

When invaders go unnoticed: the case of *Gracilaria vermiculophylla* in the British Isles

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Abstract – Our knowledge of non-native algae in benthic estuarine habitats is relatively poor, especially compared to algal introductions along open shores or on floating structures. *Gracilaria vermiculophylla* is a widespread macroalgal invader in the temperate estuaries of the Northern Hemisphere, and, here, we expand its documented range within northeastern Ireland and England. Established populations occur within two inlets in the border counties, Carlingford Lough (Counties Louth and Down) and Dundrum Bay (County Down), but *G. vermiculophylla* is absent from open coasts between these sites. Repeated surveys in Dundrum Bay showed variable abundances, with an increase in biomass between 2013 and 2016. Three populations were discovered in England, where this species had not previously been identified: Christchurch Harbour (Dorset), Brownsea Island in Poole Harbour (Dorset), and Kingsbridge Estuary (Devon). The Irish and English thalli belong to the most common, invasive *cox1* haplotype 6. Using a combination of morphological observations and 10 microsatellite loci, we found that the population at Carlingford Lough included both reproductive haploid gametophytes and diploid tetrasporophytes and genetic signatures of sexual reproduction, but the populations at Christchurch and Brownsea displayed signatures of partial clonality. Genetic diversity was higher along the south coast of England as compared

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to the Irish population, consistent with patterns of diversity previously described for the European coasts. Finally, we also note the occurrence of a putative *G. vermiculophylla* population in Wales at Porthmadog, Gwynedd. As the sites in which we have now documented *G. vermiculophylla* in the British Isles also host shellfish aquaculture activities, our study is further evidence for the role of aquaculture in the spread of invasive species.

Algae / biological invasion / *cox1* / estuaries / *Gracilaria vermiculophylla* / microsatellites / reproductive mode

INTRODUCTION

Biological invasions can profoundly impact native communities and ecosystems, though studies in the marine environment lag behind their terrestrial and aquatic counterparts (Molnar *et al.*, 2008). Traditionally, marine invasion research has been focused on estuarine habitats in which human activities, such as shipping and oyster aquaculture, are common (Preisler *et al.*, 2009). Estuaries tend to have lower native species richness than open coasts (Elton, 1958), and are semi-closed, allowing for the retention of small populations (Wasson *et al.*, 2005), thereby facilitating successful invasions.

Estuarine habitats, however, lack a strong tradition of phycological research, particularly for marine macroalgae. Although hard substratum is required for spore attachment, free-floating macroalgal populations derived from attached thalli are not uncommon in these habitats due to hydrodynamic conditions in which vegetative propagation (a common characteristic of biological invaders, e.g., Kolar & Lodge, 2001) can maintain biomass (Norton & Mathieson, 1983). Macroalgal invaders have been often overlooked and understudied largely as a result of the lack of diagnostic morphological characters with which to easily discriminate among species or even genera (e.g., Gurgel & Fredericq, 2004).

The invasion of the red seaweed *Gracilaria vermiculophylla* (Ohmi) Papenfuss can be considered as a case study of an invader going unnoticed. Recently, Hu & Lopez-Bautista (2014) reviewed the myriad of studies that have addressed phenotypic patterns of broad environmental tolerance exhibited by *G. vermiculophylla*. In addition to eurytolerance (Weinberger *et al.*, 2008), the capacity to undergo extensive vegetative fragmentation has facilitated the invasion of soft-sediment habitats (Krueger-Hadfield *et al.*, 2016). Krueger-Hadfield *et al.* (2016) found that in the native range, along Chinese, South Korean, and Japanese coastlines, both haploid gametophytic and diploid tetrasporophytic thalli were predominantly fixed to hard substratum by holdfasts, and genetic signals of vegetative propagation were negligible. In contrast, in the non-native range, tetrasporophytic thalli dominated, reaching up to 100% cover in sampled quadrats (Thomsen *et al.*, 2009; Nejrup & Pedersen, 2010; Byers *et al.*, 2012) and multiple signals of vegetative fragmentation were detected (Krueger-Hadfield *et al.*, 2016, see also Gerstenmaier *et al.*, 2016). The combination of eurytolerance and vegetative fragmentation has enabled this alga to profoundly transform estuarine ecosystems, where it has been shown to have both positive (e.g., Kollars *et al.*, 2016) and negative effects on native communities (e.g., Freshwater *et al.*, 2006).

Despite the scale of the invasion, populations of *Gracilaria vermiculophylla* are still being documented in bays and estuaries throughout the Northern Hemisphere

where this seaweed was often previously recorded as a native gracilarioid (Krueger-Hadfield *et al.*, 2017, S.A. Krueger-Hadfield, *unpubl. data*). For example, in Virginia, *G. vermiculophylla* was assumed to be the native congener *G. tikvahiae* (Thomsen *et al.*, 2006), largely due to the phenotypic plasticity observed in the latter species (Hanisak *et al.*, 1988). Due to a lack of diagnostic features and equivocal taxonomy in the Gracilariales (Gurgel & Fredericq, 2004), the current distribution of this non-native ecosystem engineer is still poorly known.

In Atlantic Europe, *Gracilaria vermiculophylla* is reported to be spreading rapidly (Nyberg *et al.*, 2009). Since 2008, we have been following the development of populations of a gracilarioid alga in Carlingford Lough, northeastern Ireland that was presumed to be *G. vermiculophylla*, but never definitively identified (C.A. Magill & C.A. Maggs, *unpubl. data*). Recently, we discovered a morphologically similar species at three sites on the south coast of England. The aims of this study were to (1) confirm the identity of this gracilarioid alga in Ireland and England using the DNA barcode marker *cox1*; (2) document its spread and abundance in northeastern Ireland; and (3) investigate the reproductive mode of populations that are dominating shallow subtidal algal communities in the British Isles.

