

## Research Article

# Everywhere you look, everywhere you go, there's an estuary invaded by the red seaweed *Gracilaria vermiculophylla* (Ohmi) Papenfuss, 1967

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## Abstract

*Gracilaria vermiculophylla* (Ohmi) Papenfuss, 1967 is a red alga native to the northwest Pacific, but over the last 100 years, this ecosystem engineer has invaded estuaries throughout the Northern Hemisphere. Despite previous studies documenting populations in estuarine habitats in British Columbia, California, and Baja California, our knowledge of its distribution along the North American Pacific is incomplete. In 2017, we surveyed 61 sites from Chusini Cove (Prince of Wales Island, Alaska) to Bayside Park (San Diego Bay, California), spanning more than 23° of latitude. We documented 33 new sites where *G. vermiculophylla* is currently found, but may have been previously overlooked, or not recognized. Many of these locations were areas of intensive shellfish aquaculture, particularly oysters, and are also important flyover stops for migrating birds, possibly contributing to the spread of this seaweed. Our study fills in the significant gaps in the distribution of *G. vermiculophylla* in North America, and, as such, will be an important contribution to future studies on the ecological and evolutionary consequences of this widespread marine invasion along this coastline.

**Key words:** Algae, Gracilariales, *Gracilariopsis andersonii*, haplodiplontic life cycle, invasion, non-native, eastern Pacific, Rhodophyta

## Introduction

Ecosystem engineers play key ecological roles by structuring species assemblages through modifying, maintaining, or creating habitats (Crooks 2009). Many seaweeds fulfill these roles in nearshore marine ecosystems, such as kelps in subtidal habitats or fucoids in intertidal habitats (Lüning 1990). Therefore, it is unsurprising that many macroalgal invaders act as ecosystem engineers and cause profound changes in recipient habitats (reviewed in Williams and Smith 2007).

The red seaweed *Gracilaria vermiculophylla* (Ohmi) Papenfuss, 1967 is native to the northwestern Pacific, but has invaded virtually every temperate estuary in the Northern Hemisphere over the last 100

years (Kim et al. 2010; also reviewed in Krueger-Hadfield et al. 2017a). In particular, this alga has dramatically transformed the soft-sediment communities to which it has been introduced, as these habitats were historically devoid of macroalgal cover (e.g., Byers et al. 2012). For example, along the eastern coast of the United States, it creates structural complexity for invertebrates (e.g., Wright et al. 2014), and it has formed a novel mutualism with the tube worm *Diopatra cuprea* Bosc, 1802 (Thomsen and McGlathery 2005; Kollars et al. 2016). In other regions, such as the Baltic, the *G. vermiculophylla* invasion has been shown to negatively affect native fucoids by competing for space and light (Hammann et al. 2013). Studies on the influence of this invader, however, have focused on the eastern coast of the United States (Thomsen and McGlathery 2005; Byers et al. 2012; Ramus et al.

2017) and populations around the Jutland Peninsula (Germany and Denmark; Weinberger et al. 2008; Hammann et al. 2013; Thomsen et al. 2013), whereas other invaded regions have been less well studied.

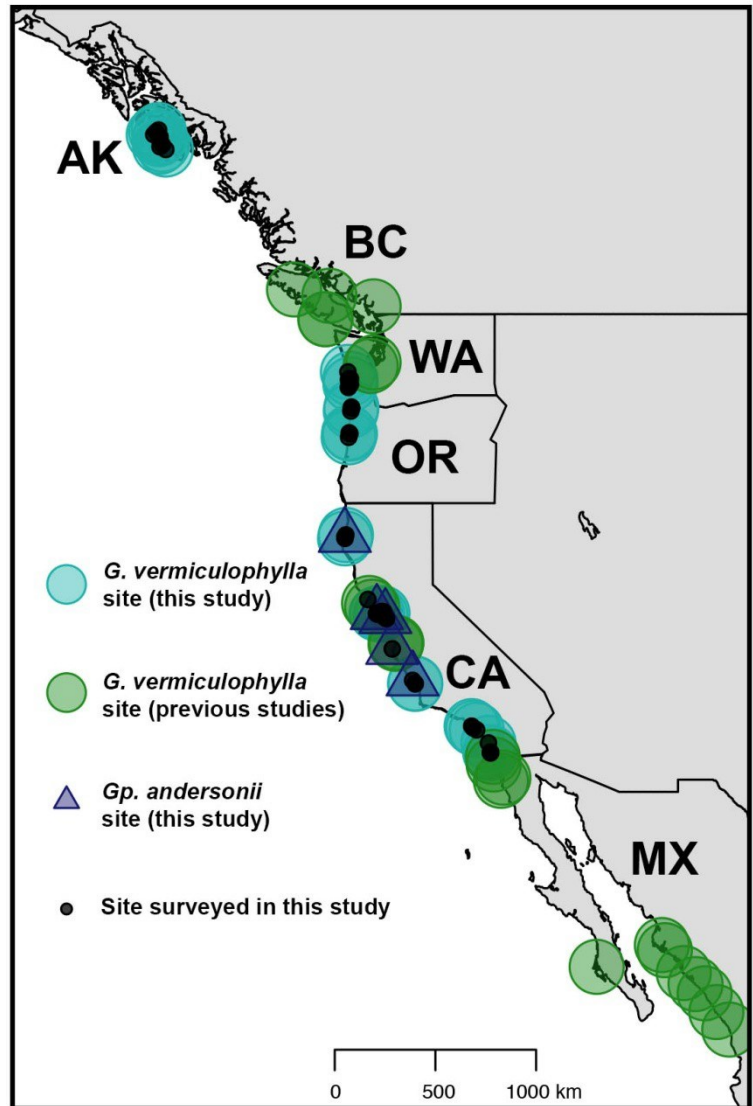
The paucity of studies in these other regions is due, in large part, to a lack of robust distributional data because this species is difficult to identify due to few diagnostic features (Gurgel and Fredericq 2004). For example, in the bays and estuaries of Virginia, *G. vermiculophylla* had previously been identified as the native congener *G. tikvahiae* (Thomsen et al. 2006; Thomsen et al. 2009). Likewise, Krueger-Hadfield et al. (2017b) was the first to document *G. vermiculophylla* in the British Isles, where this seaweed was restricted to soft sediment habitats. These habitats were not included in the extensive, rocky shore algal surveys carried out throughout the British Isles. Macroalgal diversity is comparatively low in soft-sediment habitats because these habitats have limited hard substratum which is necessary for spore recruitment. As a consequence of reduced macroalgal diversity, there has been less phycological study of macroalgae in the estuarine habitats into which *G. vermiculophylla*, among other seaweeds, has been introduced throughout the Northern Hemisphere.

