from Japan and international shipping are major invasion vectors (Carlton, 1987; Wonham & Carlton, 2005), and are well documented in terms of biogeography and timing. *Ulva australis* is a common fouling alga on the oyster *Magallana gigas* (Thunberg) cultivated on rafts in Japan (Miyazaki, 1938). Successful introductions of oyster seed from Japan to BC and Puget Sound started at the beginning of the 19th century and lasted to the 1970s (Ruesink *et al.*, 2005). Oysters originated from the main Japanese farming centres *i.e.* Miyagi-Sendai (East Japan), Nagasaki-Kumamoto and Hiroshima (SW Japan) (Fujiya, 1970). Maritime transport between Japan and the USA is well-documented in terms of historical changes in port hierarchy and geographic specializations (Maurette, 1922; Ducruet, 2015). Yokohama and Osaka-Kobe have been the main Japanese ports involved in the trade with Western and Eastern overseas countries since the mid-19th century (Maurette, 1922). However, natural dispersal of *U. australis* may also have contributed to its wide amphi-Pacific distribution as tsunamis are known to occur on the scale of geological times (Dawson & Stewart, 2007), particularly in seismically active coasts like those of Japan (Garrett *et al.*, 2016). The 2011 Great East Japan Earthquake and Tsunami was responsible for the arrival of huge amounts of marine debris on North-eastern Pacific shores, including derelict boats fouled with *U. australis*. Use of combined haplotypes *atp*I-H and *trn*A-N (Hanyuda *et al.*, 2016) elucidated the biogeographic origin of these *U. australis* stranded on the Oregon shores since their combined haplotypes matched those found in Iwate Pref.-Northern Japan (Hanyuda *et al.*, 2018). Finally, the amphi-Pacific map of these combined haplotypes in *U. australis* highlighted the wide distribution of the unique haplotype

H5 (Hanyuda *et al.*, 2016, Fig. 1). This surprisingly fully matches the aquaculture history of oyster transfers from the East coast of Japan (Miyagi Pref.) to Australasia and Americas (Ruesink *et al.*, 2005 and references therein for Australia, New Zealand and Americas). Known secondary transfers of oysters within American countries, *e.g.* USA to Chile (Ruesink *et al.*, 2005) do not contradict the view of contemporary oyster transfers as a major vector for *U. australis* within the Pacific region.

Challenging the introduction history of* Ulva australis *in France

In Europe (France and the Netherlands), two combined haplotypes H5 and H42 (Fig. 3) were previously found, but their distinct geographic distribution suggested repeated introduction events (Hanyuda *et al.*, 2016). Haplotype H5 was found in the Netherlands (fig. 1 in Hanyuda *et al.*, 2016) in *U. australis* collected in 2005 within the Oosterschelde in the vicinity of Yerseke, which has been the main *Magallana gigas* culture centre since the mid 1960s in the Netherlands (Haydar & Wolff, 2011). Haplotype H5 was found along the Miyagi (Japan), BC and west USA coasts (fig. 1 in Hanyuda *et al.*, 2016), regions known to be the biogeographic primary sources of imported *M. gigas* into the Oosterschelde (Ruesink *et al.*, 2005). At Concarneau, 80% of *U. australis* specimens harboured the same H5 (Table 1, Fig. 3), in line with this interpretation. Indeed, the rebound of the French oyster industry was linked to the national RESUR operation based on massive and repeated imports of *M. gigas* adults from BC and spat from Miyagi in the 1970s (Grizel & Héral, 1991).

However, all other combined haplotypes we found at Concarneau (H42 and H51) and Roscoff (H42 and H44) (Table 1) conflict with the above view of the oyster trade as a unique vector of introduction of *U. australis* into Brittany. Previous results obtained from Roscoff and Thau by Hanyuda *et al.* (2016) (only H42 found in specimens collected in 2005 and 2002 at Roscoff and Thau, respectively, Fig. 3) also strengthen this conflict and furthermore

