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Invasion of novel habitats uncouples haplo-diplontic life cycles

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Abstract

Baker's Law predicts uniparental reproduction will facilitate colonization success in novel habitats. While evidence supports this prediction among colonizing plants and animals, few studies have investigated shifts in reproductive mode in haplo-diplontic species in which both prolonged haploid and diploid stages separate meiosis and fertilization in time and space. Due to this separation, asexual reproduction can yield the dominance of one of the ploidy stages in colonizing populations. We tested for shifts in ploidy and reproductive mode across native and introduced populations of the red seaweed *Gracilaria vermiculophylla*. Native populations in the northwest Pacific Ocean were nearly always attached by holdfasts to hard substrata and, as is characteristic of the genus, haploid–diploid ratios were slightly diploid-biased. In contrast, along North American and European coastlines, introduced populations nearly always floated atop soft-sediment mudflats and were overwhelmingly dominated by diploid thalli without holdfasts. Introduced populations exhibited population genetic signals consistent with extensive vegetative fragmentation, while native populations did not. Thus, the ecological shift from attached to unattached thalli, ostensibly necessitated by the invasion of soft-sediment habitats, correlated with shifts from sexual to asexual reproduction and slight to strong diploid bias. We extend Baker's Law by predicting other colonizing haplo-diplontic species will show similar increases in asexuality that correlate with the dominance of one ploidy stage. Labile mating systems likely facilitate colonization success and subsequent range expansion, but for haplo-diplontic species, the long-term eco-evolutionary impacts will depend on which ploidy stage is lost and the degree to which asexual reproduction is canalized.

Keywords: Baker's Law, biological invasion, biphasic life cycle, fragmentation, *Gracilaria*, haploid–diploid, population genetics, seaweed, uniparental reproduction

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Introduction

Mating systems affect the distribution of genetic diversity within and among populations. For example, outcrossing species tend to be more genetically diverse and exhibit less genetic differentiation among populations than inbreeding species (Hamrick & Godt 1996;

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Duminil *et al.* 2009). The life history traits affecting mating systems and dispersal are evolutionarily labile, affect genetic structure by influencing the drift/migration equilibrium and vary within and across taxa (Lynch 1984; Bierzychudek 1985; Kolar & Lodge 2001; Barrett 2002; Auld & de Casas 2012; van Kleunen *et al.* 2015). In the particular case of the colonization of novel habitats, the number of potential mates is often exceedingly low or even zero. Thus, initial colonization will act as a sieve on the extant variation in reproductive traits (Barrett 2002; Barrett *et al.* 2008; van Kleunen *et al.* 2008; Burns *et al.* 2011; Hao *et al.* 2011; Pannell 2015). Baker (1955) formalized this argument and proposed that species with an enhanced capacity for uniparental reproduction (i.e. selfing, asexuality or both) following long-distance colonization events will have an increased likelihood of successful establishment. Although Baker (1955) focused on dispersal from continents to oceanic islands, recent studies have demonstrated colonization at any spatial scale is facilitated by uniparental reproduction (e.g. Pannell & Barrett 1998; Pannell *et al.* 2015).

At the conclusion of his seminal essay, Baker (1955) suggested an extension of his ideas to both biological introductions and haplo-diplontic species. Since then, many studies have evaluated correlations between life history traits and mating systems in introduced, diplontic (diploid) species (Kolar & Lodge 2001; Crawford *et al.* 2009; Billliard *et al.* 2012; de Groot *et al.* 2012; van Kleunen *et al.* 2015; Krueger-Hadfield *et al.* 2015). Indeed, higher rates of uniparental reproduction are found in introduced populations across taxonomic groups (van Kleunen *et al.* 2008; Burns *et al.* 2011; Hao *et al.* 2011) and within the same species (Ward *et al.* 2011; Petanidou *et al.* 2012). However, despite Baker's (1955) suggestion, few studies have explicitly tested Baker's prediction in haplodiplontic organisms (or, haploid–diploid) in which there are both prolonged, multicellular haploid *and* diploid stages.

Rates of uniparental (i.e. selfing and asexual reproduction) vs. biparental reproduction (i.e. outcrossing) are of critical evolutionary importance, but the mechanisms by which these evolutionary processes operate fundamentally differ between diplontic and haplodiplontic life cycles (see reviews in Crawford *et al.* 2009; de Groot *et al.* 2012; Billliard *et al.* 2012; Krueger-Hadfield *et al.* 2015). Unlike diplontic life cycles in which fertilization immediately follows meiosis, haplo-diplontic life cycles have a prolonged haploid stage and as a consequence meiosis and fertilization are spatially and temporally separated (Fig. 1A diplontic vs. B haplodiplontic life cycles). There are two possible ways for a haplo-diplontic species to self through either intra- or intergametophytic selfing depending on whether the species is monoicious or dioicious, respectively (Klekowski 1969; Soltis & Soltis 1992; Beukeboom & Perrin 2014). Intragametophytic selfing occurs when gametes from the same haploid gametophyte unite and results in instant homozygosity. Intergametophytic selfing occurs when haploid gametophytic siblings (i.e. those derived from the same diploid sporophyte) mate. In addition to selfing, when asexual reproduction is frequent, species with biphasic life cycles will tend to become dominated by only one of the two ploidy stages (e.g. Maggs 1988; Klekowski 2003). For example, in macroalgae, the haploid stage is often bypassed in environments in which fertilization may be difficult, such as low salinities (Gabrielson *et al.* 2002), as well as across other environmental gradients (Hwang *et al.* 2005; Krueger-Hadfield *et al.* 2013a). Similar patterns of ploidy dominance have also been described in mosses (Patiño *et al.* 2013; Laenen *et al.* 2016), ferns (Klekowski 2003; de Groot *et al.* 2012) and fungi (Billliard *et al.* 2012; Gladieux *et al.* 2015). Thus, the differences between diplontic and haplo-diplontic life cycles generate unique predictions for Baker's Law and the consequences of the colonization of novel habitats.

Biological invasions are specific cases of colonization events in which Baker's Law predicts shifts in reproductive mode between native and introduced populations (Pannell *et al.* 2015), including those species with biphasic life cycles. The deliberate and accidental introduction of haplo-diplontic seaweed species can have enormous ecological and economic impacts (Williams & Smith 2007; Andreakis & Schaffelke 2012). One factor that likely facilitates the rapid spread of macroalgal invaders is their capacity for uniparental reproduction. All three clades of seaweeds exhibit extensive diversity in life cycle types and, presumably, mating system variation, although the latter has been less well studied (but see, Valero *et al.* 2001; Billot *et al.* 2003; Engel *et al.* 2004; Guillemin *et al.* 2008a; Krueger-Hadfield *et al.* 2013b). The release from obligate sexual reproduction and the potential for rapid expansion through asexual reproduction have been shown to enable macroalgal range expansions into novel environments, such as along salinity (Gabrielson *et al.* 2002), temperature (Hwang *et al.* 2005), tidal (Fierst *et al.* 2010; Krueger-Hadfield *et al.* 2013a) and latitudinal gradients (Krueger-Hadfield *et al.* 2013a), and is the main method of propagation in aquaculture (Guillemin *et al.* 2008a).

