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RESEARCH ARTICLE

Clonality contributes to the spread of *Avrainvillea lacerata* (Bryopsidales, Chlorophyta) in Hawaiʻi

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Abstract

The relative rates of sexual versus asexual reproduction influence the partitioning of genetic diversity within and among populations. During range expansions, asexual reproduction often facilitates colonization and establishment. The arrival of the green alga *Avrainvillea lacerata* has caused shifts in habitat structure and community assemblages since its discovery in 1981 offshore of Oʻahu, Hawaiʻi. Field observations suggest this species is spreading via vegetative reproduction. To characterize the reproductive system of *A. lacerata* in Hawaiʻi, we developed seven microsatellite loci and genotyped 321 blades collected between 2018 and 2023 from three intertidal sites at Maunalua Bay and ʻEwa Beach. We observed one to four alleles at multiple loci, suggesting *A. lacerata* is tetraploid. Each site was characterized by high genotypic richness ($R > 0.8$). However, clonal rates were also high, suggesting the vegetative spread of *A. lacerata* plays a significant role. The importance of clonal reproduction for the persistence of *A. lacerata* in Hawaiʻi is consistent with the ecological data collected for this species and observations of other abundant macroalgal invaders in Hawaiʻi and other regions of the world. These data demonstrate the necessity for implementing appropriate population genetic methods and provide insights into the biology of this alga that will contribute to future studies on effective management strategies incorporating its reproductive system. This study represents one of the few that investigate green algal population genetic patterns and contributes to our understanding of algal reproductive system evolution.

KEYWORDS
clonality, invasion, life cycle, non-native, polyploid, seaweed

Abbreviations: MLG, multilocus genotype; PCR, polymerase chain reaction; RFU, relative fluorescence units; SNP, single nucleotide polymorphism; SSR, single sequence repeat.

INTRODUCTION

The reproductive system describes the relative rates of sexual and asexual reproduction, governing genetic diversity through the transmission of genes from one generation to the next (Barrett, 2011). Sexual reproduction can have varying effects on genetic diversity. For instance, cross-fertilization between unrelated individuals often confers greater standing genetic diversity (Hamrick & Godt, 1996). A, inbreeding decreases genetic diversity through reduced effective recombination and increased levels of homozygosity (Charlesworth & Charlesworth, 1987; Charlesworth & Wright, 2001; Ellstrand & Elam, 1993). Contrary to inbreeding, asexual reproduction tends to favor the maintenance of parental heterozygosity and may even increase heterozygosity via the accumulation of mutations over time (Marshall & Weir, 1979; Reichel et al., 2016; Stoeckel & Masson, 2014). Many eukaryotes are partially clonal, simultaneously engaging in both sexual and asexual reproduction such that the relative rates of each reproductive mode are integral to our understanding of population structure. Yet, most of our understanding of the variation in reproductive modes among populations is based on angiosperms (e.g., Whitehead et al., 2018) or animals (e.g., Olsen et al., 2020) and primarily focused on sexual reproduction (Lane et al., 2011; Rushworth et al., 2022).

Traits that influence the reproductive system can rapidly shift in response to changes in environment or demography (Barrett, 2008; Eckert et al., 2010). Biological invasions are excellent examples of anthropogenically mediated range expansions (Pannell et al., 2015) and are among the main threats to biodiversity (Bellard et al., 2016; Dueñas et al., 2021; Kolar & Lodge, 2001). Following an invasion, individuals in small, recently established populations often exhibit shifts to uniparental modes of reproduction (Pannell et al., 2015). There are often only a few founding individuals, limiting mating opportunities. A capacity to undergo asexual reproduction, self-fertilization, or both can provide reproductive assurance (i.e., Baker's Law; Baker, 1955; Pannell, 2015). Patterns of geographic parthenogenesis (originally described by Vandel, 1928; see also Bierzychudek, 1985; Lynch, 1984) have been extended to include invasions (Pannell et al., 2015) whereby asexual reproduction can maintain high fitness genotypes that otherwise could be lost in small populations through sexual reproduction and genetic drift (Haag & Ebert, 2004). Moreover, asexual reproduction favors population growth, as mating is prevented between poorly adapted individuals (Antonovics, 1968; Peck et al., 1998). Yet, most population genetic theory has been developed for obligate sexual reproduction, and we lack the appropriate tools to accurately assess and interpret rates of asexual reproduction in natural populations (see Arnaud-Haond et al., 2007; Stoeckel et al., 2021).