The west coast of North America (hereafter, WNA) has poorly resolved distributional data for seaweed invasions (Miller 2004; Miller et al. 2012), including *G. vermiculophylla* (Krueger-Hadfield et al. 2017a). Bellorin et al. (2004) first reported *G. vermiculophylla* from Baja California in 1979, and, using molecular markers, identified this species from collections in Elkhorn Slough, California (as *Gracilaria* sp., Goff et al. 1994). Saunders (2009) provided molecular evidence that *G. vermiculophylla* was present in British Columbia, and, made a case for the non-native status of this species along the coastline of WNA as opposed to a circum-Pacific distribution. First, extensive floristic surveys by his team, particularly around British Columbia, had failed to detect this species. However, *G. vermiculophylla* has been present in many other estuaries for much longer than appreciated in the literature because it has been misidentified on the basis of morphological characters, and molecular studies confirming the species identification are few and recent (see discussions in Saunders 2009; Krueger-Hadfield et al. 2016a, 2017a, b). Second, this seaweed can survive adverse conditions (e.g., Nyberg and Wallentinus 2009; Hammann et al. 2016; see also Sotka et al. 2018), suggesting that it could easily be transported long distances with oysters. Third, *G. vermiculophylla* is negatively buoyant and would not likely have passively dispersed across the Pacific Ocean.

Using a combination of genotyping and phenotyping, Krueger-Hadfield et al. (2016b, 2017a) and

Sotka et al. (2018) provided the most robust data set to date supporting *G. vermiculophylla* as a non-native species in WNA. There was a striking ecological shift from hard to soft-substratum habitats in the course of the invasion that correlated with a shift from sexual to asexual reproduction (Krueger-Hadfield et al. 2016b). Shifts to uniparental reproduction are common among colonizing, including invading, species (Kolar and Lodge 2001; Pannell et al. 2015). Non-native, WNA thalli had greater tolerance to heat, cold, and low salinity stress when compared to native thalli, but were similar to other non-native populations along the coastlines of Europe and the eastern United States (Sotka et al. 2018). Krueger-Hadfield et al. (2017a) used molecular evidence to identify sites located in Matsushima, Mango-ku, and Akkeshi Bays (Japan) as the source of the Northern Hemisphere invasion in both Pacific and Atlantic basins. These bays were the prominent sites of oyster exportation from Japan to estuaries worldwide (Barrett 1963; Byers 1999; Ruesink et al. 2005). In addition, Krueger-Hadfield et al. (2017a) provided preliminary data on herbarium specimens that were collected as the earliest records of *G. vermiculophylla* throughout the Northern Hemisphere and that match documented oyster introductions (i.e., pre-1970s; reviewed in Ruesink et al. 2005). Together these data support the status of *G. vermiculophylla* as a non-native in WNA, and indicate anthropogenic movement of thalli during activities associated with oyster culture as the likely vector of the primary anthropogenic introduction.

Little is known about the ecological and evolutionary consequences of the *G. vermiculophylla* invasion along the WNA coastline, thus, systematic, baseline surveys of the abundance and distribution of this seaweed are needed in order to begin to understand community-wide impacts across this region. Populations along the WNA coastline are of particular interest because they show high levels of genetic differentiation that likely represent multiple, separate primary invasions from Japan (Krueger-Hadfield et al. 2017a). Without records of the presence of non-native species, such as *G. vermiculophylla*, key eco-evolutionary patterns may go unrecognized. For example, previous researchers recorded dramatic changes in the abundance of a gracilarioid species present in Bodega Bay, California as a result of the introduction of a non-native crab (Grosholz and Ruiz 2009). Other investigations of the interactions between native seagrasses and unknown gracilarioid species in Tomales Bay (Huntington and Boyer 2008a, 2008b), plus the work of Grosholz and Ruiz (2009), were likely working with *G. vermiculophylla*. Correct identification of the gracilarioid species is important



**Figure 1.** Map of known *Gracilaria vermiculophylla* distribution along the WNA coastline based on this study (turquoise circles) and previous work (green circles) by Bellorin et al. (2004), Saunders (2009), Kim et al. (2010), Miller et al. (2012), García-Rodríguez et al. (2013), and Krueger-Hadfield et al. (2017a). Sites surveyed in this study are shown with additional black dots. Sites in which *Gracilariopsis andersonii* was sampled are shown with blue triangles (AK: Alaska; BC: British Columbia; WA: Washington; OR: Oregon; CA: California; MX: Mexico).

as *G. vermiculophylla* has not co-evolved with the native species in Tomales and Bodega bays. Without knowledge of the distribution of this widespread invader, its consequences on native species along the WNA coast may go undocumented.