MATERIALS AND METHODS

Study sites and surveys of distribution and abundance

Carlingford Lough, Co. Louth (south shore), Co. Down (north shore), northeastern Ireland: The Lough is a glacial fjord between Northern Ireland and the Republic of Ireland (Table 1; Fig. 1) and is used extensively for aquaculture (mostly oysters and mussels), with about 24% of the seabed licensed, and about 9% occupied (AFBI,

Table 1. Sampling sites and dates, showing the number of thalli sampled and genotyped and the collectors for sites in the British Isles to which *Gracilaria vermiculophylla* has been introduced

| Site | Latitude, Longitude | Date | No. of thalli sent to the USA | No. of thalli sequenced with <i>cox1</i> | No. of thalli genotyped with <i>μsats</i> | Collectors |
|------------------------------------------------|----------------------------------|------------|----------------------------------------|---------------------------------------------------|-------------------------------------------------|----------------------------------------------|
| Carlingford Lough, Co. Louth, Ireland | 54° 2'5.72" N; 6° 9'42.20" W | 2.09.2014 | 22 | 8 | 22 | C.A. Maggs, C.L. Magill |
| River Glaslyn, Porthmadog, Wales | 52° 55.33602' N; 4°7.17906' W | 23.07.2017 | 1 | 0 | 0 | F. St. P. D. Bunker |
| Batson Creek, Kingsbridge Estuary, Devon | 50°14'34.5" N; 3°46'8.5" W | 21.08.2015 | 1 | 1 | 0 | S.A. Krueger- Hadfield, N. Mieszkowska |
| Brownsea Island Lagoon, Dorset | 50°41'20.9" N; 1°58'43.6" W | 9.10.2016 | 16 | 8 | 14 | C.A. Maggs |
| Christchurch Harbour, Dorset | 50°43'5.13"N, - 1°45'37.08"W | 11.10.2015 | 100 | 8 | 32 | C.A. Maggs |

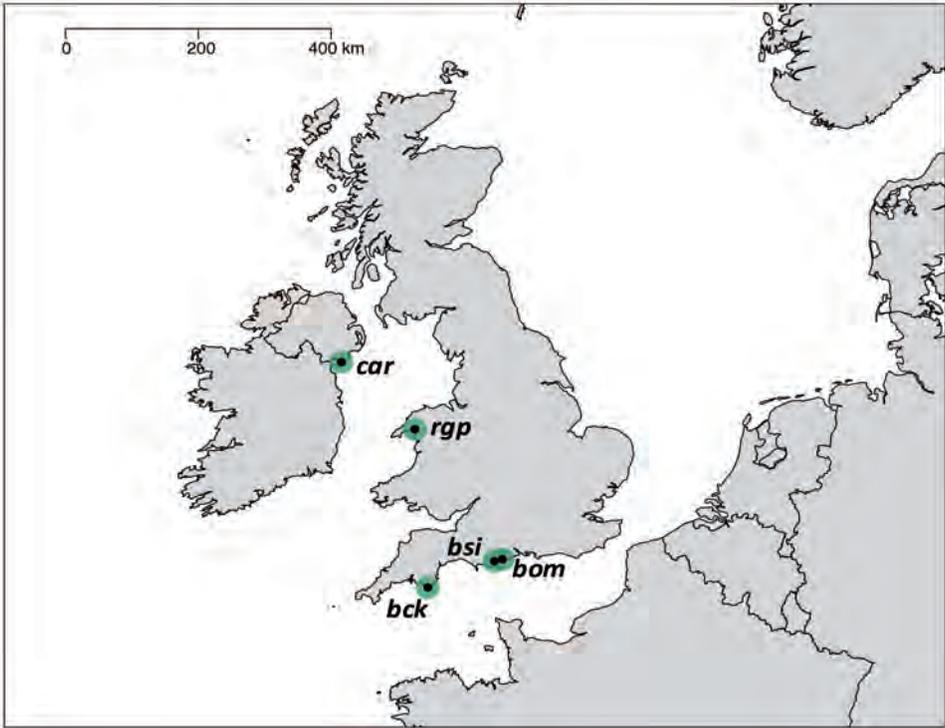
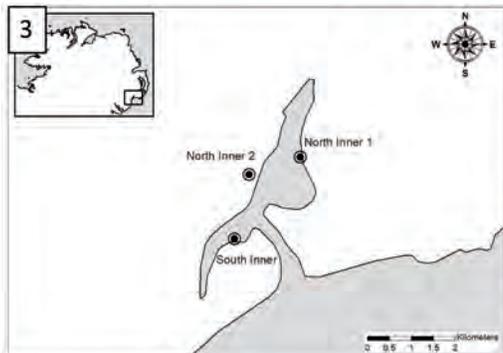
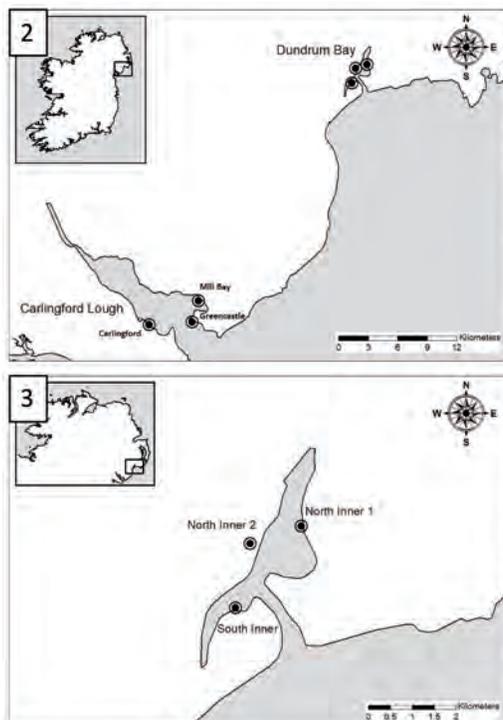


Fig. 1. Collection map of sites surveyed in the British Isles. Information on sampling for DNA studies is provided in Table 1 for the sites abbreviated here: *car*, Carlingford Lough and Dundrum Bay; *rgp*, Glaslyn, Porthmadog; *bck*, Batson Creek Kingsbridge Estuary; *bsi*, Brownsea Island; *bom*, Christchurch Harbour.

2015). The seabed and shoreline substrata of the Lough change from silty sediment in the inner Lough to boulders, cobbles and patches of bedrock in the mouth (AFBI, 2015). Salinity in the area of our surveys typically averages c. 25 PSU, but a minimum value of 4.8 PSU has been recorded, resulting from the influence of freshwater from streams and other discharges, particularly at low tide (AQUAFACT, 2011). Much of Carlingford Lough is designated as a Special Area of Conservation (SAC) under the EU Habitats Directive, and potential adverse impacts of aquaculture are monitored (AFBI, 2015).

A survey of the coastline between the sites where *Gracilaria vermiculophylla* had been identified along the northeastern shore of Carlingford Lough eastwards from Warrenpoint and northwards on the South Down coastline to Dundrum was carried out in September 2013 (Figs 2-4). Ten suitable locations where the shore could be accessed directly from the road without crossing private property were surveyed, and presence or absence of *G. vermiculophylla* was recorded.