The lack of population genetic data is particularly acute for macroalgae despite these taxa constituting a large portion of non-native species in marine environments (Schaffelke et al., 2006; Williams & Smith, 2007). Macroalgal invasions have concomitant negative consequences for local communities via the overgrowth and smothering of native species, alterations of surrounding habitats, and decreases in available food sources for herbivores (Schaffelke et al., 2006). Despite the continuous increase in the number of macroalgal invasions, most of the knowledge on invasive macroalgae is limited to what we have learned from high-profile invaders, such as *Caulerpa taxifolia* (Deveney et al., 2008) and *Sargassum muticum* (Engelen et al., 2015), and has mainly focused on ecological rather than evolutionary processes (see discussion in Sotka et al., 2018), especially with regard to reproductive system variation (see Krueger-Hadfield, 2020; Krueger-Hadfield et al., 2021). Algae display tremendous variations in their life cycles (Bell, 1994) and are predicted to be partially clonal (Otto & Marks, 1996). Yet, there were too few data to test this hypothesis and this has not improved substantially in the past ~30 years (see, as examples, Krueger-Hadfield et al., 2021; Krueger-Hadfield 2024).

We investigated the reproductive mode of the green alga *Avrainvillea lacerata*. In 1981, specimens morphologically identified as *A. amadelpha* were documented in Hawaiʻi off the western shores of Oʻahu (Brostoff, 1989). Nearly 40 years later, molecular assessments with the inclusion of type material revealed this alga to be *A. lacerata* (Lagourgue et al., 2023; Wade, 2019). Since the initial discovery, new observations of *A. lacerata* have been documented around Oʻahu, both in the intertidal as well as the mesophotic (Cox et al., 2017; Spalding et al., 2019). This alga has now been observed on the neighboring islands of Kauaʻi and Maui (Smith et al., 2002; Wade, 2019). The invasion of *A. lacerata* has led to habitat and community change as it forms extensive mounds, some up to 30 m wide (Littler et al., 2004, 2005; Peyton, 2009). The mounds alter the benthos through increased sedimentation, which modifies hard substrate to resemble soft sediment habitats (Foster et al., 2019; Littler et al., 2004). The alga's ability to engineer habitat structure, its complex branching and holdfast morphology (Littler & Littler, 1992; Olsen-Stojkovich, 1985), and its possible herbivore-deterring secondary metabolites (see Hay et al., 1990) have all influenced the surrounding ecosystem by contributing to significant shifts in surrounding invertebrate (Foster et al., 2019; Longenecker et al., 2011), algal (Peyton, 2009; Smith et al., 2002), and fish communities (Langston & Spalding, 2017).

Although the ecological consequences of the *Avrainvillea lacerata* invasion on Hawaiian ecosystems have been documented (e.g., Foster et al., 2019;

Peyton, 2009; Smith et al., 2002; Veazey et al., 2019), there is a lack of basic knowledge regarding the reproductive system of both this species and the genus more broadly. For example, the life cycle is assumed to be diploid, in which somatic development only occurs in the diploid phase. However, this assumption is based on *A. lacerata*'s phylogenetic position relative to other green algae in the Bryopsidales and in the genus *Avrainvillea* (Littler & Littler, 1992; Olsen-Stojkovich, 1985). Polyploidy has been suggested for some bryopsidalean macroalgae (Kapraun, 1994; Kapraun & Nguyen, 1994; Kapraun & Shipley, 1990; Varela-Álvarez et al., 2012), but no studies have investigated the extent of polyploidy throughout this order using genetic tools. Moreover, the life cycle influences the reproductive system (see Krueger-Hadfield, 2020; Otto & Marks, 1996), but there are no population genetic data describing the prevailing reproductive mode or overall reproduction in the genus *Avrainvillea* (Littler & Littler, 1992; Olsen-Stojkovich, 1985). Field observations suggest the alga is predominately spreading through the vegetative growth of mounds, and reproductive structures have never been observed (Smith et al., 2002). To our knowledge, no detailed phenology study exists to complement studies of reproduction in *A. lacerata*.

In this study, we developed seven microsatellite loci to genotype *Avrainvillea lacerata* blades and describe its reproductive system in Hawai‘i. Based on field observations of large mounds suggesting vegetative spread and the lack of reproductive thalli, we hypothesized that *A. lacerata* is primarily spreading through asexual reproduction. Further, we sampled over multiple years, to quantify the clonal rate at the sites sampled (see discussion in Becheler et al., 2017). These microsatellites will facilitate future studies investigating the invasion history of this alga and could eventually help others to discern the source of the invasion from its native range (see as example Krueger-Hadfield et al., 2017). Not only will quantitative data on the reproductive system aid in the management of this invader, the data will test the Otto and Marks (1996) hypothesis of a correlation between the reproductive mode and life cycle, expanding the available population genetic data for macroalgae across marine biomes.