Krueger-Hadfield et al. (2017a) reported established populations of *G. vermiculophylla* in Puget Sound, Bodega Bay, and Tomales Bay, as well as thalli in San Diego Bay. Here, we expand upon those observations to provide a baseline distributional survey of the invasion along the entire WNA coastline. In this study, we surveyed 61 estuarine and rocky intertidal sites from Alaska, Washington, Oregon, and California in the summer of 2017 in an effort to improve resolution of the distribution of *G. vermiculophylla* along

the WNA coastline. This survey is the foundation for on-going studies of the eco-evolutionary factors that have facilitated this invasion and the influence this invasive ecosystem engineer has on the native flora and fauna across its non-native range.

## Methods

### Study sites and field surveys

We surveyed 61 sites from Chusini Cove, Prince of Wales Island, Alaska (55.80588N; -133.17063W) to Bayside Park, San Diego Bay, California (32.628576N; 117.108039W) from May to July 2017 (Figure 1, Figure 2, Table S1). Sites were chosen using the following

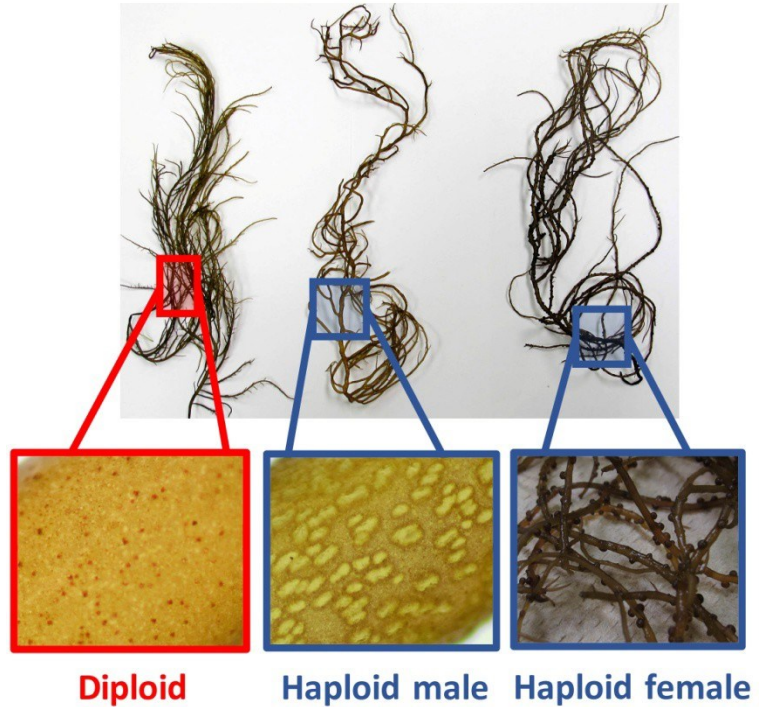




**Figure 2.** A - *Gracilaria vermiculophylla* habitat at Kaguk Cove (akg), Alaska (photo credit: T.A. Stephens); B - *G. vermiculophylla* thalli at Gray's Harbor (bdw), Washington. Circled in yellow is another invasive species, the orange striped anemone *Diadumene lineata* (photo credit: W.H. Ryan); C - *G. vermiculophylla* at Netarts Bay (onb), Oregon (photo credit: S.A. Krueger-Hadfield); D - *G. vermiculophylla* on the shore along Seminary Drive at Strawberry Point in Richardson Bay (sfs), California (photo credit: S.A. Krueger-Hadfield); E - *G. vermiculophylla* at Agua Hedionda (ahd), California (photo credit: S.A. Krueger-Hadfield); F - *Gracilariopsis andersonii* at Bolinas Wharf (bow), California (photo credit: S.A. Krueger-Hadfield); G - Patch of *G. vermiculophylla* at Vance Street in Humboldt Bay (hvs), California (photo credit: S.A. Krueger-Hadfield); H - *Gp. andersonii* at Asilomar State Beach (asi), California (photo credit: S.A. Krueger-Hadfield).

criteria: Google Earth® to identify suitable habitats based on site characteristics from previous extensive sampling of *G. vermiculophylla* populations (Krueger-Hadfield et al. 2016b, 2017a, b; Sotka et al. 2018), by referring to anecdotal records of oyster culture in the literature (e.g., Barrett 1963), by analyzing previous work on the distribution of other invasive species associated with oyster transport and cultivation (e.g., Byers 1999), and by referring to field guides where gracilarioid thalli were reported (Dawson 1965; Abbott and Hollenberg 1976; Silva 1979; Dawson and Foster 1982; Silberstein and Campbell 1989; Stewart 1991; Zimmerman and Caffrey 2002; Druehl and Clarkson 2016).

At each site, we recorded the presence or absence of gracilarioid thalli. If present, we haphazardly sampled thalli separated by at least 1 m in order to standardize the sampling across populations as well as reducing the chances of sampling the same genet (i.e., individual) twice (Krueger-Hadfield et al. 2016b). For each thallus, we recorded whether it was fixed via a holdfast to hard substratum or if it was free-floating (*sensu* Krueger-Hadfield et al. 2016b). Here, we use the term fixed to refer to fixation to the substratum by a holdfast in order to distinguish these thalli from those that may be attached, or glued, to worm tubes, and, therefore, anchored, but not fixed to the hard substratum (Krueger-Hadfield et al. 2016b).



**Figure 3.** The diagnostic reproductive features of diploid tetrasporophytes (shown in red) and haploid male and female gametophytes (shown in blue) of *Gracilaria vermiculophylla* (photo credit: S.A. Krueger-Hadfield).

Reproductive state was determined using a dissecting microscope (40x, Figure 3). Thalli were dried in silica gel as voucher specimens, and for subsequent DNA extractions.