Near Carlingford, a stretch of shore approximately 1 km long was surveyed in 2013 (Figs 2, 4). Five equidistant vertical transects ~ 200 m apart were walked from low to high shore in the two hours around low tide. Quadrats (1 m²) were placed centrally on the transect approximately every 10 m (n = 9-11). Presence or absence of *Gracilaria vermiculophylla* was recorded and any *G. vermiculophylla*



Figs 2-4. **2.** Study sites in Dundrum Bay and Carlingford Lough in North-east Ireland (showing position in Ireland, inset). Dot on the southwestern shore of Carlingford Lough and three dots in Dundrum Bay indicate where shore sampling was undertaken. Dots on the northeastern shore of Carlingford Lough are areas where *G. vermiculophylla* was found in a larger-scale coastal survey. **3.** Detail of Dundrum sampling sites. **4.** A view of the site sampled at Carlingford Lough taken in 2011 (photo credit C.L. Magill).



thalli inside the quadrat area were removed and stored in separate labelled bags. The sampled *G. vermiculophylla* was returned to the lab where it was picked clean by hand, rinsed, and blotted dry before weighing to the nearest 0.1 g with an Ohaus Adventurer AR3130 (Ohaus, Nanikon, Switzerland).

Inner Dundrum Bay, Co. Down, northeastern Ireland: This inlet (Figs 2-3) is part of a designated SAC (AQUAFACT, 2012). The substratum is sedimentary, consisting of a mixture of sand and finer muds. Three main rivers flow into the Inner Bay, and recorded salinity ranges from 24-36 PSU (AQUAFACT, 2012). Dundrum Bay Oysters was established in 1986: *Mytilus edulis* is cultivated in the south Inner Bay and *Magallana (Crassostrea) gigas* in the north in shellfish-designated waters (AQUAFACT, 2012; DAERA, 2015). The same gracilarioid species as in Carlingford Lough, again provisionally identified as *G. vermiculophylla*, was found at Dundrum by Northern Ireland Environment Agency staff in 2012 (C. Beer, *pers. comm.*).

The biomass of *Gracilaria vermiculophylla* was surveyed in September/October 2013 and repeated in August 2016 at three sites: North Inner 1 (NI1), North Inner 2 (NI2) and South Inner (SI) (Fig. 2; 54°15'57.88"N; 5°49'6.64"W). At NI1, six transects ~ 150 m apart were made from the high shore to the permanent channel which remains at low tide (freshwater inflow, Blackstaff River) along approximately 1 km of shore. Quadrats (1 m²) were placed approximately every 40 m until the channel was reached (n = 5-13, dictated by the curvature of the channel; Table 1). *G. vermiculophylla* thalli were recorded and collected as previously described. At NI2, there was little evidence of *G. vermiculophylla* apart from a few loose-lying thalli and no further sampling was undertaken. At SI, *G. vermiculophylla* was absent from approximately 0.24 km² of shore apart from a defined patch of c. 1054 m² (0.001 km²).

High density patches at NI1 and SI were investigated in more detail. At NI1, nine equally spaced 1 m² quadrats were sampled in an area of ~ 400 m². At SI, 20 equally spaced 1 m² quadrats were sampled in an area of ~ 1054 m². For comparison with published studies of this species elsewhere, wet to dry mass conversion values were obtained. Individual fresh thalli were weighed to the nearest 0.1 g with the Ohaus balance, then dried in a domestic dehydrator (Andrew James), initially set at 50°C, with temperature reduced when the alga dried until ambient temperature was reached. Dried samples were then re-weighed.

Christchurch Harbour, Dorset: This estuary has the Stour and Hampshire Avon rivers feeding into it, and exchange with coastal waters is via a narrow (~ 45 m) channel at Mudeford (Table 1; Figs 1, 5). There are no current or previous aquaculture activities. The substratum is mostly fine mud with patches of pebbles and occasional bedrock outcrops. Continuous monitoring of water quality takes place nearby at the Mudeford ferry pontoon as part of the NERC Macronutrient Cycles project (<http://www.nerc.ac.uk/research/funded/programmes/macronutrient/>) and is reported online (<https://stormcentral.waterlog.com/SiteDetails.php?a=120&site=363&pa=NOCstor ms>). Salinity normally varies with the tidal cycle from 13-32 PSU, but during heavy rainfall periods is approximately 0.

Surveys in Christchurch Harbour were carried out from Barn Bight to the open channel to determine the distribution of *Gracilaria vermiculophylla* between 2015 and 2017 (Figs 6-7). Biomass estimates were made in August 2016 by clearing random 0.5 × 0.5 m quadrats. All *G. vermiculophylla* within the quadrat was removed, washed carefully in clean seawater, shaken vigorously to remove water and weighed with a domestic digital scale (Tefal) accurate to 1 g.



Figs 5-7. **5.** Christchurch Harbour, Dorset (Google Earth), showing (yellow line) distribution of *Gracilaria vermiculophylla* on intertidal and subtidal sediment from October 2015 to March 2017 with position of high-density patch (star) in Barn Bight sampled in August 2016. **6.** The high density patch of *Gracilaria vermiculophylla* at Barn Bight (photo credit: C.A. Maggs). **7.** A general view of the shoreline in Christchurch Harbour (photo credit: C.A. Maggs).

Brownsea Island Lagoon, Poole Harbour, Dorset: The artificial non-tidal saline lagoon was separated from the sea by construction of a sea wall in the 1850s (Table 1; Figs 1, 8-10). The lagoon is now connected to the rest of Poole Harbour via sluice channels. The lagoon bed is firm mud with some areas of small pebbles. The salinity in the lagoon when measured by Herbert *et al.* (2010) was 22-29 PSU. Poole Harbour was the first site in England to import oysters (*Magallana (Crassostrea) gigas*), brought from Arcachon, France, for fattening by the Poole Oyster Company in 1890 (Humphreys *et al.*, 2014). More recently, oysters have been cultivated on the north coast of Brownsea Island, Poole Harbour since at least 2002 (Wordsworth, 2005). The lagoon was omitted from lagoon surveys in England in the 1980s and 1990s, but, in November 2009, Herbert *et al.* (2010) found large floating clumps of “*Gracilariopsis longissima*”.



Figs 8-10. **8.** Brownsea Island lagoon general view (photo credit: C.A. Maggs). **9.** A bed of *Gracilaria vermiculophylla* (photo credit: C.A. Maggs). **10.** A view of *Gracilaria vermiculophylla* thalli underwater with the common free-floating morphology (photo credit: C.A. Maggs).

Distributional surveys of putatively *G. vermiculophylla* thalli were made in the lagoon in October and December 2016. Presence/absence of population morphologically corresponding to *G. vermiculophylla* was noted along the outer shores of Brownsea Island and along 2 km of the eastern shore of Poole Harbour in October and December 2016.