METHODS

Field sampling

We collected *Avrainvillea lacerata* blades from two sites along the southern coastline of O‘ahu. Single blades from a distinct mound were considered a putative genet (i.e., one genotype; Harper, 1980), with a blade defined as a single upright, fan-shaped apex connected to a single stipe from a clump with a discrete holdfast mound (i.e., a ramet). At each site, we haphazardly placed a 50m transect parallel to the shore during a low tide in a representative area of the intertidal zone occupied by *A. lacerata*. At each sampling point, we collected a single upright blade from distinct mounds separated by at least 1m. In May 2018 and June 2019, we collected 50 blades from Maunalua Bay (21.279880, −157.728160) and 50 blades from the ‘Ewa Beach site, Lagoon East (21.304310, −158.036240; site 13, Cox et al., 2017; Figure 1).

Our site in Maunalua Bay was protected, and the substrate was composed of soft sediment. Increased coastal development has led to increased sedimentation (Peyton, 2009). There has also been an increase in invasive species, such as *Avrainvillea lacerata* in this area (Peyton, 2009). Concomitant with all the changes, there has been a decrease in the native seagrass *Halophila hawaiiana* (Peyton, 2009). In 1985, *A. lacerata* was collected in Maunalua Bay (Brostoff, 1989). By 2003, there were extensive mounds that covered up to 100% of the soft sediment (Peyton, 2009). Despite a large-scale removal effort, mounds of *A. lacerata* have grown back, albeit in a much patchier distribution than before the removal. We predicted the occurrence of more unique genotypes at our site in Maunalua Bay as compared to the site ‘Ewa (see below) from (i) regrowth of mounds that were left behind following the removal, (ii) the arrival of new genotypes from sexual reproduction, or (iii) the arrival of new genotypes from nearby sites, including the subtidal or mesophotic.

‘Ewa Beach Lagoon East, by comparison, is a more exposed site with greater wave exposure and hard substratum. This site also has greater native algal diversity (see Cox et al., 2017). In 2008, *Avrainvillea lacerata* mounds were observed in low abundances (Cox et al., 2017), but now it is one of the most abundant algae, forming extensive mounds. Due to greater wave action and the competition for space, we predicted recruitment of *A. lacerata* would be more difficult at this location compared to the Maunalua Bay site. Thus, we expected to find a higher occurrence of vegetative growth of mounds leading to the dominance of fewer genotypes.

In addition to comparing population genetic structures between the two sites at Maunalua Bay and ‘Ewa Beach, we wanted to make comparisons between two sites that were ~870m apart but differed in their environmental characteristics. In May 2021 at ‘Ewa Beach, we collected 30 blades each from Lagoon East and a second site called Kaloi (21.305800, −158.028400; site 2, Cox et al., 2017; Figure 1). Kaloi differs from Lagoon East in substrate angularity and topographic relief and is a flat bench with higher wave exposure. Lagoon East is more wave protected in comparison to Kaloi and has an angular carbonate bench with greater topographic relief.