challenge the initial suggestion that *U. australis* could have been introduced into the Thau lagoon as early as the 1970s *via* oyster *M. gigas* transfers in 1971-1976 (Verlaque *et al.*, 2002). Based on the distribution map of the combined haplotypes H42 and H44 in Japan and Korea (fig. 1 in Hanyuda *et al.*, 2016), it is suggested that *U. australis* found at Concarneau, Roscoff and Thau originated from Kobe (Japan) and/or Southeastern Korea. A closer examination of microsatellite genotypes (fig. 2 in Hanyuda *et al.*, 2016) identifies Kobe Port as the most likely origin of *U. australis* populations at Roscoff, although the genotype found at Thau was more geographically spread (from Kobe to Kumamoto in Japan and in Southeastern Korea). The finding of a new H51 haplotype at Concarneau does not contradict this view. According to Hanyuda *et al.* (2016) this may reflect a mutation that arose since the first introductions or, more likely, insufficient sampling in the native range as further exemplified by the study of tsunami marine debris (Hanyuda *et al.*, 2018). These results indicate maritime transport between Japan (at least) and France as another vector for the introduction of *U. australis* into Mediterranean and Atlantic waters. Aguilar-Rosas *et al.* (2008) and Couceiro *et al.* (2011) similarly suggested the arrival of *U. australis* was linked to maritime vessel traffic in Baja California and the Strait of Gibraltar, respectively.

We thus speculate that these repeated and independent events have been linked to the maritime trade between France and Southeast Asia in the late 19th or early 20th centuries. The maritime history of Japan was profoundly reorganized during the Meiji era (1867–1912) and a new foreign trade policy was initiated for 1868 (Horie, 1952) that actively promoted cooperation and business with the USA and Colonial Countries. The opening of the Suez Canal on November 1869 also offered new shipping routes between Europe and Asia (Fletcher, 1958; Berneron-Couvenhes, 2007), and favoured maritime companies from leading European countries *i.e.* Great Britain, France, The Netherlands, Italy and Germany (Miller, 2012). For instance, the ‘Compagnie des Messageries Maritimes’ set up in 1851 at Marseille

operated several ship lines among Yokohama, Kobe, Shanghai, Hong-Kong, Singapore, Calcutta, Mumbai and Marseille (Berneron-Couvenhes, 2007; Metzger, 2009). Historical analysis of global maritime transports since the late 19th century *de facto* highlighted the leading role of Yokohama and Kobe ports together with the emerging role of Chinese ports in the early 20th century (Ducruet, 2013), and the leading role of Marseille and Le Havre, as the main French port at that time (Ducruet & Marnot, 2016). Long-term port activities are under the control of regional conflicts and world wars (Ducruet & Marnot, 2016). Numerous wrecks from trading ships from Japan from WWI are known from the Mediterranean and the Atlantic (https://uboat.net/wwi/ships_hit/; see also Hiroaki, 2015). Changes in the northeastern Asian port hierarchy between Japan, Korea and China have also been noticeable since the 1980s (Ducruet *et al.*, 2011; Ducruet, 2013), so contemporary European records of *U. australis* may be linked to vessel traffic originating from ports within the Bohai and Yellow Seas. More phylogeographic studies along Korean and Chinese coasts within the native area of *U. australis* are required to test these hypotheses.

The patchy records of *U. australis* along the French coasts suggest that its presence is underestimated, mostly due to historical misidentifications as *Ulva* species show great morphological diversity and plasticity. The use of molecular markers for population genetics and biogeography is a key tool contributing to a better interpretation of the introduction history of *U. australis*. Using markers alongside systematic field sampling using large sample sizes and analysing herbarium specimens will lead to a much deeper understanding of the biogeographic origin of introduced populations, vector hierarchy and timing of introductions. Including an interdisciplinary exploration of human maritime activities is crucial since coastal macroalgal biodiversity has been heterogeneously reshaped over centuries by transport and aquaculture activities.

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Supplementary information

Supplementary information can be found online at http://dx.doi.org

Supplementary table S1. Primers used for amplification and sequencing.

Supplementary table S2. List of specimens deposited in GenBank.

Supplementary table S3. List of Genbank sequences at *tuf*A used in the haplotype network.

Supplementary table S4. List of Genbank sequences at ITS2.

Supplementary fig. S1. Conditions of PCR amplification cycle for each DNA region.