Kingsbridge Harbour, Devon: This is a ria which extends inland for 8 km to Salcombe and has a variety of commercial and tourism uses including fishing and yachting (Table 1; Figs 1, 11; CEFAS, 2009). Oysters (*Magallana (Crassostrea) gigas*) have been cultivated in the Salcombe-Kingsbridge Estuary since at least 2003, at Charleton Creek and Frogmore Creek, where seed from commercial hatcheries is re-laid (CEFAS, 2009). Freshwater input from rivers is low, and salinity is typically 33-35 PSU but occasionally drops to 22-23 PSU (Environment Agency, 2016). Intertidal substrata are predominantly muddy in the inner estuary with few rocks, to which only fucoids were attached at the time of the survey.

Distributional surveys were made throughout the ria driving along coastal roads and investigating accessible sites at low tide in August 2015. Only a single gracilarioid thallus was found.

River Glaslyn, Porthmadog, Wales: During Natural Resource Wales monitoring of the Penlleyn and Sarnau SAC in July 2017, a putative population of *Gracilaria vermiculophylla* was observed at one site on 23 July 2017 (Figs 1, 12). The habitat was muddy sand in the upper shore below the saltmarsh, and at the edge of a tidal channel. Salinity is low and variable with freshwater input from the River Glaslyn,



Fig. 11. The Batson Creek boat launch where *Gracilaria vermiculophylla* was sampled in the Kingsbridge Estuary on 21 August 2015 (photo credit: S.A. Krueger-Hadfield).



Fig. 12. *Gracilaria vermiculophylla* growing in muddy sand at Glaslyn, Porthmadog on 23 July 2017 (photo credit: F. St. P.D. Bunker).

as indicated by the presence of *Fucus ceranoides*. There are no aquaculture activities in this part of Cardigan Bay, but yacht moorings are adjacent to the site at a large marina at Pwllheli. Boat traffic between Ireland and Anglesey was thought to have spread the carpet sea squirt *Didemnum vexillum* (Griffith *et al.*, 2009, R. Holt, *pers. comm.*).

Statistics

Simple linear regression and t-tests on biomass data (g wet mass m⁻²) were carried out in *R* ver. 3.3.0 (R Core Team, 2016).

Morphological studies

Thallus samples from each of the sites described above were examined under dissecting microscopes (× 40) for the presence of reproductive structures. If present, cystocarps and tetrasporangial axes were sectioned by hand using a razor blade and photographed on a Leitz Dialux microscope with a digital camera (Figs 13-15).

Sample collection and processing for DNA

Carlingford: Thalli were collected in September 2014 at distances > 1 m apart and stored in silica gel for subsequent DNA extraction (n = 22). Voucher specimens were deposited in the Natural History Museum, London.

Christchurch Harbour: In October 2015, 100 thalli were collected individually at Barn Bight. Thalli were stored individually in small plastic bags with a moist paper towel and shipped live to the Grice Marine Laboratory, SC, USA. A small piece of each thallus was dried in silica gel as both voucher specimens and for subsequent DNA extractions. Voucher herbarium specimens were deposited in the Natural History Museum, London and at the University of Alabama at Birmingham. We randomly chose 32 thalli for fragment analysis.

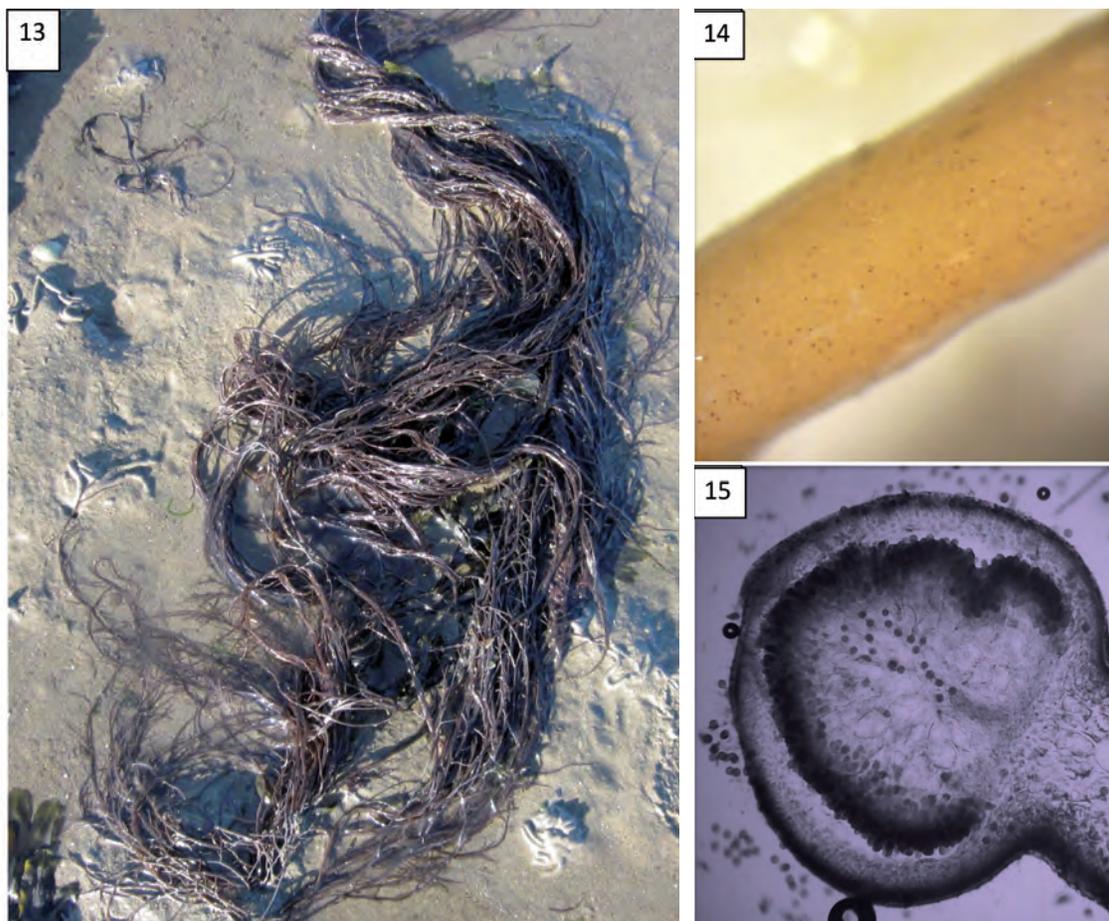
Brownsea Island lagoon: In December 2016, 16 individual thalli were collected randomly at 3 m intervals along the lagoon shoreline taking the closest thallus to the distance marker, bagged separately and shipped live to the University of Alabama at Birmingham, AL, USA. Voucher specimens were deposited in the Natural History Museum, London and at the University of Alabama at Birmingham. Only 14 thalli were used for fragment analysis as two DNA extractions failed.