The red macroalga *Gracilaria vermiculophylla* (Ohmi) Papenfuss has spread from its native distribution in the northwestern Pacific Ocean to virtually every high-salinity, temperate estuary in Europe and North America within the last few decades (Bellorin *et al.* 2004; Guillemin *et al.* 2008b; Weinberger *et al.* 2008; Saunders 2009). Initial records of the expansion to the introduced range date back as early as the late 1970s (Bellorin *et al.* 2004),

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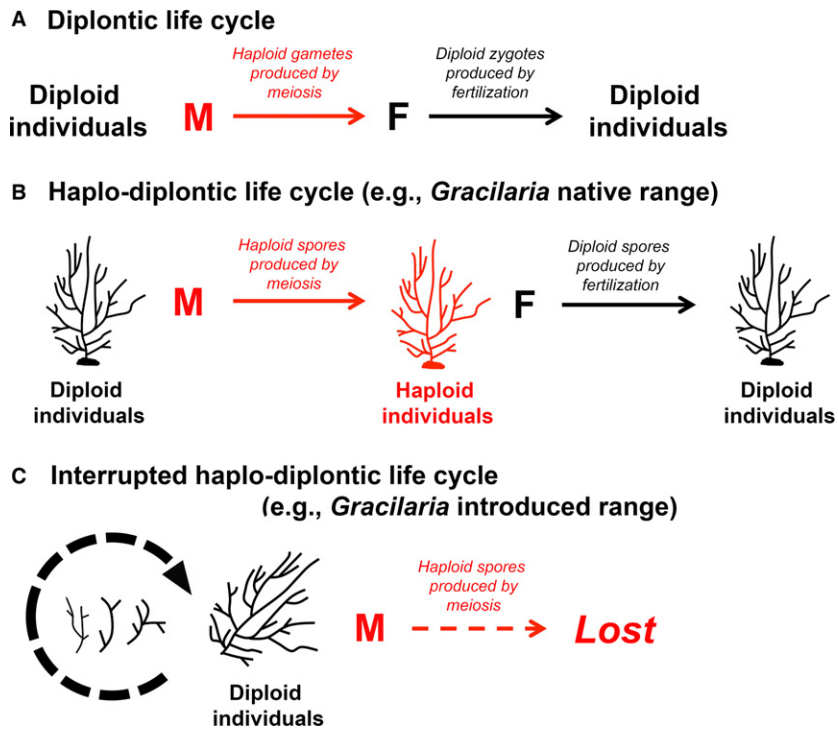

Fig. 1 (A) Diplontic life cycle. Fertilization (F) rapidly follows meiosis (M) in monophasic life cycles, such as exhibited by animals. Gametes are produced by meiosis and the haploid stage is very reduced or unicellular. (B) Haplo-diplontic life cycle of native *Gracilaria vermiculophylla*. Meiosis and fertilization are spatiotemporally separated in biphasic or haplo-diplontic life cycles. The mature diploid produces haploid spores via meiosis. These haploid spores settle and become free-living, macroscopic, haploid individuals. Haploids produce gametes, and after fertilization, the diploid spores settle and become the next generation of diploids. All individuals had holdfasts in the native range of *G. vermiculophylla*, fixing the thalli to hard substratum (see Fig. 2A). (C) Uncoupled haplo-diplontic life cycle of introduced *G. vermiculophylla*. Thalli were typically diploid, free-floating and maintained by fragmentation (dashed black line showing smaller fragments). Haploid spores rarely settle and germinate in the soft-sediment habitats of the introduced range and, thus, fertilization cannot be completed. Populations rely, instead, on fragmentation.

although cryptic invasions were likely occurring earlier. Based on oyster aquaculture records (Barrett 1963; Ruesink *et al.* 2005) and genetic data (S.A. Krueger-Hadfield, A.E. Strand and E.E. Sotka, *unpublished data*), there were at least three primary invasions into each of the three continental margins in the Northern Hemisphere, followed by secondary spread along each coastline. The abundance of *G. vermiculophylla* has increased to upwards of 80–100% cover of benthic habitats in some locations (Thomsen *et al.* 2009a; Nejrup & Pedersen 2010; Byers *et al.* 2012). Introduced populations can outcompete native macroalgae (Hammann *et al.* 2013a), provide novel habitat for invertebrates (Nyberg *et al.* 2009) and alter community structure, species interactions and trophic pathways (Byers *et al.* 2012). The ecological success of *G. vermiculophylla* in introduced habitats has been attributed to its high stress tolerance (Yokoya *et al.* 1999; Raikar *et al.* 2001), herbivore resistance (Hammann *et al.* 2013b) and, in some areas, an absence of other macroalgal competitors (Byers *et al.* 2012).

Using multilocus microsatellite genotyping of native and introduced populations of the red alga *Gracilaria vermiculophylla*, we tested two predictions from Baker's Law: (i) introduced populations primarily undergo uniparental reproduction which is distinct in the introduced range in comparison with the native range and (ii) asexual reproduction in introduced populations correlates with increased heterozygosity and the reduced frequency or loss of one free-living ploidy stage. To our knowledge, this represents the first test of explicit Baker's Law in a haplo-diplontic species in the context of biological invasions.

Materials and methods

Gracilaria life cycle

Gracilaria vermiculophylla alternates between free-living haploid (dioicious gametophytes) and diploid (tetrasporophyte) stages (Figs 1B and S1, Supporting information). Nonmotile, very short-lived male gametes are

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released and fertilize an egg retained on the haploid female thallus. The zygote is mitotically amplified thousands of times within the cystocarp, producing thousands of genetically identical diploid carpospores. The carpospores disperse and germinate into the free-living diploid. The diploid sporophytic thallus produces haploid tetraspores via meiosis that disperse and germinate into male or female haploid gametophytes. Although the typical Florideophyte red algal life cycle is often referred to as triphasic, there are only two free-living stages, the diploid tetrasporophytes and haploid gametophytes. Moreover, the cystocarp contains carpospores that are genetically identical to the next free-living stage, the tetrasporophyte. Thus, genetically, the red algal life cycle is biphasic.

In addition to the sexual life cycle described above, *Gracilaria* species are also able to reproduce vegetatively through thallus fragmentation (Kain & Destombe 1995). These free-floating thalli can grow indefinitely and propagate naturally without holdfasts. Guillemin *et al.* (2008a) documented extensive fragmentation in cultivated stands of *Gracilaria chliensis* in southern Chile. Contrary to hard substratum populations, sporic recruitment is unlikely in soft-sediment habitats where reproduction will be mainly vegetative (Stokke 1957; Guillemin *et al.* 2008a).