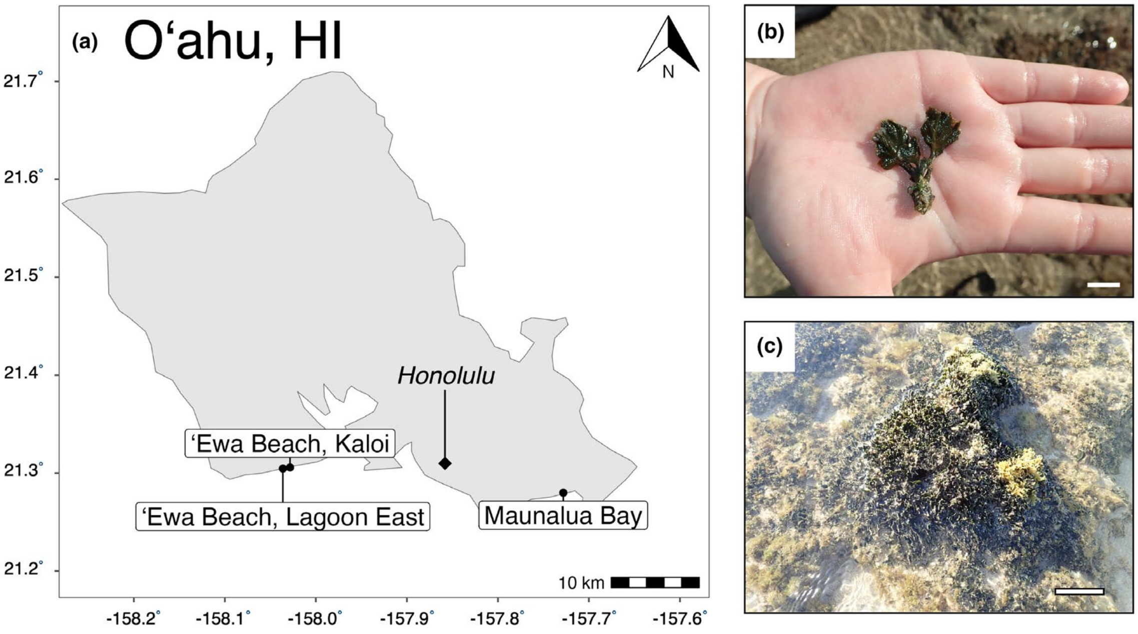

FIGURE 1 Sampling sites of *Avrainvillea lacerata* on O‘ahu, HI. (a) Map of *A. lacerata* sampling sites on O‘ahu, HI. Honolulu is included as a locational reference. (b) One thallus of *A. lacerata* with multiple blades connected to a single holdfast. Scalebar: 1 cm. Photo credit: H. Spalding. (c) Example of an *A. lacerata* mound from which blades were sampled. Scalebar: 10 cm. Photo credit: H. Spalding. [Color figure can be viewed at wileyonlinelibrary.com]

In addition to the population-level sampling, we wanted to assess diversity within single *Avrainvillea lacerata* mounds, which result from lateral vegetative growth (Littler et al., 2005), presumably of a single genet. In May 2022, we collected blades from eight mounds at Lagoon East. From each mound, we collected five blades for a total of 40 blades. We repeated this sampling in June 2023, when we sampled blades from eight separate mounds in ‘Ewa, Lagoon East. Each clump had between one and three holdfasts fused together, and each holdfast seemed to be associated with a group of blades. From each holdfast within a clump, two to three blades were sampled.

All blades were rinsed in seawater and preserved in silica gel (ACTÍVA Flower Drying Art Silica Gel, Marshall, TX, USA).

DNA extraction

We extracted DNA from approximately 5–6 mg of each dried *Avrainvillea lacerata* blade. We homogenized blades with one 2.8 mm ceramic bead using a bead mill (BeadMill24; Fisher Scientific, Waltham, MA, USA) at a speed of $4\,\mathrm{m \cdot s^{-1}}$ for 45 s, until each blade was homogenized into a fine powder. Some blades required up to four bursts with the bead mill. We used the OMEGA biotek E.Z.N.A.® Plant DNA DS Kit (Norcross, GA, USA) according to the manufacturer's instructions. We eluted DNA in a single volume of 150 µL using the provided elution buffer.

Microsatellite locus identification

Single sequence repeat (SSR)-enriched genomic sequence data were generated by Microsynth ecogenics GmbH (Balgach, Switzerland). We identified putative loci from the SSR-enriched library and followed Schoebel et al. (2013), with modifications as implemented in Ryan et al. (2021) and Heiser et al. (2023), which we summarize here. We used MSATCOMMANDER 1.0.8-beta (Faircloth, 2008) to design primers for di-, tri- and tetranucleotide repeat motifs separately. A minimum of eight repeats were selected and the following primer melting temperatures (T_m): minimum of 58°C, optimum of 60°C, and maximum of 62°C. For dinucleotides, we identified 408 sequences with eight or more repeats; 95 of those had primers assigned, and 16 were potentially duplicated in the library. For trinucleotides, we identified 1712 sequences with eight or more repeats; 376 of those had primers assigned, and 102 were potentially duplicated in the library. For tetranucleotides, we identified 153 sequences with six or more repeats; 22 of those had primers assigned, and two were potentially duplicated in the library. We had 79, 274, and 20 potential loci with di-, tri-, and tetranucleotide repeat motifs, respectively.