Representative thalli from each site were pressed on herbarium paper, retained in the Krueger-Hadfield lab at the University of Alabama at Birmingham, and donated to the University Herbarium at the University of California at Berkeley.

#### *DNA extraction and cox1 sequencing*

Total genomic DNA was isolated from 5–10 mg of dried thallus following the manufacturer’s instruction for the Nucleospin® 96 plant kit (Macherey-Nagel, Düren, Germany), except for the cell lysis buffer in which samples were left at room temperature for one hour (Krueger-Hadfield et al. 2011). DNA was eluted in a single step in 100 µL molecular grade water.

The mitochondrial gene *cox1* was amplified for 124 thalli 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). We did not distinguish between haploid and diploid thalli for the mitochondrial sequencing because we wanted to obtain mitochondrial haplotypes across the site as a whole. Thus, if a site had both haploid and diploid thalli, we chose a subset of thalli to

sequence. PCR amplification was performed on a total volume of 25 µL, containing 0.5 U of *taq* DNA polymerase, 2.5 mM of each dNTP, 2 mM MgCl<sub>2</sub>, 1 x reaction buffer, 250 nM of each primer and 5 µL of DNA and PCR conditions previously described (Krueger-Hadfield et al. 2017a). Approximately, 5 µL of PCR product using 1 µL of Orange G loading dye were visualized on 1.5% agarose gels stained with GelRed (Biotium, Fremont, CA, USA).

One µL of ExoSAP-It (Affymetrix, Santa Clara, CA, USA) was added to 7 µL of PCR product, and incubated for 15 minutes at 37 °C followed by 15 minutes at 80 °C. Four microliters of 2 µM primer was added to each product and sequenced in the forward direction commercially by Eurofins Genomics (Louisville, KY, USA). Sequences were edited using *4Peaks* (Nucleobytes, The Netherlands), aligned with the haplotypes from Yang et al. (2008), Kim et al. (2010), and Krueger-Hadfield et al. (2017a) using *Muscle* (Edgar 2004) in *Seaview* ver. 4.6 (Gouy et al. 2010) with default parameters. We assigned haplotype numbers to ~ 1100 base pair fragments according to Kim et al. (2010) and Krueger-Hadfield et al. (2017a). We did not include the haplotype designations of Gulbransen et al. (2012) and Liu et al. (2016) because their sequences covered only a portion of this larger ~ 1100 bp fragment. We defined new haplotypes using *DnaSP*, ver. 5.10.1 (Librado and Rozas 2009).



### Analysis of mitochondrial sequences

A median joining network (Bandelt et al. 1999; Woolley et al. 2008), including haplotypes from Yang et al. (2008), Kim et al. (2010), and Krueger-Hadfield et al. (2017a), was constructed using *PopART* (<http://popart.otago.ac.nz>).

We used rarefaction to estimate haplotype diversity using haplotype data reported in Yang et al. (2008), Kim et al. (2010), Krueger-Hadfield et al. (2017a), and this study in order to quantify the effects of sampling effort on haplotype diversity in native and WNA populations, and to confirm the hypothesis that WNA populations were non-native (Saunders 2009; Krueger-Hadfield et al. 2017a). We used ESTIMATES ver 9.1.0 (Colwell 2013) to calculate the non-parametric estimator Chao2. This estimator can be useful in predicting the eventual asymptote in haplotype diversity for a given population by including the effects of rare haplotypes on the total haplotype diversity (Gotelli and Colwell 2001; Blakeslee et al. 2008). The Chao2 estimator is one of the most robust estimators for these types of analyses (Foggo et al. 2003), and has been used previously to determine whether haplotype diversity differs between native and non-native regions (e.g., Blakeslee et al. 2008).

## Results

### Species identification

Gracilarioid thalli were found at 41 of the 61 surveyed sites from Alaska to California. At 20 sites, there were no gracilarioid thalli and we did not record the presence of other algal species (e.g., *Ulva*). Using genetic barcoding, we confirmed the presence of *G. vermiculophylla* at 27 of the 41 surveyed sites in which we found gracilarioid thalli (Table S1), including two sites in Elkhorn Slough (ekb, elk) and one site in Tomales Bay (tmb) that have also been previously reported (Bellorin et al. 2004; Hughey 2013; Krueger-Hadfield et al. 2016b; 2017a). There were an additional nine sites in which there were putative *G. vermiculophylla* thalli based on habitat and the presence of gracilarioid thalli (see also Krueger-Hadfield et al. 2017b), though these records need to be confirmed by sequencing (Table S1). For example, at Los Alamitos Bay, in southern California, all thalli in the Marine Stadium Park were completely bleached, and we were unable to sequence *cox1*. However, based on habitat and morphology, this probably represents another site with *G. vermiculophylla*. Finally, at six sites, we found *Gracilariopsis andersonii*, the native species. In general, at the mouths of bays or on rocky shores, we found abundant populations of the native species *Gp. andersonii*. *G. vermiculophylla*,

on the other hand, was restricted to the inner parts of bays, often on muddy substratum, and sometimes mixed with seagrasses. We did not find mixed assemblages of *G. vermiculophylla* or *Gp. andersonii*.

### Attachment and reproductive state

*G. vermiculophylla* populations were found as either fixed via a holdfast (i.e., fixed) or free-floating. At four populations (Table S2), we were unable to determine whether *G. vermiculophylla* or *Gp. andersonii* thalli were fixed or free-floating as they were completely buried in sand or mud, but some thalli had holdfasts suggesting fixation to pebbles or small stones prior to detachment as we extracted them from burial.