Sample collection

We sampled the breadth of the known Northern Hemisphere range across 30 native and 35 introduced sites (Table S1, Supporting information). We sampled 23 Japanese sites across all the major islands from Kyushu to Hokkaido as well as seven Chinese and South Korean sites. In the introduced range, we sampled sites covering the latitudinal distribution along the western coast of North America (n = 4), eastern coast of the United States (n = 21) and Europe (n = 10). Approximately 30 algal thalli were sampled from each site (Table S1, Supporting information) and were collected at least one metre apart in order to standardize the sampling of attached and unattached thalli and minimize the chances of sampling the same genet twice. Thalli were dried in silica gel as both voucher specimens and for subsequent DNA extractions.

The species identity of sampled thalli was confirmed with amplification of 10 species-specific microsatellite markers (Kollars *et al.* 2015) and, in some cases, DNA barcoding (Kim *et al.* 2010). The 10 microsatellite loci utilized in this study did not amplify in the congenerics *Gracilaria pacifica*, *G. tikvahiae* or *G. gracilis*.

When possible, ploidy was determined through observations of reproductive material under a dissecting microscope (40×, Fig. S1, Table S1, Supporting information). All thalli were genotyped blind in order to reduce bias in calling alleles based on known ploidy. Nevertheless, all phenotypically haploid material produced only one allele at the 10 microsatellite loci (n = 45) and all phenotypically diploid material produced at least one heterozygous locus (n = 185; also see Kollars *et al.* 2015). Therefore, when only dried samples were provided, we assigned ploidy based on the multilocus microsatellite genotype and considered an individual thallus as diploid if heterozygous at any of 10 loci genotyped (see also Guillemin *et al.* 2008b; Krueger-Hadfield *et al.* 2011).

Sites were characterized as attached if the thalli were fixed to hard substratum via a holdfast (Fig. 2A). All sites in which the thalli were drifting atop soft-sediment habitats without holdfasts were characterized as free-floating (Fig. 2B,C). Once detached from the substratum, *Gracilaria* thalli are unable to form new holdfasts and, thus, cannot re-attach (Guillemin *et al.* 2008a).

The binomial law was used to estimate the probability of detecting haploid–diploid ratios (including both reproductive and vegetative plants) deviating from the null hypothesis of $\sqrt{2}$:1 (Table S1, Supporting information), which is expected at demographic equilibrium when haploids and diploids are equal in fitness and sex ratios are equal to 1:1 (Destombe *et al.* 1989; Thornber & Gaines 2004). Krueger-Hadfield & Hoban (2016) simulated various sampling strategies for haplo-diplontic seaweeds and found sample sizes of 20 were sufficient to detect large deviations from $\sqrt{2}$:1.

DNA extraction and microsatellite amplification

Total genomic DNA was isolated following Kollars *et al.* (2015) or using 5 – 10 mg of dried tissue and the Nucleospin® 96 plant kit (Macherey-Nagel, Düren, Germany; Table S1, Supporting information). We followed 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, 2013b). Replicate control samples of the same thalli were run from chelex and MN Nucleospin extracts to ensure no shifts in fragment sizes between the two extraction methods. Simplex PCRs, fragment analysis and genotyping are described elsewhere for all loci (Kollars *et al.* 2015), with the addition of a new locus, Gverm_7969 (GenBank Accession no. KT232099, Table S2, Supporting information).

Microsatellite loci whose average rounding error was below 10% of the repeat size, as assessed by TANDEM (Matschiner & Saltzburger 2009), are useful for subsequent analyses. For example, a dinucleotide locus should exhibit rounding errors <0.2 in order to be considered a good locus. All loci were within this rounding error except Gverm_6311 (Table S2, Supporting information). We manually checked poorly binned alleles,

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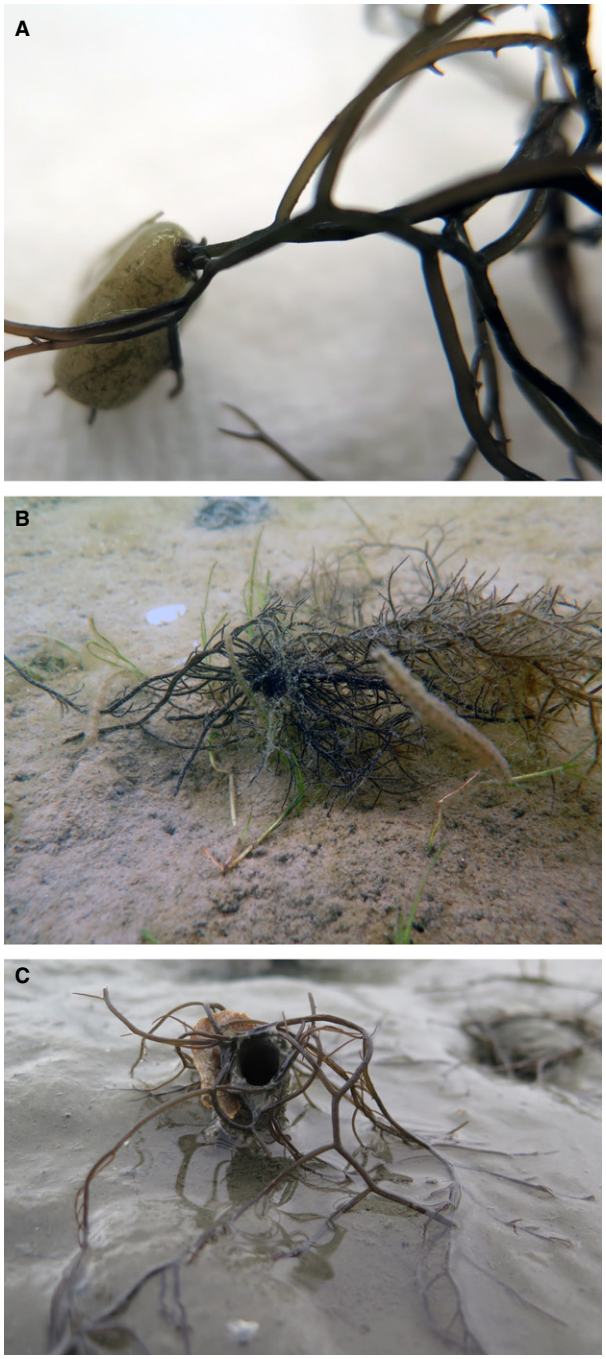

Fig. 2 (A) Native populations of *Gracilaria vermiculophylla* are attached to hard substratum via a holdfast. Here, there are two upright, thalli emerging from the small discoid holdfast. (B) In contrast, the majority of introduced populations of *G. vermiculophylla* drift as free-floating thalli above soft-sediment habitats. (C) In the southeastern United States, thallus fragments are incorporated into the mucus tube caps of the polychaete genus *Diopatra*. (Photo credits: S.A. Krueger-Hadfield).

following Krueger-Hadfield *et al.* (2013b), and are confident we did not artificially over- or underestimate allelic richness.