We followed Schoebel et al. (2013) and used R version 3.5.2 (R Core Team, 2018) to combine the primer and microsatellite sequences into one file. For the dinucleotides, we had 76 unique reads remaining after merging the files. For trinucleotides, we had

267 unique reads remaining after merging the files. For tetranucleotides, we had 20 unique reads remaining after merging the files. After removing duplicated forward and reverse primer sequences, we had 239 unique reads remaining. We then combined the files with unique reads.

We calculated the absolute difference between the forward and reverse T_m for each primer pair and sorted the result from smallest (0°C) to largest (2.51°C). We, then, sorted the putative loci by the forward penalty score, reverse penalty score, and the pair penalty score. Lastly, we calculated and sorted by the absolute difference between penalty scores divided by the pair penalty from smallest to largest to ensure that the difference between the forward and reverse penalties was as small as possible. We selected the top 162 loci from each of these categories and combined them in one file. We ranked the resulting 94 loci according to the combined scores from all five categories.

Finally, we conducted a BLAST search in Geneious Prime v.2022.2.2 (Biomatters Ltd., Auckland, New Zealand) using the SSR-enriched library to ensure that only one primer pair was binding to the same locus, no primer pair was binding to more than one locus, and repeat regions were not within the primers. A total of 49 candidate loci were initially screened using seven blades (see Table S1 in the Supporting Information).

Microsatellite locus screening, PCR conditions, and fragment analysis

Candidate loci were amplified using simplex polymerase chain reactions (PCRs) with a final volume 15 μL:2 μL of diluted DNA (1:100), 250 nM of each forward and reverse oligo, 1X of GoTaq® Flexi DNA Green Buffer (Promega, Cat # M891A), 2 mM of $MgCl_2$, 250 μM of each dNTP (Promega, Cat # R0192), 1 mg · mL^{-1} of bovine serum albumin (BSA; Fisher Bioreagents, Cat # BP9706-100i), and 1 U of Promega GoTaq® Flexi DNA Polymerase. We used the following PCR program: 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, with a final elongation at 72°C for 5 min. Approximately 5 μL of each PCR product was screened on 1.5% agarose gels stained with GelRed (Biotium, Fremont, CA, USA, Cat #41002-1). Each locus was then categorized based on the amplification profile: one band, multiple bands, or no amplification (Table S1). We considered loci to be good candidates if they amplified well across the seven blades and only had one band per thallus. Based on these criteria, 12 candidate loci were selected for screening using the capillary sequencer.

We assigned dyes—6FAM, NED, VIC, PET—to each forward oligo for the 12 loci (Table S1). We performed fragment analysis of all samples at the Heflin Center for Genomic Sciences at the University of Alabama at Birmingham. We diluted 1 μL of PCR product in 10 μL of molecular grade H_2O and then added 1.5 μL diluted PCR product to 9.7 μL HiDi formamide (Applied Biosystems) and 0.35 μL GS500 LIZ (Applied Biosystems, Cat # 4322682).

We scored alleles using GENEIOUS PRIME v.2022.2.2 (https://www.geneious.com). In scoring alleles, we implemented strict criteria: e scored alleles that (a) exhibited 500 or more relative fluorescence units (RFUs) and (b) had a peak architecture common to all other alleles for a particular locus. For each allele we scored, we recorded the RFU in addition to the base pair size. Following this, we binned raw allele sizes manually into integers, ensuring rounding did not artificially increase allelic diversity. A considerable proportion of blades displayed more than two alleles in at least one of the loci studied. To confirm this, we ran re-extracted DNA from a subset of blades and repeated PCRs.

Assigning polyploid genotypes

We observed between one and four alleles at each locus per blade (Table S1). We could easily assign a tetraploid genotype for blades with a single allele (e.g., AAAA) or four alleles observed at a locus (e.g., ABCD). However, for blades with two or three alleles at a single locus, we first determined allele dosage. GenoDive (Meirmans, 2020) was used to correct for unknown allele dosage through imputation of missing alleles using maximum likelihood based on estimated allele frequencies observed in the genotyped specimens prior to allele dosage estimation (see Preston et al., 2022). As we obtained greater genotypic diversity than expected based on raw alleles observed from blades collected from the same mound (see Results), we developed a method whereby we used a multinomial kernel to compute the likelihood of each possible genotype and used the RFU for each allele as a proxy for the “dosage” (see Appendix S1 in the Supporting Information). Unlike with GenoDive, which assigns genotypes based on the assumption that the population of study is at Hardy–Weinberg proportions, using RFU values as a proxy for dosage allowed us to assign genotypes without prior assumptions regarding the population. For each blade, we assigned the genotype that corresponded with the maximum posterior probability.