Supplementary fig. S2. Schematic diagram of *tuf*A sequence variability in *U. australis*

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Author contributions

P.-G. Sauriau: original concept, fieldwork, analysis of molecular data, drafting and editing manuscript; M. Dartois: original concept, fieldwork, molecular analysis, analysis of molecular data, drafting and editing manuscript; V. Becquet: molecular analysis, drafting and editing manuscript; F. Aubert: fieldwork, laboratory analysis; V. Huet: fieldwork, laboratory analysis; M. Bréret: macroalgae taxonomy, laboratory analysis; A. Viricel: analysis of molecular data, drafting and editing manuscript; E. Pante: original concept, molecular analysis, analysis of molecular data, drafting and editing manuscript.

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Table 1: Combined haplotypes (H) of chloroplast (cH) and mitochondrial (mH) haplotypes found in *Ulva australis* following numbering by Hanyuda *et al.* (2016). *: new combination neither described by Hanyuda *et al.* (2016) nor Hanyuda *et al.* (2018), -: no record.

Chloroplast haplotypes	Mitochondrial haplotypes	Combined haplotypes	Site (number of samples)		
			Roscoff (31)	Concarneau (10)	La Tranche (0)
cH3	mH3	H5	-	8	-
cH11	mH25	H42	28	1	-
cH11	mH26	H44	3	-	-
cH4	mH3	H51*	-	1	-

Fig. 1: Median-joining network of t*uf*A haplotype (659 bp) in *Ulva australis*. Grey: *U. fenestrata* as the outgroup with MK456404 as the holotype of *U. fenestrata* (Hughey *et al.*, 2019) and HQ610325 from Saunders & Kucera (2010); tick mark: one mutation; *U. australis* *tuf*A haplotype labels and Genbank accession numbers: see Supplementary figure S2 and table S3.

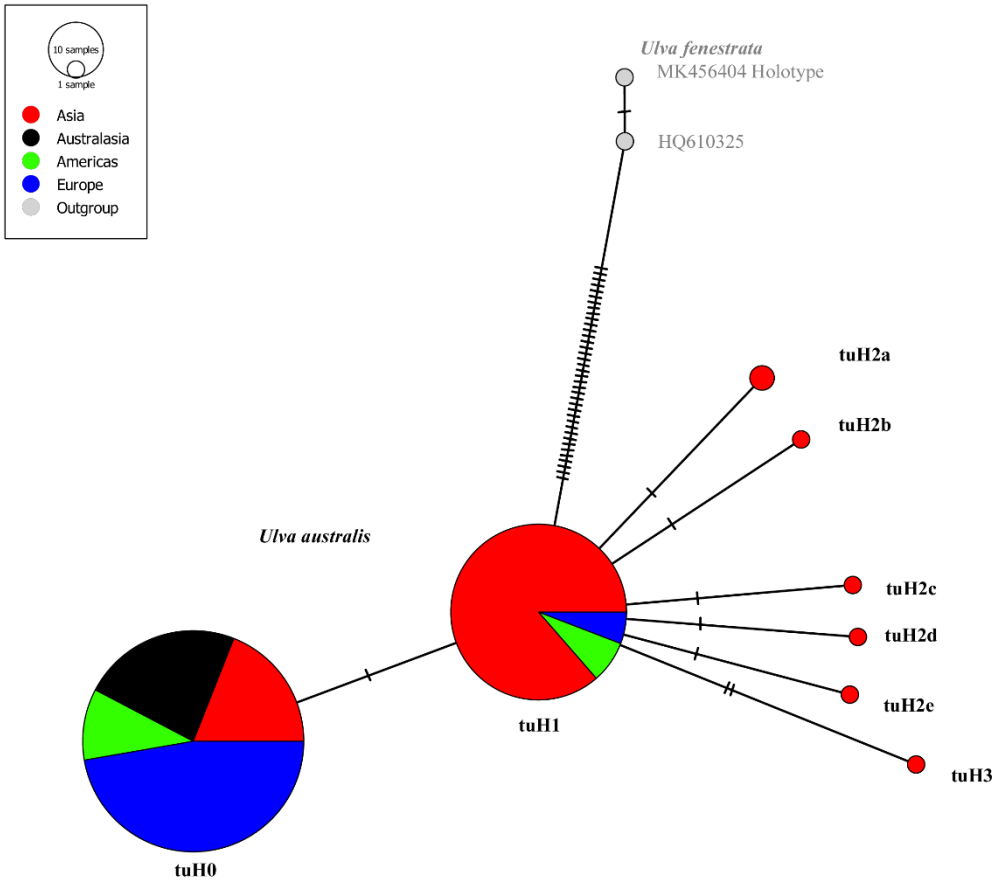

Fig. 2: Map of sampling sites showing the relative occurrence of the *tuf*A haplotypes (659 bp) detected for *Ulva australis.* Labels tuH0-tuH3 are named based on the number (0-3) of substitutions in *tuf*A sequences (see Supplementary figure S2). Other markers than *tuf*A and studies based on morphological observations: red and blue cross, respectively.