Microsatellite data analyses

The frequency of null alleles was directly estimated from the rate at which haploid gametophytes did not amplify after discounting technical errors (Krueger-Hadfield *et al.* 2011). Due to extensive clonality in the introduced range, we did not estimate null alleles for diploids as the random mating assumptions of ML-NullFreq were violated (Kalinkowski & Taper 2006; Krueger-Hadfield *et al.* 2011, 2013b). In *G. vermiculophylla*, null allele frequency estimates ranged from 0 to 0.01 across all 10 loci used to genotype 679 haploids (Table S2, Supporting information). Thus, null alleles did not constitute a problem for assigning genotypes or calculating other population genetic metrics, such as *F*-statistics.

Custom R (R Core Team 2015) routines were written in order to determine ploidy based on the multilocus genotype and create input files for downstream analyses. The number of repeated identical multilocus microsatellite genotypes (MLGs) was computed using a custom R routine, following Parks & Werth (1993) and Arnaud-Haond *et al.* (2007). Then, the frequency of different MLGs was calculated as: R (G−1) / (N−1), where G is the number of unique multilocus genotypes and N is the total number of studied individuals (Dorken & Eckert 2001). P_{sex}, which is the probability for a given MLG to be observed in N samples as a consequence of different sexual reproductive events, was calculated for each repeated MLG. If P_{sex} was >0.05, duplicated multilocus genotypes were considered as different genets. If P_{sex} was smaller than 0.05, the duplicated MLGs were considered as ramets (or clones) of the same genet.

Multiple MLGs may belong to the same clone, but due to the existence of somatic mutation or scoring errors, they could be artificially labelled as unique MLGs (Douhovnikoff & Dodd 2003). This can lead to an overestimation of the number of genets in the sample. This potential bias can be investigated by inspecting the frequency distribution of genetic distances among pairs of MLGs (Douhovnikoff & Dodd 2003; Meirmans & van Tienderen 2004). Somatic mutations or scoring errors at a significant rate should be reflected in the existence of a peak in the frequency distribution of genetic distances at very low genetic distances (Douhovnikoff & Dodd 2003; Meirmans & van Tienderen 2004; Arnaud-Haond *et al.* 2005, 2007). The genetic distances between all pairs of MLGs were computed using the R package POPPR ver. 2.0.2 (Kamvar *et al.* 2014, 2015). The maximum number of allelic differences across the 10 loci used in this study was 40.

Fragmentation of thalli in other species in the Order Gracilariales has been well documented (Kain &

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Destombe 1995; Guillemin *et al.* 2008b), yet clonal 'amplification' is likely not equal across MLGs. Deviations from Hardy–Weinberg and linkage equilibria may occur at certain loci because a genotype may be disproportionately amplified, rather than different evolutionary processes at work (Sunnucks *et al.* 1997; Halkett *et al.* 2005a). Thus, many studies remove repeated MLGs before calculating heterozygosity and other *F*-statistics in order to avoid distorting these estimates (e.g. Sunnucks *et al.* 1997; Halkett *et al.* 2005a; Krueger-Hadfield *et al.* 2011). However, unlike modular species for which clonal processes are a mode of ramification, such as seagrasses (Arnaud-Haond *et al.* 2007), in the introduced *G. vermiculophylla* populations, fragmentation is the hypothesized reproductive mode and not a mode of ramification. Therefore, we performed the following analyses on two types of data sets. The first data set was analysed using all sampled, diploid thalli, including repeated MLGs (i.e. all thalli). The second data set included only diploid MLGs for which $P_{sex} > 0.05$ (i.e. one thallus per genotype based on P_{sex}).

Linkage disequilibrium was evaluated using the single multilocus estimate $\bar{r}_d$ (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 data sets in which sex and recombination were 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 data set. In addition to physical linkage on a chromosome, disequilibria 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 average expected heterozygosity (H_E) and observed heterozygosities (H_O) were calculated using GENALEX ver. 6.5 (Peakall & Smouse 2006, 2012). An estimate of the mean expected number of alleles (A_E) was computed using the program HP-RARE ver. 1.0 (Kalinkowski 2005) on the smallest sample size of 10 diploids (i.e. 20 alleles). Tests for Hardy–Weinberg equilibrium and *F*-statistics were performed on diploid thalli using FSTAT ver. 2.9.3.2 (Goudet 1995). F_{is} was calculated for each locus and over all loci according to Weir & Cockerham (1984) and significance was tested by running 1000 permutations of alleles among individuals within samples.

Summary statistic means (e.g. H_o) were compared between native and introduced regions using a two-tailed *t*-test or Wilcoxon signed-rank test depending on violation of normality and homoscedasticity assumptions in order to assess broadscale patterns. Within-population comparisons (LD, heterozygote excess, heterozygote deficiency) were made using Fisher's exact tests implemented in R. Bonferroni correction was applied to correct for multiple tests comparing summary statistics (Sokal & Rohlf 1995). It was possible to calculate LD in 29 of the 32 introduced diploid subpopulations, so within-population tests were performed on 29 introduced sites for LD, whereas the heterozygote excess or deficiency was measured at 32 introduced sites. In the other three sites, the number of genotypes was too low (<5) to allow for a robust estimate of LD.

Results

We sampled and genotyped 588 diploid and 337 haploids from 30 native sites in China, South Korea and Japan and 934 diploids and 232 haploids from the 35 introduced sites along the North American and European coastlines. In the introduced range, only 10% of the diploid thalli exhibited a single heterozygous locus compared to 37% of the diploid thalli in the native range. The frequency of diploids identified solely by the microsatellite MLG may have been slightly underestimated because some rare diploids may be fixed homozygotes across the 10 loci utilized, particularly in the native range in which diploids were less heterozygous (also see *Population signals of vegetative reproduction*). However, given the consistency of the match between phenotype- and genotype-based assessments of ploidy, we considered this a rarity.

The number of alleles per locus ranged from 3 to 52 (Table S2, Fig. S2, Supporting information), for a total of 170 alleles. The frequency distributions of the number of different alleles between all MLG pairs within a site were unimodal in all populations without trends towards higher frequencies at very low differences as might be expected if somatic mutations or scoring errors were common (Fig. S3, Supporting information).