In fixed *G. vermiculophylla* populations, we found diploid tetrasporophytes as well as male and female gametophytes. In sites with free-floating thalli, we found sterile or tetrasporophytic thalli, characteristic of other populations in this species (Krueger-Hadfield et al. 2016b, 2017b).

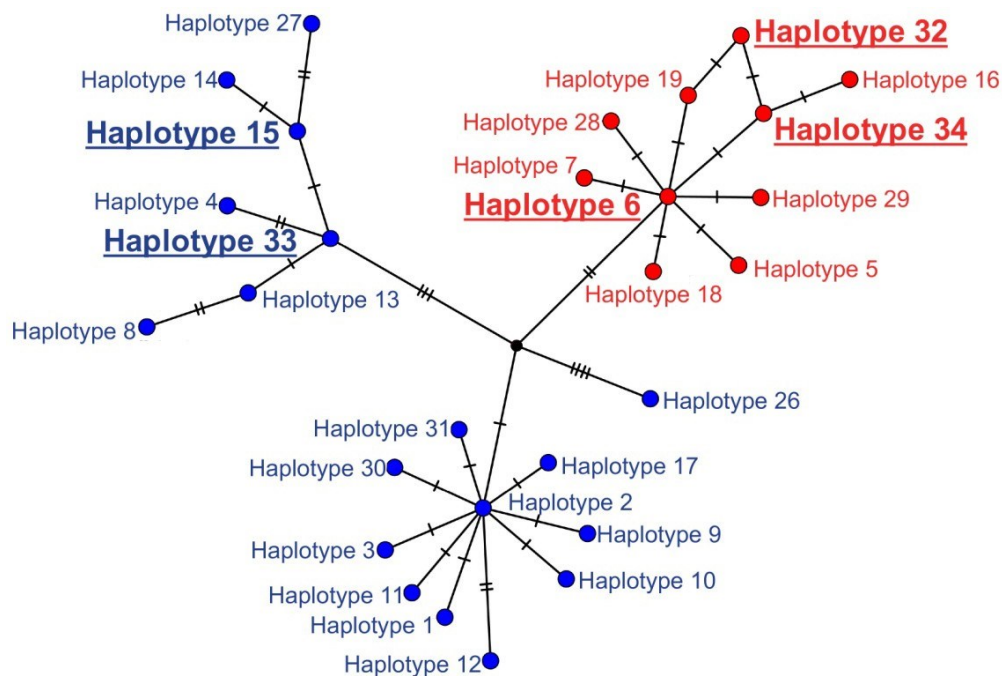
### Haplotype diversity

We identified five haplotypes among the sequenced specimens, two of which were previously described by Yang et al. (2008), Kim et al. (2010), and Krueger-Hadfield et al. (2017a; Table S3). Most *G. vermiculophylla* specimens (116 of 124; 94%; Table S1) belonged to haplotype 6. At Bayview in Morro Bay (mbv), we collected four thalli that belonged to haplotype 15. We also uncovered three new haplotypes at two sites in Morro Bay (mbm, mbv). The new haplotypes 32 and 34 were found at mbv, and one thallus each from mbm and mbv belonged to haplotype 33.

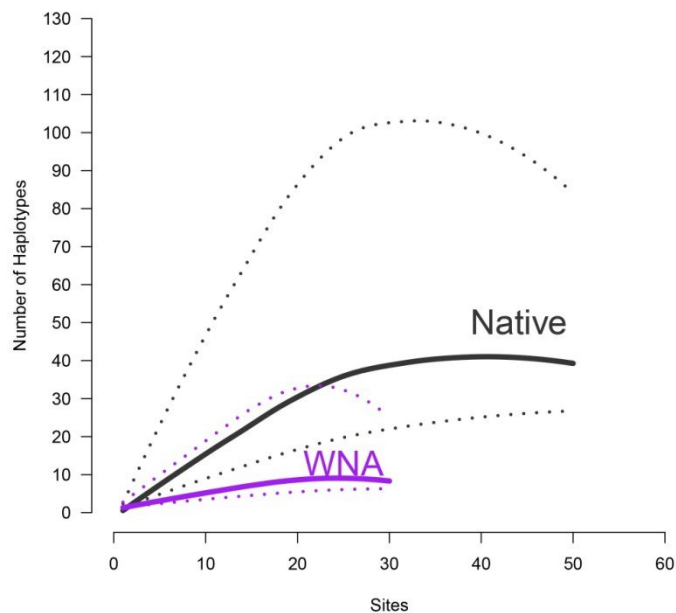
There were two main haplotype groups based on the “C/T” haplotypes from Krueger-Hadfield et al. (2017a). The “T” group was less divergent and there were fewer haplotypes than the “C” group (Figure 4), as previously described by Krueger-Hadfield et al. (2017a). Haplotypes 32 and 34 belong to the “T” haplotype group, whereas haplotype 33 belongs to the “C” haplotype subgroup that includes haplotypes 4, 8, 13, 14, 15, and 27 (Figure 4; Table S3). Haplotype estimates (Chao2) for the expected, maximum number of haplotypes were about five times greater in the native range (native: ~ 50 [95% CI, 25–102]; WNA: ~ 9 [95% CI, 6–32]; Figure 5).

## Discussion

*Gracilaria vermiculophylla* populations span more than 33° of latitude from Alaska, through Washington, Oregon, California, and Baja California (Bellorin et al. 2004; García-Rodríguez et al. 2013; Krueger-Hadfield



**Figure 4.** Median joining network based on mitochondrial *cox1* haplotypes from Kim et al. (2010), Krueger-Hadfield et al. (2017a), and this study. Red and blue haplotypes have either a “T” or a “C”, respectively, at the 945<sup>th</sup> bp and follow the designation of the two haplotype groups from Krueger-Hadfield et al. (2017a). Haplotypes shown in bold and underlined represent haplotypes detected from sequenced specimens in this study.