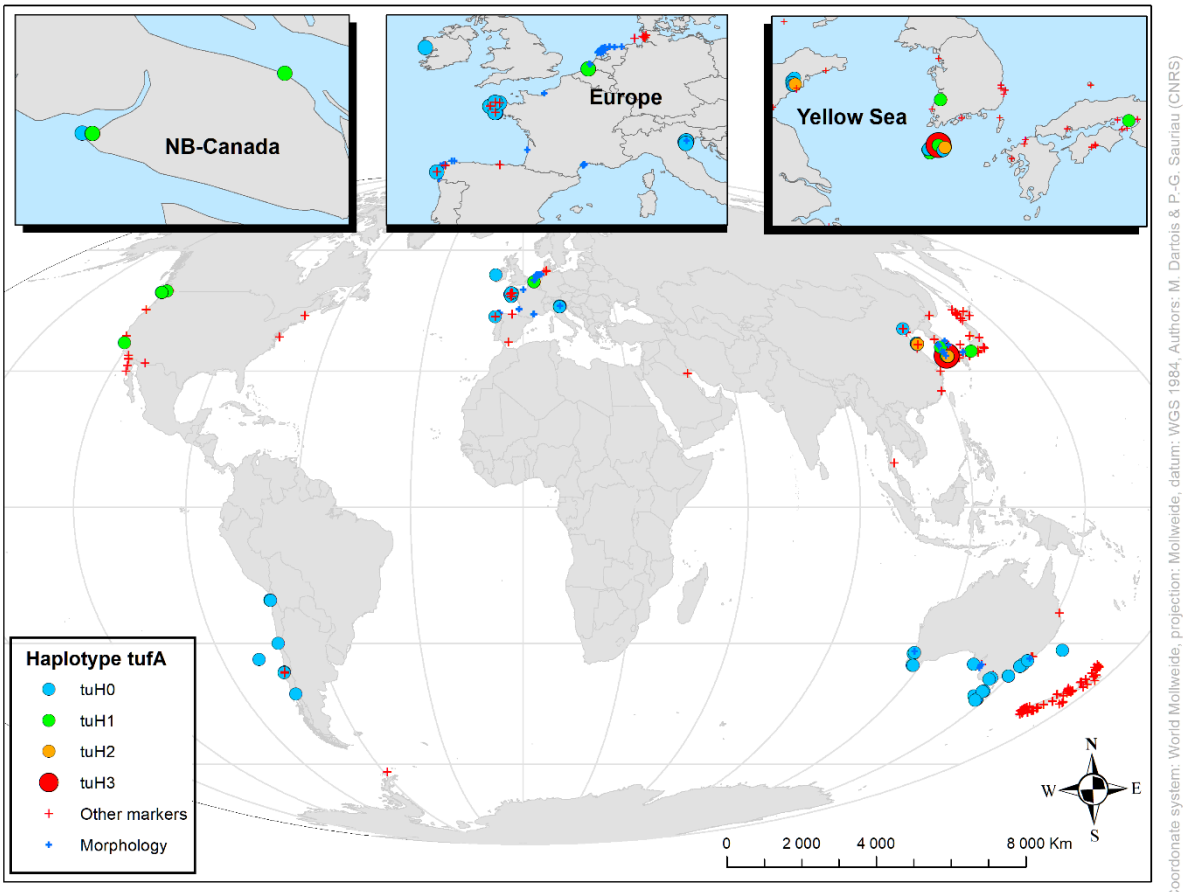

Fig. 3: Map of combined *atp*I-H and *trn*A-N haplotypes in western Europe. Square: this study, circle: data from Hanyuda *et al.* (2016), red and blue crosses: as in Fig. 2.