Variation in habitat and ploidy between introduced and native ranges

In our survey of 30 native sites, nearly all *Gracilaria vermiculophylla* thalli were attached to hard substratum (i.e. pebbles, rocks or shells) via a holdfast (Fig. 2A). In contrast, 32 of 35 introduced sites were composed of free-floating thalli with few to no holdfasts. Thalli drifted on intertidal and shallow subtidal mudflats (Fig. 2B) or were glued to mucus tubes created and decorated by polychaetes in the genus *Diopatra* (Table 1). The thalli anchored by *Diopatra* worm tubes were not fixed by holdfasts, but rather glued to the tube caps (i.e. thalli

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Table 1 Population genetic statistics of native and introduced populations of *Gracilaria vermiculophylla* based on 10 microsatellite loci of 1522 diploids and 569 haploids. The means of each statistic and within-population tests were computed on the diploid subpopulations including repeated genotypes

	All thalli including repeated genotypes[1]			Thalli with repeated genotypes removed[2]		
Statistic	Introduced	Native	*P*	Introduced	Native	*P*
Proportion diploid	0.811	0.578	***	0.802	0.603	***
Proportion of repeated multilocus genotypes	0.362	0.092	***	—	—	—
Genotypic richness (*R*)	0.610	0.908	***	—	—	—
Number of copies of repeated genotypes	5.7	2.3	***	4.4	0.9	***
Allelic richness (A_E)	2.1	2.3	NS	2.3	2.5	NS
Expected heterozygosity (H_E)	0.293	0.281	NS	0.313	0.215	NS
Observed heterozygosity (H_O)	0.341	0.215	***	0.331	0.215	***
Inbreeding coefficient (F_{is})	−0.234	0.223	***	−0.111	0.238	***
Linkage disequilibrium (LD, $\bar{r}_D$)	0.172	0.039	***	0.082	0.026	**
Within-population tests[3], %						
Populations with significant LD	62	7	***	35	7	NS
Populations with significant heterozygote excess	34	0	**	6	0	NS
Populations with significant heterozygote deficit	9	40	NS	9	47	**

[1]Bonferroni adjusted $\alpha = 0.006$; NS: $P > 0.006$; ***: $P < 0.0001$.
[2]Bonferroni adjusted $\alpha = 0.007$; NS: $P > 0.006$; ***: $P < 0.0001$
[3]Bonferroni adjusted $\alpha = 0.017$ for both data sets including all thalli and with repeated genotypes removed; NS: $P > 0.017$; **: $P < 0.005$; ***: $P < 0.0001$.

did not recruit to the tubes via spore dispersal and settlement; Thomsen *et al.* 2009b; Byers *et al.* 2012). Therefore, we considered these sites as free-floating. In the *Diopatra*-associated populations, we sampled a single thallus fragment per worm tube with each worm tube separated by 1 m.

The difference between hard-substrate native habitats and the soft-substrate introduced habitats was mirrored by profound shifts in haploid–diploid ratios (Fig. 3A, Table 1). On average, the native populations were 58% diploid. The majority of the native populations exhibited haploid–diploid ratios indistinguishable from the expected ratio of $\sqrt{2}$:1 (diploid proportion: 33–67%; Tables S1 and S3, Supporting information). These results were generally consistent with the inclusion and exclusion of repeated MLGs. There were seven Japanese sites (Jonai, Wajiro, Jigozen, Shinori, Akkeshi, Hirakawa and Tenjinjima) and one South Korean site (Jindo) with significant diploid bias regardless of the inclusion or exclusion of repeated MLGs (70–97% diploid). The only haploid bias was found in the South Korean site at Odo-2-ri, but this bias disappeared when the repeated haploid MLGs were removed from the analysis.

In contrast, introduced sites were, on average, 81% diploid, a significantly greater frequency than that found within the native range (Fig. 3A, Table 1) when assessed both with and without repeated MLGs. Twenty-seven introduced sites were significantly diploid-biased when repeated MLGs were included (diploid proportion: 87–100%; Tables S1 and S3, Supporting information). Thirteen of these 27 sites were diploid-fixed (i.e. 100% of thalli were diploid).

There were two exceptions to these general patterns in the introduced range. First, three sites (Oakland Beach, Rhode Island; Horsens and Nyborg, Denmark) were near or at haploid fixation and had high proportions of repeated haploid MLGs (86–94% of the thalli; Table S3, Supporting information). Second, two introduced sites (Elkhorn Slough, California; and Ape Hole Creek, Maryland) were found exclusively attached to hard substratum during the surveys performed in this study (Table S1, Supporting information). These two sites were composed of haploid–diploid ratios indistinguishable from $\sqrt{2}$:1 (Table S1, Supporting information). A third introduced site at Magotha Road in Virginia was composed of nearly equal proportions of attached and free-floating thalli. This site was characterized by shells and small pebbles enabling sporic recruitment.

Population genetic signals of vegetative reproduction

Several population genetic statistics were consistent with greater rates of asexual fragmentation in introduced relative to native sites. These analyses were performed on diploids only given the lack of haploid thalli in the introduced sites. There were significantly more

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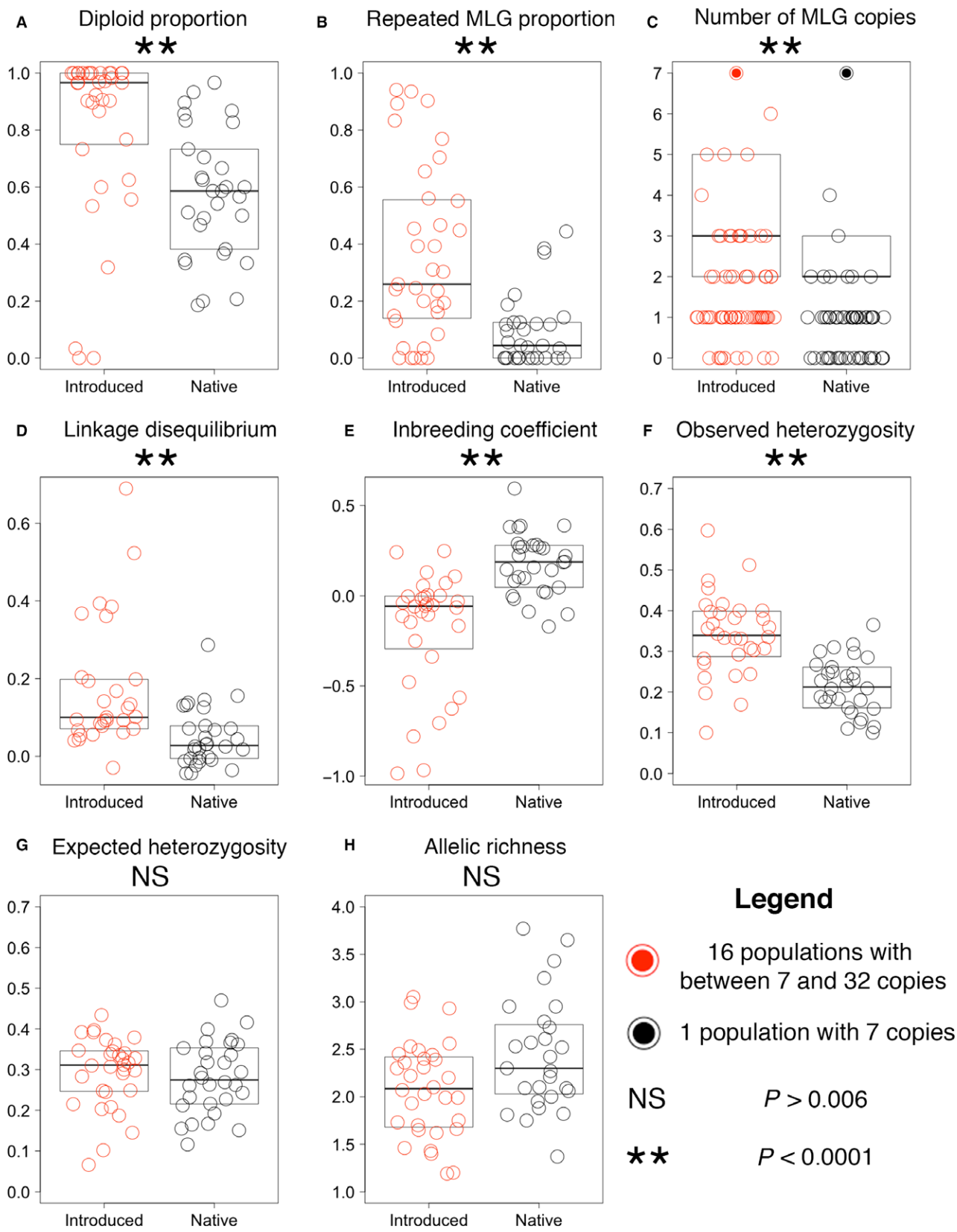