**Figure 5.** Haplotype estimation curves for native (dark grey) vs WNA (purple) *G. vermiculophylla*. The native Chao2 estimator suggests a maximum of ~ 40 haplotypes (95% CI, 25–102, dashed grey line) in the native range vs. ~ 9 haplotypes (95% CI, 6–32, dashed purple line) in WNA. Haplotype data were obtained from Kim et al. (2010), Krueger-Hadfield et al. (2017a), and this study.

et al. 2017a), and exhibit a more continuous distribution along WNA than previously reported. This study fills in significant gaps in the distribution of *G. vermiculophylla* along the WNA coast, and will

be an important contribution to future studies of this widespread marine invader. As previously discussed in the literature (Rueness 2005; Saunders 2009; Krueger-Hadfield et al. 2017a, b), oyster spat exports

from Asia and secondary introductions from sites within and between coastlines have likely contributed to the spread of this seaweed, as well as many other seaweeds (Mineur et al. 2007; Mineur et al. 2012). Below, we discuss the genetic structure of populations along the WNA using mitochondrial haplotypes, a brief history of the invasion, and scope for future work.

#### *Distribution of gracilarioid seaweeds along the WNA coastline*

In this study, generally, gracilarioid thalli present at the mouth of bays were identified as *Gp. andersonii*, but as we surveyed deeper into the estuaries or lagoons, *G. vermiculophylla* was the only gracilarioid species present among our sampled specimens, either fixed to hard substratum via a holdfast or free-floating. We suspect this pattern holds true in Oregon and Washington as well as California, but we did not sample the mouths of bays and estuaries in Oregon or Washington in the present study. The patterns found in our study confirmed the description of gracilarioid seaweed distributions, such as habitat type, provided in guidebooks when taxonomic revisions are taken into account (e.g., Dawson and Foster 1982; Druehl and Clarkson 2016).

The southeastern Alaskan coast is highly complex, with a few thousand kilometers of shoreline of potentially suitable habitat (see <http://shorezone.org>). On Prince of Wales Island, there are many sheltered or semi-sheltered bays in which *G. vermiculophylla* was observed, growing high in the intertidal (approx. + 0.3–1 m MLLW), at a similar tidal height as *Fucus* sp. and *Mastocarpus* sp. *G. vermiculophylla*, however, did not grow on the same substrate as these other macroalgal species. Instead it was most often present on muddy or fine-sand habitat (sometimes with pebbles intermixed) above seagrass beds, but rarely overlapping them.

In San Francisco Bay, we found *Gp. andersonii* inside the bay rather than restricted to exposed rocky shores or at the mouth of the bay. We found *Gp. andersonii* at Crown Beach, a sandy beach with some rocks. By contrast, we only found *G. vermiculophylla* on muddy shores with some hard substratum and the upper-mid intertidal species *Fucus* and *Mastocarpus*. Silva (1979) described *Gp. andersonii* (as *G. sjoestedtii*) occurring on outer coasts as well as along the muddy shores of bays, including through the central San Francisco Bay, whereas, “*G. verrucosa*” co-occurred with *Gp. andersonii* at several sites throughout the central bay. Without sequencing herbarium specimens from these collections, it is not clear to which taxon this “*G. verrucosa*” belonged as many gracilarioid taxa had been lumped prior to

subsequent revision. For example, Abbott (1985) described *G. pacifica* in order to distinguish it from *G. verrucosa*. Abbott (1983) also described *G. papenfussii* to distinguish it from *Gp. andersonii*. Our surveys suggest the occurrence of both *Gp. andersonii* and *G. vermiculophylla* in the San Francisco Bay, but not *G. pacifica* or *G. papenfussii*. Surveys should be conducted throughout San Francisco Bay to determine where other populations of *G. vermiculophylla* and other gracilarioid species may be located throughout the bay.

Wehrenberg (2010) compared *Gp. andersonii* from an exposed rocky shore to a population from a protected estuarine sand flat, just inside the mouth of Elkhorn Slough. The rocky shore population included reproductive haploid and diploid thalli, whereas the sand flat was composed of thalli without reproductive structures. This mirrors patterns in *G. chilensis* (Guillemin et al. 2008b) and *G. vermiculophylla* (Krueger-Hadfield et al. 2016b), where rocky shore populations in each species were sexual with a mix of haploids and diploids. In our study, soft-sediment populations were composed of sterile material that was overwhelmingly diploid, though some soft sediment sites did have thalli with tetrasporangial sori, suggesting meiosis had not been entirely lost.

Inside Elkhorn Slough, previous reports have described *Gp. andersonii* (as *Gp. lemaneiformis*) becoming “luxuriant” in the spring and carpeting the mudflats (Silberstein and Campbell 1989; Zimmerman and Caffrey 2002). We have not found *Gp. andersonii* inside the slough on multiple surveys (Kollars et al. 2015; Krueger-Hadfield et al. 2016b, 2017a, this study). Rather, we have consistently found *G. vermiculophylla* at Azevedo Pond within the upper part of the slough. On our surveys in 2017, we found an established population just downstream from the sluice at the southern end of Azevedo Pond, as well as at the Kirby Park boat launch. The latter population at Kirby Park was not found on previous surveys in 2013 and 2015 (S.A. Krueger-Hadfield, pers. obs.), but Hughey (2013) had previously recorded the presence of *G. vermiculophylla* there.

We do not, however, know whether fixed populations of *G. vermiculophylla* are the “donor” populations for the nearby free-floating thalli in the non-native range. However, with the detailed distribution data uncovered in this study, we can now test hypotheses about genetic differentiation among sites with fixed and free-floating thalli.