Kingsbridge Estuary: In August 2015, a single, vegetative, gracilarioid thallus was collected from the Batson Creek Boat Park. A small part was dried in silica gel for subsequent DNA extraction and the rest of the thallus was preserved as an herbarium voucher at the University of Alabama at Birmingham.

Porthmadog: A population morphologically identified as *Gracilaria vermiculophylla* was observed on 23 July 2017 (Table 1). Samples were not sequenced, but based on habitat and morphology, the occurrence of *G. vermiculophylla* in Wales is highly likely. A specimen was preserved as an herbarium voucher and sent to the University of Alabama at Birmingham.

DNA extraction

Total genomic DNA was isolated using 5-10 mg of dried tissue and the Nucleospin[®] 96 plant kit (Macherey-Nagel, Düren, Germany). We followed



Figs 13-15. **13.** Individual *Gracilaria vermiculophylla* thallus growing on pebble in muddy sand (Carlingford, March 2011) (photo credit: C.L. Magill). **14.** Tetrasporophyte with tetrasporangia (Carlingford, October 2013) (photo credit: C.A. Maggs). **15.** Transverse section of cystocarp showing basal constriction, central columella and layer of mature carposporangia (Carlingford, October 2013) (photo credit: C.A. Maggs).

manufacturer's instructions except for the cell lysis step in which lysate was left at room temperature for one hour (Krueger-Hadfield *et al.*, 2011). DNA was eluted in 100 μ l of molecular grade water.

Mitochondrial sequencing

The mitochondrial gene *cox1* was amplified using the primer sets 43F (Geraldino *et al.*, 2006) and 880R (Yang *et al.*, 2008) and 622F (Yang *et al.*, 2008) and 1549R (Geraldino *et al.*, 2006) for 8 thalli each from Carlingford Lough, Christchurch Harbour and Brownsea Island lagoon and for the single thallus sampled in the Kingsbridge Estuary. PCR amplification was performed on a total volume of 25 μ l, containing 0.5 U of *Taq* DNA polymerase, 2.5 mM of each dNTP, 2 mM

MgCl₂, 1 × reaction buffer, 250 nM of each primer and 5 µl of DNA, with PCR conditions described in Yang *et al.* (2008). Approx. 5 µl of PCR product with 1 µl of Orange G loading dye were visualized on 1.5% agarose gels containing ethidium bromide or GelRed (Biotium, Fremont, CA, USA). 1 µl of Exo-SAP-IT (Affymetrix, Santa Clara, CA, USA) was added to 7 µl PCR product and incubated for 15 min at 37°C followed by 15 min at 80°C. 4 µl 2 µM primer was added to each product and sequenced commercially by Eurofins Genomics (Louisville, KY, USA). Sequences were edited using *Apeaks* (Nucleobytes, The Netherlands). Consensus sequences were made from the 43F-880R and 622F-1549R *cox1* sequences using *Seaview* ver. 4.6 (Gouy *et al.*, 2010). We aligned the 25 consensus *cox1* sequences with the haplotypes from Kim *et al.* (2010) and Krueger-Hadfield *et al.* (2017) using *Muscle* (Edgar, 2004) in *Seaview* with default parameters.

Microsatellite genotyping and fragment analysis

Ten microsatellite loci (Kollars *et al.*, 2015; Krueger-Hadfield *et al.*, 2016), specific to *G. vermiculophylla* were used to genotype 22 thalli from Carlingford Lough, 32 thalli from Christchurch Harbour and 14 thalli from Brownsea Island lagoon following the amplification protocols described in Krueger-Hadfield *et al.* (2016). Alleles were scored manually using *Genemapper* ver. 4 (Applied Biosystems, Foster City, CA, USA) and allele sizes were binned with *Tandem* ver. 1.08 (Matschiner & Saltzburger, 2009) following Krueger-Hadfield *et al.* (2013). Previously, Krueger-Hadfield *et al.* (2016) found low frequencies of null alleles (less than 1% across loci in the haploids; see Krueger-Hadfield *et al.*, 2013 for a discussion on calculating null allele frequencies in haploid-diploid species). We used appropriate internal positive and negative controls in order to minimize genotyping error from run to run and across different machines.

For vegetative thalli without reproductive structures from our morphological analyses (above), we determined ploidy based on the multilocus genotype following Krueger-Hadfield *et al.* (2013, 2016). A thallus was considered diploid if at least one of the 10 microsatellite loci was heterozygous. We confirmed the ploidy of all reproductive material using the same methodology. In order to compare with previously published analyses, we focused here only on diploid thalli as they dominate the non-native range (Krueger-Hadfield *et al.*, 2016). We did not calculate population genetic metrics in the haploid thalli.

The number of repeated identical multilocus microsatellite genotypes (MLGs) was computed using *RClone* ver. 1.0 (Bailleul *et al.*, 2015) in *R* ver. 3.3.0 (R Core Team, 2016). When the same multilocus genotype (MLG) is detected n times in a sample of N sampling units, the probability, P_{sex} , that the repeated MLGs originate from different sexual reproductive events (i.e., from different zygotes and, therefore, different genets), is derived from a binomial expression (see Arnaud-Haond *et al.*, 2007). If the P_{sex} p -value was > 0.05 , each of the duplicated MLGs were considered as different genets and retained in the data set. If the P_{sex} p -value was < 0.05 , the duplicated MLGs were considered as ramets (or clones). We kept only one representative of each repeated MLG that exhibited P_{sex} p -values < 0.05 . See Krueger-Hadfield *et al.* (2013) and Krueger-Hadfield *et al.* (2016) for further examples of using P_{sex} in order to determine clonal membership in red seaweeds.

Based on clonal membership as determined by P_{sex} , the genotypic richness, R , was calculated as: $R = (G-1) / (N-1)$, where G is the number of unique multilocus genotypes (MLG) and N is the total number of studied individuals (Dorken & Eckert, 2001).

We calculated the following genetic indices for the total data set, including repeated genotypes (i.e., repeated MLGs with $P_{sex} < 0.05$), and for a ' P_{sex} -ed' data set, which included unique MLGs (i.e., only thalli with $P_{sex} > 0.05$; Krueger-Hadfield *et al.*, 2016). Linkage disequilibrium was evaluated for each site using the single multilocus estimate (Agapow & Burt 2001) and implemented in the *R* package *poppr* ver. 2.0.2 (Kamvar *et al.*, 2014; 2015). In order to test for departure from random associations between loci, the observed data set was compared to 1000 simulated datasets in which sex and recombination was imposed by randomly reshuffling the alleles among individuals for each locus (Agapow & Burt, 2001) followed by Bonferroni correction (Sokal & Rohlf, 1995). The two alleles of the same locus were shuffled together to maintain associations between alleles within loci in the randomized dataset. In addition to physical linkage on a chromosome, disequilibrium may be due to a lack of recombination caused by clonal propagation or selfing (mating system) or to differences in allele frequencies among populations (spatial genetic structure).