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Fig. 3 Boxplots of the main summary statistics for introduced and native sites for all thalli across both ranges, including repeated genotypes. The summary statistics were calculated on the diploid data set (with the exception of the proportion diploid which included all sampled individuals from 65 populations). The Bonferroni corrected $\alpha = 0.006$. (A) The proportion of diploid thalli, (B) the proportion of repeated multilocus genotypes (MLGs), (C) the number of copies of a repeated genotype (not shown in plot: the red enclosed circle denotes 16 introduced populations with 7–32 copies of a MLG and the black enclosed circle denotes 1 native population with 7 copies of a MLG), (D) the multilocus estimate of linkage disequilibria $\bar{r}_D$, (E) estimate of multilocus inbreeding coefficient F_{is}, (F) observed heterozygosity H_O, (G) expected heterozygosity H_E and (H) expected allelic richness A_E, based on a sample size of 10 diploid individuals (i.e. 20 alleles).

unique genotypes (i.e. genotypic richness) within native sites than introduced sites (91% vs. 61%, respectively; Table 1). Similarly, there were greater proportions of repeated diploid MLGs in the introduced vs. native sites (36% vs. 9%, respectively; Fig. 3B, Tables 1 and S3, Supporting information). Two South Korean sites (Daecheon and Jindo) and one Japanese site (Nagasaki-machi) had between 37% and 44% repeated genotypes; all other native sites showed less than 25% repeated MLGs. The introduced sites exhibited more copies of repeated MLGs than did the native sites (average of 5.7–2.3, respectively; Fig. 3C, Table 1).

Introduced sites exhibited significantly higher multilocus linkage disequilibrium (LD) as compared to native sites ($\bar{r}_d$ 0.172 vs. 0.039, respectively; Table 1, Fig. 3D, Table S4, Supporting information). Only two native sites (7%) showed significant multilocus LD, whereas 19 introduced sites (62%) were characterized by values of $\bar{r}_d$ greater than zero ($0.067 < \bar{r}_d < 0.523$; Tables 1 and S4, Supporting information). Removing repeated MLGs resulted in a decrease of $\bar{r}_d$ values in both native and introduced sites (0.003 vs. 0.008; Table S4, Supporting information), but 10 sites retained significant estimates of LD in the introduced range ($0.056 < \bar{r}_d < 0.583$).

Introduced sites were characterized by negative F_{is} values, indicating an excess of heterozygotes (average −0.234; Fig. 3E, Tables 1 and S4, Supporting information). Over 60% of the introduced sites displayed significantly negative F_{is} values, or heterozygote excess, compared to only 7% of sites in the native range. Removing repeated MLGs reduced the percentage of introduced sites with significantly negative F_{is} values to 35%, but the average over all introduced sites remained negative (−0.111; Table S4, Supporting information). In the native range, regardless of analysing all thalli or one thallus per genotype, over 40% of native sites showed significantly positive F_{is} values (average 0.2; Fig. 3E, Tables 1 and S4, Supporting information). Introduced sites also exhibited significantly higher observed heterozygosities, on average, compared to the native range (0.341 vs. 0.215, respectively; Fig. 3F, Tables 1 and S3, Supporting information).

Overall, the lower levels of genotypic diversity, greater linkage disequilibrium, negative F_{is} values and higher H_O are all signals consistent with greater rates of vegetative reproduction (i.e. fragmentation of thalli) in the introduced relative to the native range.

Allelic diversity

Although we detected lower genotypic diversity in the introduced range, we found no significant differences in allelic diversity between the introduced and native sites. Average expected heterozygosity was 0.293 and 0.281 (Fig. 3G, Tables 1 and S3, Supporting information) and the average number of alleles was 2.1 and 2.4 (Fig. 3H, Tables 1 and S3, Supporting information) in the introduced and native ranges, respectively. Nevertheless, there was a trend in which allelic diversity appeared to be more impacted by bottlenecks during invasion than expected heterozygosity due to the loss of rare alleles (Nei *et al.* 1975). These results were consistent when all diploid thalli and one-thallus-per-genotype data sets were analysed (Tables 1 and S3, Supporting information).

Discussion

As predicted by Baker's Law, the long-distance colonization and subsequent range expansion of the *Gracilaria vermiculophylla* along the coastlines of North America and Europe correlated with greater rates of uniparental reproduction in introduced relative to native populations. As predicted for haplo-diplontic organisms (Table 1), the increase in asexual fragmentation was associated with the dominance of a single ploidy stage. Below, we discuss these patterns and their implications for other colonizing species with biphasic life cycles.

Shifts in ploidy ratio

Across native *Gracilaria vermiculophylla* sites, haploid–diploid ratios were approximately equivalent to $\sqrt{2}$:1, with slight diploid biases. This pattern is typical of the Order Gracilariales (Kain & Destombe 1995). Although studies have described variation in macroalgal haploid–diploid ratios (reviewed in Fierst *et al.* 2005), relatively few have investigated the demographic and ecological

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mechanisms generating or maintaining variation among and within species (but see, Destombe *et al.* 1989; Kain & Destombe 1995; Thornber & Gaines 2004; Krueger-Hadfield 2011; Guillemin *et al.* 2013, 2014). The variation in haploid–diploid ratios across native populations was likely driven by differences in the fertility and survivorship of each ploidy stage (see DeWreede & Klinger 1988; Destombe *et al.* 1989; Richerd *et al.* 1993; Thornber & Gaines 2004; Fierst *et al.* 2005), but the biological and ecological factors maintaining these ratios have yet to be tested in *G. vermiculophylla*.