It is also unclear whether *G. vermiculophylla* is outcompeting native gracilarioid thalli in these ecosystems since *G. vermiculophylla* and the native species *Gp. andersonii* appear to occupy different niches within these estuaries (see also Wehrenberg 2010).



However, Gorman et al. (2017) found that *G. vermiculophylla* thalli grew at similar rates to the native *G. tikvahiae* in Long Island Sound, but with the former better able to survive temperature and osmotic stress than the latter (see also Kim et al. 2016). These results suggest that *G. vermiculophylla* may be able to outcompete *G. tikvahiae* in Long Island Sound. *Gracilaria tikvahiae* has become increasingly hard to find in the mudflats of Charleston Harbor, South Carolina that are now dominated by *G. vermiculophylla* (S.A. Krueger-Hadfield, *pers. obs.*). Along the WNA coastline, similar studies between *G. vermiculophylla* and *Gp. andersonii* are warranted to determine the interaction between *G. vermiculophylla* and native species. As Byers (1999) showed with displacement of the native snail *Cerithideopsis californica* by the invasive mud snail *Battillaria attramentaria*, these processes can be gradual, but nevertheless detrimental to overall ecosystem health. It is possible that similar, gradual processes are occurring due to the interaction of *G. vermiculophylla* and native species throughout the Northern Hemisphere.

#### Haplotype diversity along the WNA coastline

As found in previous studies (Kim et al. 2010; Krueger-Hadfield et al. 2017a), the majority (> 90%) of *G. vermiculophylla* thalli belonged to haplotype 6, the dominant haplotype in the northern part of the native range and throughout the non-native range. At Morro Bay, California, we found four thalli that belonged to haplotype 15. This “C” haplotype had previously only been found in the native range in Tokyo Bay in Japan (Kim et al. 2010), and at non-native sites in British Columbia and Virginia (Krueger-Hadfield et al. 2017a). We also uncovered three new haplotypes at Morro Bay. The haplotypes 32 and 34 fall into the “T” clade described by Krueger-Hadfield et al. (2017a) which is found north of ~ 35°N in the native range, and includes haplotype 6. The “C” haplotype 33 fell into the same “C” clade as haplotype 15. These “C” haplotypes were found south of ~ 35°N latitude in southern Japan and South Korea (Krueger-Hadfield et al. 2017a).

Haplotypes 32–34, from this study, have not yet been found in the native range (Kim et al. 2010; Krueger-Hadfield et al. 2017a). However, the haplotype estimation curves for the native vs. WNA ranges (Figure 5) support previous suggestions that WNA populations were non-native (e.g., Saunders 2009; Krueger-Hadfield et al. 2017a; Sotka et al. 2018). There are approximately five times as many haplotypes estimated for the native range as compared to those in WNA. Thus, the haplotypes found exclusively in the non-native range (haplotype 19 from Kim et al. 2010; haplotype 29 from Krueger-Hadfield et al.

2017a; and haplotypes 32, 33, 34 from this study) are likely among the missing haplotypes in the native range that will be discovered with future sampling and sequencing efforts (see also Miura et al. 2006; Blakeslee et al. 2008). For example, haplotype 15 was found in Tokyo Bay by Kim et al. (2010), and the new “C” haplotype 33 is in the same subgroup as haplotype 15, suggesting that this haplotype may be found in southern Japan with additional sampling.

Although Krueger-Hadfield et al. (2017a) found that most haplotypes originated in three bays in northeastern Honshu and southern Hokkaido Islands, Japan, there were exceptions along the WNA coastline. Thalli from some sites appeared to be a mixture of northern and southern Japanese genetic clusters (see Figures 3 and S3 from Krueger-Hadfield et al. 2017a), indicating the introduction of thalli from multiple Japanese source sites. Here, we have identified another non-native area, in Morro Bay, where a mixture of “C” and “T” haplotypes occurred. Genotyping with microsatellite loci will allow comparisons of patterns of genetic diversity and differentiation between WNA and Japan, and among WNA sites.

#### Oysters, birds, and algae

Elton (1958) described oyster introductions as “the greatest agency of all that spreads marine animals to new quarters of the world.” Previous studies (Rueness 2005; Saunders 2009) implicated oyster imports as the vector leading to the spread of *G. vermiculophylla*, and Krueger-Hadfield et al. (2017a) identified Matsushima, Mango-ku, and Akkeshi bays as the main sources of the Northern Hemisphere *G. vermiculophylla* invasion using microsatellite genotyping. Though the majority of exported Pacific oysters originated from the Miyagi Prefecture in Japan, and Matsushima Bay in particular, other areas including Hiroshima, Kumamoto, and Tokyo contributed to oyster exports (Barrett 1963).

We have found *G. vermiculophylla* thalli in many of the bays and lagoons to which Japanese oysters have been introduced and cultured (Byers 1999). This included bays surveyed by Byers (1999) which did not have the invasive gastropod *B. attramentaria*, but did have *G. vermiculophylla* (Table S1).

We have found *G. vermiculophylla* thalli fixed via holdfasts to oyster shells in the native and non-native range (S.A. Krueger-Hadfield, *pers. obs.*), suggesting that spores, sporelings, or thalli may have hitchhiked on oyster shells. It is also entirely plausible, however, that *G. vermiculophylla* was used as packing material for Japanese oyster stock, and could have been subsequently thrown into the bays and lagoons along the WNA coastline after oysters were delivered.

*G. vermiculophylla* is one of the dominant macrophytes in Matsushima and Akkeshi Bays (Krueger-Hadfield et al. 2017a) along with *Zostera japonica*, a seagrass that was introduced to Washington State likely as packing material for adult Japanese oysters (Fisher et al. 2011). The global invader *Sargassum muticum* was also observed in oyster shipments (Critchley and Dijkema 1984). Similarly, the baitworm trade, among other fisheries, uses the fucoid *Ascophyllum nodosum* to keep the worms alive during shipment (Fowler et al.