For each site, the expected heterozygosity (H_E), observed heterozygosity (H_O), and the inbreeding coefficient G_{IS} (an analogue of F_{IS}) were calculated in *Genodive* ver. 2.0b23 (Meirmans & Van Tienderen, 2004). The mean expected number of alleles based on the smallest sample size ($N = 10$ diploids) using rarefaction (A_E) was calculated using the *R* package *diversity* (Keenan *et al.*, 2013). Finally, the expected number of MLGs at the lowest common sample size ($N = 10$; *eMLG*) using rarefaction and the evenness of genotypic distribution ($E.5$) were calculated using the *R* package *poppr* ver. 2.0.2 (Kamvar *et al.*, 2014, 2015).

RESULTS

Identification of thalli from Ireland and southern England

When aligned to the trimmed 1203 bp *cox1* sequence, the sample collected in 2015 at Batson Creek Boat Park in the Kingsbridge Estuary and each of the 8 thalli collected from Carlingford Lough, Christchurch Harbour and Brownsea Island lagoon were 100% similar to the *cox1* haplotype 6 of *Gracilaria vermiculophylla* (Kim *et al.*, 2010; Krueger-Hadfield *et al.*, 2017). Haplotype 6 is the most common haplotype throughout the non-native range in the Northern Hemisphere (Kim *et al.*, 2010; Krueger-Hadfield *et al.*, 2017), as well in as the source region of the invasion in Northeastern Japan (Krueger-Hadfield *et al.*, 2017). A single sequence for each site was deposited in GenBank (Brownsea: MF943131; Christchurch: MG943132; Carlingford: MF943133).

Distribution and abundance in north-eastern Ireland and in Dorset

At Carlingford and Dundrum, distribution along the transects was patchy and biomass was variable, but low overall compared to southern England (Table 2). At Dundrum, there was a noticeable increase in biomass along the transects between 2013 and 2016 (Table 2), and the biomass in the high-density patch was statistically indistinguishable across years (t-test, $p > 0.05$; Table 3). Individual thallus wet weight ranged from 5-800 g. There was a direct relationship between wet and dry mass of thalli ($n = 17$) from Dundrum ($R^2 = 0.998$, $Df = 1,15$, $p = 0.0001$). The wet to dry mass conversion equation derived from the regression is $y = 0.1299 * x - 0.7645$.

Table 2. Distribution and biomass of *Gracilaria vermiculophylla* in shore transects at Carlingford Lough in September 2013 and Dundrum Bay in October 2013 & September 2016. n = the number of quadrats in transect

| Transect | Biomass (g wet mass m ⁻²), mean ± SD | | |
|----------|--------------------------------------------------|------------------|---------------------|
| | Carlingford Lough 2013 | Dundrum Bay 2013 | Dundrum Bay 2016 |
| 1 | 2.5 ± 4.4 (n = 11) | < 1 (n = 8) | 7.2 ± 12.2 (n = 7) |
| 2 | 14.2 ± 22.7 (n = 11) | 0 (n = 5) | 14.2 ± 16.7 (n = 6) |
| 3 | 7.8 ± 14.6 (n = 10) | < 1 (n = 8) | 15.4 ± 36.9 (n = 9) |
| 4 | 0 (n = 9) | < 1 (n = 9) | 38.9 ± 75.9 (n = 8) |
| 5 | 0 (n = 9) | < 1 (n = 12) | 0 (n = 8) |
| 6 | – | < 1 (n = 13) | 28 ± 59.8 (n = 10) |

Table 3. Biomass of *Gracilaria vermiculophylla* in high-density patches at Dundrum Bay. n = number of quadrats

| Location | Biomass, g wet mass m ⁻² , mean ± SD (range) | |
|-----------------------|---------------------------------------------------------|------------------------|
| | 2013 | 2016 |
| North Inner 1 (n = 9) | 186 ± 398 (0-1236) | 152 ± 249 (0-750) |
| South Inner (n = 20) | 121 ± 240 (0-1028) | Few loose-lying thalli |

Table 4. Biomass of *Gracilaria vermiculophylla* in Christchurch Harbour, 29 August 2016

| Location | Biomass, wet mass g m ⁻² , mean ± SD (range) |
|------------------------------------------|---------------------------------------------------------|
| Barn Bight, high density patch (n = 7) | 1760 ± 760 (588-2932) |
| Barn Bight, general distribution (n = 4) | 442 ± 83 (332-516) |

At Christchurch Harbour, Dorset, *Gracilaria vermiculophylla* was absent inland of Barn Bight, and in 2015, it was distributed continuously from Barn Bight to the pier near the entrance to the sea at Mudeford (Fig. 3), with biomass of 332 to 2932 g m⁻² from the mid-intertidal into the shallow subtidal zone (Table 4). It also comprised a major element of the strandline algae. In 2017, it was abundant in the small tidal lagoon inside Hengistbury Head, and along the north side of Christchurch Harbour (Fig. 3).

In Poole Harbour, Dorset, *Gracilaria vermiculophylla* was abundant in Brownsea Island lagoon, but absent from the outer coastline of Brownsea Island. In Holes Bay, a brackish-water inlet off the eastern shore of Poole Harbour, sparse populations of *G. vermiculophylla* were identified by morphology.

Variation in ploidy ratios and reproductive mode at Carlingford, Christchurch, and Brownsea

The number of alleles ranged from 3 to 7 depending on the locus. A total of 39 alleles were detected across the three sites in Ireland and England.

At Carlingford, 12 of the 22 thalli exhibited one allele at each of the 10 microsatellite loci and were considered haploid (Table 5). Surveys at Carlingford also uncovered reproductive thalli, including female gametophytes with cystocarps (Fig. 7). Upon re-examination of the silica gel preserved material, three of these