In contrast to the native populations, the ecological shift from hard substratum to soft-sediment habitats during the invasion likely resulted in diploid dominance and, in some sites, diploid fixation. The spores produced by meiosis (i.e. haploid tetraspores) and fertilization (i.e. diploid carpospores) require hard substrata in order to recruit and generate holdfasts (Kain & Destombe 1995). If spores cannot settle because hard substrata are absent or extremely rare (i.e. soft-bottom environments), then they will not grow and mature. Vegetative reproduction (i.e. fragmentation) of existing thalli will become the dominant reproductive mode (e.g. Kain & Destombe 1995; Guillemin *et al.* 2008a; Nelson *et al.* 2015). Martín *et al.* (2011) found the gravel fraction in Bahía Bustamante in Argentina was large enough for spores of *Gracilaria gracilis* to settle due to the significant percentages of holdfasts. However, few thalli of *G. vermiculophylla* from the introduced range had holdfasts when sampled in soft-sediment habitats (<5%). Although sexual reproduction and the recruitment of haploid and diploid spores may occur, fragmentation was the dominant reproductive mode in these soft-sediment habitats.

It is uncertain why haploids, rather than diploids, were consistently lost in the introduced range as theoretically, asexual fragmentation and loss of sexual reproduction could favour either haploid or diploid dominance due to demographic stochasticity. It is possible that initial founders were largely diploid and this bias was maintained by fragmentation and the proximity of other diploid-dominated sites during population expansion. Alternatively, diploid stages may dominate because diploid thalli in the genus *Gracilaria* tend to grow faster and are more robust to environmental stress than haploids (e.g. Guillemin *et al.* 2013). It is noteworthy that free-floating populations of other *Gracilaria* species tend to be dominated by either diploid (Rao 1973; Nelson 1989; Aguilar-Rosas *et al.* 1993; Kain & Destombe 1995; Guillemin *et al.* 2008a), or vegetative, possibly sterile, thalli (Stokke 1957; Kain & Destombe 1995; Iyer *et al.* 2004; Guillemin *et al.* 2008a). We require more data to tease apart the relative importance of fitness, founder effects and demographic stochasticity in driving the diploid bias in the introduced range.

There were three exceptional sites in the introduced range (Oakland Beach, Rhode Island and two Danish populations) in which thalli bore cystocarps and were, thus, phenotypically female haploids (S.A. Krueger-Hadfield, *personal observation*; Nejrup & Pedersen 2012). These sites were also remarkable in that very few unique genotypes were found across the 30–35 thalli genotyped (2-4 MLGs based on P_{sex}) with as many as 32 copies of a single MLG. It appears that these sites were initially poorly sampled, possibly with preferential sampling of the more charismatic, reproductive female thalli. Return surveys in 2015 at Oakland Beach and Horsens, Denmark, found reproductive haploid *and* diploid thalli, almost all of which were attached to pebbles (S.A. Krueger-Hadfield and E.E. Sokta, *unpublished data*).

Shifts in reproductive mode

Detecting hallmarks of asexual reproduction can be difficult as infrequent recombination events can remove the genetic signatures of clonality (Halkett *et al.* 2005b). Nevertheless, we detected multiple signals of greater rates of clonality in the introduced populations, including higher levels of heterozygosity (H_O and F_{is} were significantly larger and negative, respectively), greater linkage disequilibrium and more repeated genotypes than native populations. Relatively, few studies have investigated increased heterozygosity levels in asexual vs. sexual populations within the same species (but see Dorken & Eckert 2001; Halkett *et al.* 2005a; Guillemin *et al.* 2008a). Our study not only broadens the range of organisms, but also the ecological contexts in which these predictions of asexual genetic signatures apply (i.e. following an invasion of novel environments).

Based on analyses of population structure with these microsatellite genotypes (S.A. Krueger-Hadfield, A.E. Strand, E.E. Sotka, *unpublished data*), oyster transfer records (Barrett 1963; Ruesink *et al.* 2005) and the geographic breadth of the invasion (Bellorin *et al.* 2004; Guillemin *et al.* 2008b; Weinberger *et al.* 2008; Saunders 2009), the uncoupling of the biphasic life cycle leading to diploid dominance has occurred independently during the invasions each continental margin in the Northern Hemisphere. Diploid dominance was consistent with the dearth of hard substratum in nearly all North American and European estuaries in which *G. vermiculphylla* now resides as well as the general lack of thalli bearing holdfasts. The presence of holdfasts in free-floating populations would have indicated recruitment of sexually produced spores to hard substrata with subsequent detachment as *Gracilaria* thalli are unable to form new holdfasts (Guillemin *et al.* 2008a). Indeed, in the two populations in which there was abundant hard

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substratum (Elkhorn Slough, California and Ape Hole Creek, Maryland), all thalli were attached via holdfasts to gastropod shells or small rocks. The populations at Elkhorn Slough and Ape Hole Creek had few free-floating thalli and displayed few genetic signatures of inbreeding, specifically intergametophytic selfing as F_{is} values were not significantly different from zero (Klekowski 1969; Krueger-Hadfield *et al.* 2013b), or vegetative fragmentation (Tables S3 and S4, Supporting information). The principal difference between these two high-salinity estuaries and the other introduced habitats is the abundance of hard substrata, which allows sexual reproduction and spore recruitment to occur.

Surprisingly, almost half of the native populations exhibited significant heterozygote deficiencies consistent with inbreeding or a Wahlund effect (i.e. positive F_{is} values, Fig. 3E, Table S4, Supporting information). Without more exhaustive and spatially explicit sampling, it is not possible to distinguish between these two processes (Krueger-Hadfield *et al.* 2013b). The low estimates of null allele frequencies in the haploid thalli in this study suggested null alleles did not substantially contribute to the positive F_{is} values (Table S2, Supporting information). This result contrasts with most other natural populations of *Gracilaria* species for which the mating system has been analysed in which the populations tended to be allogamous (Engel *et al.* 2004; Guillemin *et al.* 2008a). The reasons for this discrepancy remain unclear.