2016), introducing *Ascophyllum* as well as other invertebrates living in the algae from the east coast of the United States to the San Francisco Bay (Cohen et al. 2001; Miller et al. 2004; Blakeslee et al. 2016).

The current distribution of *G. vermiculophylla* mirrors that of other species introduced with oysters, such as *S. muticum* (Engelen et al. 2015), along the WNA coastline from southeastern Alaska to Baja.

Many of these estuarine sites, however, are also important rest-stops along the flyways of migrating birds (e.g., Ward et al. 2005). Nyberg and Wallentinus (2009) hypothesized that migrating seabirds may carry small thallus fragments from one site to another leading to secondary introductions. This might be important in the secondary spread of *G. vermiculophylla* and other gracilarioid seaweeds through WNA estuaries, but is unlikely to have contributed to the invasion of the WNA coastline from the native range.

For example, *G. vermiculophylla* was found in the Bolsa Chica wetland reserve in Southern California, an important restored habitat and birding area without a history of oyster cultivation. We have seen birds use *G. vermiculophylla* thalli at other sites in the non-native range to build nests (S.A. Krueger-Hadfield, *pers. obs.*), suggesting that transport by birds may be a vector responsible for secondary introductions.

Another mechanism for secondary introduction along the WNA coastline may be the commercial cultivation of the seaweed itself. *Gp. andersonii* is cultivated as abalone feed in central California (M. Graham, *pers. comm.*, in Wehrenberg 2010). Likewise, Neushul and Neushul (1992) and West (1992) reported that the best candidate for cultivation in California was *G. lemaneiformis* (now known as *Gp. andersonii*). It is possible that these authors referred, in fact, to *G. vermiculophylla*, which was harvested in Japan for its high quality agar (Okazaki 1971). Dawson and Foster (1982) also mentioned the fishery for herring eggs on gracilarioid seaweeds in San Francisco and Tomales bays, which likely included *G. vermiculophylla*. Without surveys of historical and contemporary gracilarioid populations, it is unclear which species are involved in harvesting, or moved around between sites.

### Future directions

Without rich barcode databases and easily implemented genetic tools, species identification will not be easy for non-specialists who often depend on morphological characters alone (Saunders 2009). There are, however, useful primers available, such as 622F (Yang et al. 2008) and 1549R (Geraldino et al. 2006), that distinguish *Gracilaria* and *Gracilariopsis* species with sequencing, and could be used for relatively rapid identification by researchers. Alternatively, *rbcL* could be used to distinguish *G. vermiculophylla* from other co-occurring gracilarioid species (Guillemin et al. 2008a). Without using these molecular tools, species like *G. vermiculophylla*, will likely remain undetected in other estuarine locations.

*G. vermiculophylla* has largely flown under the radar in estuaries along WNA, despite previous authors calling attention to the lack of knowledge about its distribution along this coastline (e.g., Miller et al. 2012). This is particularly problematic in areas where ecologists and managers are actively working to assess the impacts of non-native macrophytes, such as *Zostera japonica* or *Spartina alterniflora*, on native seagrass communities, and overall ecosystem health (see Williams 2007), because *G. vermiculophylla* is probably also acting as an important ecosystem engineer at these sites. Moreover, *G. vermiculophylla* has often been misidentified as the native species *Gp. andersonii*, potentially confounding community studies that are investigating (or have investigated) the interaction of a gracilarioid seaweeds and native species (e.g., seagrasses, seaweeds, invertebrates). Developing greater awareness of these two morphologically similar species is important, as *Gp. andersonii* and *G. vermiculophylla* may have different nutrient composition, and to limit the secondary introduction of *G. vermiculophylla* to other sites through collecting and cultivating activities.

Our survey has demonstrated that *G. vermiculophylla* is abundant throughout WNA. We know this species has the propensity for profound impacts on the ecosystems to which it is introduced (e.g., Byers et al. 2012; Ramus et al. 2017). Eco-evolutionary studies are critical along the WNA coastline, where the *G. vermiculophylla* invasion has gone undetected in many sites. Based on work in other non-native regions, these populations are not likely to be innocuous invaders (e.g., Thomsen et al. 2012; Byers et al. 2012; Hammann et al. 2013; Ramus et al. 2017). In conjunction with the differences in fixed via holdfasts versus free-floating populations, future studies should also address the mating system differences across the native and non-native ranges of *G. vermiculophylla* in order to understand the

longer-term ecological and evolutionary impacts of sexual versus asexual reproduction in this seaweed. It is possible that the fixed populations along the WNA, and other coastlines (Krueger-Hadfield et al. 2016b, 2017a), may serve as source populations for free-floating populations which are sustained mainly through vegetative growth and asexual fragmentation. The site and demographic information provided here can provide the foundation for these types of studies in WNA estuaries.

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## Author contributions

SAKH conceived the study; SAKH, TS, WHR, and SH collected samples; SAKH performed genetic analyses and analyzed data; SAKH wrote the manuscript. All authors approved the final manuscript.

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## Supplementary material

The following supplementary material is available for this article:

**Table S1.** Descriptions of the sites surveyed in 2017 along the west coast of North America.

**Table S2.** Reproductive state of *G. vermiculophylla* and *Gp. andersonii* thalli from surveyed sites in 2017 along the west coast of North America.

**Table S3.** *Gracilaria vermiculophylla* haplotypes from Yang et al. (2008), Kim et al. (2010), Krueger-Hadfield et al. (2017a), and this study.

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