Table 5. Population genetic statistics of diploid thalli sampled at Carlingford Lough, Christchurch Harbour and Brownsea Island based on 10 microsatellite loci for which 22, 32 and 14 thalli were genotyped, respectively. Diploid and haploid thalli were determined by the presence of reproductive structures, the microsatellite multilocus genotype (MLG), or both. For each population, number of diploid thalli, the number of haploid thalli, the proportion diploid, the proportion repeated diploid MLGs based on P_{sex} , the genotypic richness (R), expected number of MLGs ($eMLG$), genotypic evenness ($E.5$), allelic richness (A_E), expected heterozygosity (H_E), observed heterozygosity (H_O), the inbreeding coefficient (G_{IS}), and multilocus estimate of linkage disequilibrium (\bar{r}_d) are shown. For Christchurch Harbour and Brownsea Island, there were repeated MLGs and population genetic statistics were calculated in the total data (i.e., including repeated diploid MLGs) and a P_{sex} -ed data set (i.e., only a single thallus per MLG based on P_{sex}). Significant values are shown in bold (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$). We did not analyze clonal membership in the haploid thalli (ND)

| Statistic | Carlingford Lough ($n = 22$) | Christchurch Harbour ($n = 32$) | | Brownsea Island ($n = 14$) | |
|----------------------------------------|--------------------------------------|-----------------------------------------|----------------|------------------------------------|----------------|
| | All thalli | All thalli | P_{sex} -ed | All thalli | P_{sex} -ed |
| Number diploid | 10 | 28 | 26 | 11 | 10 |
| Number haploid | 12 | 4 | ND | 3 | ND |
| Ploidy determination | Microsatellite | Microscopy & Microsatellite | | Microscopy & Microsatellite | |
| Proportion diploid | 0.45 | 0.88*** | 0.87*** | 0.79*** | 0.77*** |
| Proportion repeated diploid MLG | 0 | 0.07 | – | 0.09 | – |
| Genotypic richness (R) | 1.0 | 0.93 | – | 0.90 | – |
| Expected number of MLGs ($eMLG$) | 9 | 9.06 | 9.27 | 9.18 | 10 |
| Genotypic evenness ($E.5$) | 0.95 | 0.85 | 0.91 | 0.96 | 1 |
| Allelic richness (A_E) | 1.50 | 2.20 | 2.22 | 1.89 | 1.90 |
| Expected heterozygosity (H_E) | 0.207 | 0.272 | 0.277 | 0.358 | 0.360 |
| Observed heterozygosity (H_O) | 0.140 | 0.246 | 0.252 | 0.245 | 0.260 |
| Inbreeding coefficient (G_{IS}) | 0.323* | 0.094 | 0.092 | 0.315** | 0.278* |
| Linkage disequilibrium (\bar{r}_d) | 0.118 | 0.050* | 0.046 | 0.191* | 0.165* |

haploid thalli were found to bear cystocarps. The remaining 10 thalli from Carlingford exhibited at least one heterozygous microsatellite locus and were considered diploid. The diploid proportion was 0.45, but this was not significantly different from the null expectation of $\sqrt{2}:1$ haploid:diploid (Destombe *et al.*, 1989; Thornber & Gaines, 2004). No diploid MLGs were found more than once and there was no evidence of linkage disequilibrium (Table 5), however, there was an excess of homozygotes as indicated by significantly positive multilocus G_{IS} (0.323; Table 4).

In contrast, at Christchurch, 28 of the 32 thalli genotyped (0.88) were found to be diploid, a significant deviation from $\sqrt{2}:1$ ($p < 0.001$). We found three MLGs that were repeated twice, but none of the P_{sex} values for those pairs of repeated MLGs were < 0.05 . Thus, we considered each of these thalli as genets. One MLG was encountered four times, but only two of the four thalli exhibited P_{sex} values < 0.05 . Therefore, we only considered two of the four thalli as clones, and the other two as genets. The proportion of repeated MLGs was low (0.07) and genotypic richness was high (0.93; Table 5). We found no evidence of heterozygote excess or

deficiency, but single-locus F_{IS} estimates were variable ranging from -0.2 to 1.0 (Table 5). When we removed repeated MLGs, there was no evidence of linkage disequilibrium.

Similarly, at Brownsea, 11 of the 14 genotyped thalli were diploid (0.79; Table 5). Three thalli exhibited only one allele at each locus, and we considered these thalli haploid, however, no reproductive thalli have been sampled thus far at Brownsea. One MLG was encountered twice, and was considered a clonal replicate ($P_{sex} < 0.05$). Whether we included or excluded clones, there was evidence of significant linkage disequilibrium. Surprisingly, there were significantly positive G_{IS} values, with and without clones, suggesting an excess of homozygotes (Table 5). Yet, the single-locus G_{IS} values varied widely from -0.6 to 0.6 .

When comparing equal sample sizes, the number of expected MLGs shared at the largest sample size ($eMLG$) was similar between the three sites (Table 5). Allelic richness (A_e) and genetic diversity (H_e) were higher at the two sites in England compared to Carlingford in Ireland (Table 5). However, the evenness (E_5), as a measure of the distribution of genotype abundances, was similar among all three sites (Table 5).

DISCUSSION

Our discovery of populations of *Gracilaria vermiculophylla* in embayments in northeast Ireland, along the south coast of England, and, probably Wales, extends the already widespread known distribution of this non-native seaweed across the Northern Hemisphere (Krueger-Hadfield *et al.*, 2017). Although the coasts of the British Isles have been very well studied by phycologists since the 18th century (e.g., Stackhouse, 1801), sedimentary shores have been largely ignored. Relatively accessible rocky shores supporting high algal biodiversity have been sampled regularly (e.g., Torbay by Mrs. Griffiths, long-term correspondent of W.H. Harvey; and more recently e.g., Maggs & Hommersand, 1993), including major sampling campaigns, such as the southwest Britain Sublittoral Survey (Hiscock & Maggs, 1984) that concentrated on hard substrata. Although all the south coast harbours, estuaries and rias, including the Kingsbridge Estuary and Christchurch Harbour, were surveyed for the UK's Marine Nature Conservation Review (Dixon, 1988; Covey, 1998; Davies, 1998), the focus was on infaunal cores and the seaweeds remain poorly known. To date $> 25\%$ of 10 km grid squares in the British Isles have no records of macroalgae and proxies for collection effort are required to interpret the distribution of species richness (Blight *et al.*, 2009). More recently, floating pontoons in yacht marinas have become sites of choice for rapid sampling assessment of non-native species (Bishop *et al.*, 2015), but *G. vermiculophylla* has not been recorded as recruits on pontoons to date (S.A. Krueger-Hadfield, *pers. obs.*). As a result of the focus on hard substrata, our knowledge of which algal species may be in estuarine habitats is rather limited, even on otherwise well-studied coastlines. Moreover, without molecular identification, *G. vermiculophylla* might remain unreported or unconfirmed in many estuarine habitats (Kim *et al.*, 2010; Krueger-Hadfield *et al.*, 2017).