Extending Baker's Law to haplo-diplontic range expansions

Although Baker's (1955) penultimate paragraph discussed a capacity for uniparental reproduction in ferns and mosses, few studies have explicitly tested Baker's Law in haplo-diplontic organisms. Life history traits affecting the mating system will pass through the same ecological sieves in diploid and haplo-diplontic organisms. Therefore, labile mating systems and an enhanced capacity for uniparental reproduction should be associated with colonization success in both diploid and haplo-diplontic species. The lack of information about mating system variation and the rates of uniparental reproduction in natural populations of haplo-diplontic species is a glaring gap in the literature and limits our general understanding of mating system evolution in these species. Nevertheless, populations of haplo-diplontic species, including *Gracilaria vermiculophylla*, likely benefit and suffer from similar trade-offs of uniparental reproduction as those described for metazoans and plants (e.g. Brown & Eckert 2005; Pigneur *et al.* 2014). However, the negative effects of inbreeding depression may be more easily purged in haplo-diplontic populations when the haploid stage is exposed to selection as compared to the very reduced haploid stages in diploid life cycles (Otto & Goldstein 1992; but see Immler *et al.* 2012; Otto *et al.* 2015). Yet, although selection against highly inbred progeny has been shown to be strong in mosses (Szovenyi *et al.* 2009, 2014), the results are equivocal for brown (Barner *et al.* 2011; Johansson *et al.* 2013) and red macroalgae (Engel *et al.* 1999; Krueger-Hadfield *et al.* 2015).

The prolonged haploid stage in haplo-diplontic life cycles may also facilitate a transition to endogamous mating systems (Richerd *et al.* 1993; Otto & Marks 1996) and may enhance colonization success in haplo-diplontic organisms. Yet, for fungal species, confusion over terminology describing sexual reproduction (Billliard *et al.* 2012) and the problem of delimiting species (Gladieux *et al.* 2015) hamper a synthesis of current knowledge. For ferns (Klekowski 2003; Lott *et al.* 2003; Flinn 2006; Wubs *et al.* 2010; de Groot *et al.* 2012), mosses (Patiño *et al.* 2013; Laenen *et al.* 2016), fungi (Gladieux *et al.* 2015) and seaweeds (West *et al.* 2001; Gabrielson *et al.* 2002; Hwang *et al.* 2005; Fierst *et al.* 2010; Krueger-Hadfield *et al.* 2013a; this study), colonizing populations are often associated with higher rates of uniparental reproduction. Future directions can build on the conceptual framework provided by Pannell *et al.* (2015) outlining the contexts in which Baker's Law might apply, including (i) the colonization of oceanic islands (e.g. Patiño *et al.* 2013), (ii) metapopulations (e.g. de Groot *et al.* 2012; Krueger-Hadfield *et al.* 2013a), (iii) species invasions (this study) and (iv) range expansions (e.g. Flinn 2006; Krueger-Hadfield *et al.* 2013a; Laenen *et al.* 2016, this study).

We extend this framework for haplo-diplontic species with additional hypotheses with which to investigate these patterns. First, in haplo-diplontic species, self-fertilization can occur as intra- or intergametophytic selfing depending on whether the species is monoicious or dioicious, respectively (Klekowski 1969; Soltis & Soltis 1992). Intragametophytic selfing, or haploid selfing in fungi (Billliard *et al.* 2012), results in instantaneous homozygosity when gametes produced by the same haploid gametophyte unite. This can result in rapid purging of genetic load and may have an important selection effect on mating system traits (Crawford *et al.* 2009). In contrast, intergametophytic selfing is the result of cross-fertilization between two different haploids sharing the same diploid parent (or, diploid selfing in fungi, Billliard *et al.* 2012). As a consequence, dioecy in haplo-diplontic organisms cannot be used as a proxy for the mating system (Krueger-Hadfield *et al.* 2015) as is often the case in plants or animals (Bell 1997). Second, unlike plants and animals, future 'sexual

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reproductive assurance' will only be maintained if diploids, the stage in which meiosis occurs, are not lost from the biphasic life cycle. The reverse is not true for dioecious haploids in which sexual reproduction would require another haploid individual to enable fertilization. In this study (S.A. Krueger-Hadfield, personal observation) and in Guillemin *et al.* (2008b), reproductive structures were still found on free-floating diploid thalli, indicating sexual structures have not been entirely lost in the decades since invasion and cultivation, respectively.

Conclusions

Decades after Baker's (1955) seminal paper was published, it is now clear that his ideas apply to haplo-diplontic life cycles, such as those found in ferns (Lott *et al.* 2003; Flinn 2006; de Groot *et al.* 2012), mosses (Patiño *et al.* 2013; Laenen *et al.* 2016) and, now, explicitly in seaweeds (but see Krueger-Hadfield *et al.* 2013a). Additional empirical data will shed light on the distributions of selfing and asexual reproduction rates in natural populations across haplo-diplontic taxa (see Billliard *et al.* 2012; Krueger-Hadfield *et al.* 2015; Pannell 2015).

In the context of biological invasions, mating systems are one of the key determinants for establishment, spread and evolutionary potential and should be incorporated into risk assessment and management strategies (Hao *et al.* 2011). Our study broadens the macroalgal species for which population genetic tools have been applied to investigate reproductive mode and population structure (see Table 1 from Krueger-Hadfield & Hoban 2016) and reinforces the particular threat of macroalgal invasions in near-shore ecosystems through deliberate and inadvertent introductions (Voisin *et al.* 2005; Williams & Smith 2007; Andreakis & Schaffelke 2012). Labile mating systems allow haplo-diplontic species, including seaweeds, to expand into introduced habitats. Understanding the ecological and evolutionary outcomes from profound life history modifications resulting from natural processes (Hwang *et al.* 2005; de Groot *et al.* 2012; Krueger-Hadfield *et al.* 2013a; Patiño *et al.* 2013), aquaculture (Guillemin *et al.* 2008b) and invasions (this study) will provide excellent empirical tests of how, when and where haplo-diplontic life cycles are maintained and evolve.

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S.A.K.H., N.M.K. and E.E.S. conceived the study; S.A.K.H., N.M.K., J.E.B., M.H., R.T. and F.W. collected samples; S.A.K.H. and N.M.K. extracted D.N.A.; S.A.K.H., N.M.K., T.W.G. and D.M. generated data; S.A.K.H. and E.E.S. analysed data and wrote the manuscript; J.E.B., C.J.M. and A.E.S. contributed to discussions; and all authors approved the final manuscript.

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Data accessibility

Microsatellite primer sequences were deposited in GENBANK, Accession nos. KT232089-KT232097 and KT232099 (Kollars *et al.* 2015). Microsatellite genotypic data were deposited in DRYAD, entry doi:10.5061/dryad.fg818.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 The biphasic life cycle of *Gracilaria vermiculophylla*.

Fig. S2 Allele frequency plots for each locus.

Fig. S3 Frequency distribution of the pairwise number of allele differences between MLGs in each of native and introduced populations of *Gracilaria vermiculophylla*.

Table S1 Native and introduced population sampling information.

Table S2 The number of alleles, allele range, null allele frequency and TANDEM output (Matschiner & Saltzburger 2009), including the specified repeat size, rounding method, average rounding error and error threshold for each microsatellite locus used to genotype native and introduced *Gracilaria vermiculophylla* populations.

Table S3 Native and introduced population genetic diversity indices.

Table S4 Summary of statistical analyses for multilocus estimators of substructure.

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