Here, we have found and surveyed *Gracilaria vermiculophylla* at three areas in England and two in north-east Ireland. Most of the sites where we confirmed the presence of *G. vermiculophylla* are licensed for aquaculture of oysters, and

specifically *Magallana (Crassostrea) gigas*. Brownsea is in close proximity to an oyster farm in Poole Harbour, and we have confirmed the presence of *G. vermiculophylla*. In 2009, Herbert *et al.* (2010) found large floating clumps of “*Gracilariopsis longissima*”. Based on our identification, it appears that an invasive population of *G. vermiculophylla* has been established there for at least eight years (R. Herbert, *pers. comm.*). Misidentification of *G. vermiculophylla* in other sites close to oyster aquaculture, such as in Tomales Bay, California, has occurred (Krueger-Hadfield *et al.*, 2017), suggesting this may also be the case in Brownsea. Although Christchurch Harbour lacks aquaculture, it is close to Poole Harbour, and drift seaweeds are transported along the coast between sites. Thus, our current study provides further evidence of the significance of oyster aquaculture in the spread of invasive species (Mineur *et al.*, 2012, 2014), and *G. vermiculophylla*, in particular (Krueger-Hadfield *et al.*, 2017).

In contrast to populations at many sites throughout the non-native range in the Northern Hemisphere (Krueger-Hadfield *et al.*, 2016), we found few repeated diploid genotypes. At Carlingford, we found both gametophytes and tetrasporophytes, with few signatures of clonal fragmentation. In contrast, along the south coast of England where few thalli were fixed to hard substratum via holdfasts or bore reproductive structures, we also detected haploid thalli and few repeated diploid MLGs. Our designation of haploid thalli was based solely on the microsatellite genotype at these sites. Though all reproductive thalli in this study and others (Kollars *et al.*, 2015; Krueger-Hadfield *et al.*, 2016) have exhibited the correct ploidy based on the microsatellite MLG (i.e., one allele at each locus = haploid; two alleles at one or more loci = diploid), this has not been the case in other red seaweeds. For example, Krueger-Hadfield *et al.* (2013) found nine tetrasporophytes that were fixed homozygotes in *Chondrus crispus*, a red seaweed characterized by extremely high levels of inter-gametophytic selfing. In this study, at Brownsea, we found an excess of homozygotes which could indicate inter-gametophytic selfing. This suggests that some of the haploid thalli designated solely by microsatellite MLG may have been erroneously designated haploid and, rather, could be highly homozygous, diploid tetrasporophytes.

Nevertheless, haploid thalli have been detected in other predominantly free-floating, estuarine populations (Krueger-Hadfield *et al.*, 2016), both through observations of reproductive structures and microsatellite genotyping. The populations at Christchurch and Brownsea are not that different from many of the populations studied by Krueger-Hadfield *et al.* (2016) in terms of the site-specific population genetic metrics. Both populations were significantly tetrasporophyte-biased, but there were very few repeated MLGs (Table 4). Infrequent recombination events can remove signatures of clonality (Balloux *et al.*, 2003; Halkett *et al.*, 2005). Moreover, both the Christchurch and Brownsea populations exhibited highly variable single-locus G_{IS} estimates, ranging from strongly negative to strongly positive, perhaps indicating partial clonality (Reichel *et al.*, 2016). Recently, it has been suggested that negative values of inbreeding coefficients (F_{IS} or G_{IS}) cannot, alone, be used as evidence of the scarcity of sexual reproduction (Reichel *et al.*, 2016). In order to evaluate the reproductive mode in a population, repeated temporal sampling is necessary for generating frequency distributions of MLGs and describing population history. This is required in order to discriminate between alternative hypotheses concerning the extent of clonality when large variation in inbreeding coefficients is observed across loci (Reichel *et al.*, 2016). Thus, we hypothesize that the populations at Christchurch and Brownsea were partially clonal, whereas the population in Carlingford was predominantly sexual.

Krueger-Hadfield *et al.* (2017) found a trend along the European coastline in which genetic diversity was greatest at around latitude 45°N, located in France, and decreased at higher and lower latitudes. Though genotypic evenness was similar among the three sites, the English sites exhibited higher allelic richness and expected heterozygosities than the Irish sites. Cultivation of *Magallana (Crassostrea) gigas*, the putative vector for *Gracilaria vermiculophylla* across ocean basins (Krueger-Hadfield *et al.*, 2017), was introduced from Lisbon to the Arcachon basin in France in 1866, with subsequent introductions to the UK, in particular, by the Poole Oyster company in the 1890s (Humphreys *et al.*, 2014). It is noteworthy that the populations along the southern coast of England display higher genetic diversity. They may not only be older, but also continually receive new genotypes from Europe due to leisure craft or migrating birds (Nyberg & Wallentinus, 2009), whereas Irish populations may be younger and not receive new thalli as regularly. At Carlingford, *G. vermiculophylla* thalli have been found since 2008, at Dundrum since 2012, and, more recently in Granagh Bay, Strangford Lough (coll. & det. J. Nunn) during a BioBlitz in August 2013 (Minchin & Nunn, 2013). However, as the Dundrum Bay oyster fishery was established in 1984, it is possible that thalli have been present for longer than 10 years. More detailed sampling is necessary along European coastlines in order to understand the relationship among sites and secondary spread, particularly in Wales where we have now reported a population.

Understanding the reproductive mode and variation in ploidy ratios at estuarine sites throughout the non-native range, including the British Isles, is important as the evolutionary ecology of free-floating *Gracilaria vermiculophylla* populations is unknown. In both *G. vermiculophylla* (Krueger-Hadfield *et al.*, 2016) and *G. chilensis* (Guillemin *et al.*, 2008), though diploid thalli dominate, haploid thalli are still occasionally detected and tetrasporophytes still produce tetraspores. In estuaries with hard substratum, attached populations of *G. vermiculophylla* and *G. chilensis* are common, suggesting the haploid-diploid life cycle may be recovered following generations of vegetative fragmentation (Guillemin *et al.*, 2008; Krueger-Hadfield *et al.*, 2016, S.A. Krueger-Hadfield, *unpubl. data*). Therefore, the introduction of *G. vermiculophylla* thalli into estuaries and bays across the British Isles may facilitate spread and establishment of both haploid and diploid thalli, particularly in habitats with biogenic reefs.

The extent to which *Gracilaria vermiculophylla* and other non-native gracilarioids such as *Gracilariopsis chorda* (Mineur *et al.*, 2012), have spread in Britain is largely unknown, though they are likely to be found in similar habitats. For example, *G. chorda* was first sampled in the Gulf of Morbihan (Mineur *et al.*, 2012), an area where *G. vermiculophylla* is also common (Rueness, 2005, S.A. Krueger-Hadfield, *pers. obs.*). There are many estuaries in and around the British Isles with introduced oysters (Lallias *et al.*, 2015), such as the Plym Estuary, and it seems highly likely that *G. vermiculophylla* will soon arrive in these habitats, if it is not already present. Many non-native species are included in the rocky shore time series surveys, such as MarClim (Mieszkowska *et al.*, 2006; 2014), but these surveys could be expanded to include soft-sediment habitats in which non-native seaweeds, such as members of the Gracilariales, are prime candidates for invasion.

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