Population genetic analyses

We calculated the following summary statistics at each site and time point using GenAPoPop, software that analyzes genetic diversity and reproductive mode in autopolyploid populations (Stoeckel et al., 2024). First, we calculated genotypic richness (R) using the formula

$R = \frac{G-1}{N-1}$, where G is the number of unique multilocus genotypes (MLGs) and N is the number of blades genotyped (Dorken & Eckert, 2001). Next, we assessed genotypic evenness using pareto β (see box 4, equation 17, Arnaud-Haond et al., 2007) and D^* (see box 3, equation 11, Arnaud-Haond et al., 2007). We, then, estimated linkage disequilibrium ($\overline{r_d}$, Agapow & Burt, 2001). We calculated expected heterozygosity (H_E, Nei, 1978), observed heterozygosity (H_O, Hardy, 2016), and the inbreeding coefficient (F_{IS}, Weir & Cockerham, 1984) for each locus as well as their variances. We estimated pairwise genetic differentiation between the *Avrainvillea lacerata* sites by calculating F_{ST} and ρ_{ST} as proposed by Meirmans and Liu (2018) and Weir (1996). Finally, we used the Bayesian method implemented in ClonEstiMate (Becheler et al., 2017) and extended in GenAPoPop for polyploids (Stoeckel et al., 2024), to infer clonal rates using transition of genotype frequencies. We reported the 98% credible interval and the maximum posterior probability estimate from the distribution obtained for each population and successive temporal samples. We compared the change in genotype frequencies at the Maunalua Bay site between the two time points 2018 and 2019 in which we sampled blades. At ʻEwa Beach, Lagoon East, we inferred clonal rate using transition of genotype frequencies between samples from 2018 to 2019 and from 2019 to 2021. As *A. lacerata* is thought to be dioecious based on its phylogenetic position (Olsen-Stojkovich, 1985; Verbruggen et al., 2009), we did not infer a selfing rate because self-fertilization would not be possible if dioecious, and it have may have lead to a bias in clonal rates, especially if inbreeding occurred during sexual events. However, it should be noted that dioecy in *Avrainvillea* has not been empirically verified to the best of our knowledge. Additionally, transitions away from dioecy are possible (see as examples Heesch et al., 2021; Takahashi et al., 2023). Therefore, direct tests of dioecy in *Avrainvillea* are needed, and our method is a conservative approach at present.

Finally, we assessed genotypic richness (R) within each mound sampled as well as across all mounds in 2022 by determining the number of unique MLGs per mound. For the 2023 collection, we determined the number of MLGs observed across two to three blades sampled per holdfast because some mounds visually appeared to have more than one holdfast.

RESULTS

Microsatellite locus development and ploidy estimation

Of the 12 loci tested on the capillary sequencer, five loci produced peaks that were either too difficult to score confidently (e.g., stutters) or had poor amplification (Table S1). Seven loci amplified well and displayed clearly distinct alleles during initial simplex screening on the capillary sequencer. We amplified these seven loci in one multiplex and two duplexes (Table S2 in the Supporting Information). Across the 321 genotyped blades, 175 blades (54.5%) displayed at least one locus with three or four different alleles (Table S3 in the Supporting Information; Figure 2), 141 blades (43.9%) displayed a maximum of two alleles at all loci, and four blades (1.2%) had one allele at all seven loci. Overall, we concluded that the *Avrainvillea lacerata* blades we sampled were tetraploid (4N).

Allele dosage estimation

Blades that belonged to the same MLG based on raw alleles (i.e., the alleles observed in the traces from fragment analysis) were assigned to new MLGs after allele dosage using the maximum likelihood method implemented in GenoDive (Table 1). In contrast, blades that belonged to the same MLG based on raw alleles were more often assigned to the same MLG when using allelic RFUs as a proxy for allele dosage (Table 1). Since estimating allele dosage using maximum likelihood artificially generated greater genotypic diversity, we conducted the population genetic analyses using genotypes generated by allelic RFUs (See Table S4 in the Supporting Information as an example).

Genotypic and genetic diversity

At the Maunalua Bay site in both 2018 and 2019, all blades sampled were unique MLGs ($R = 1$, $D^* = 1$; Table 2). Observed heterozygosity (H_O) varied between 0.405 and 0.643 in 2018 and between 0.473 and 0.755 in 2019 (Table 3). Expected heterozygosity (H_E) varied between 0.499 and 0.749 in 2018 and between 0.480 and 0.750 in 2019 (Table 3). Single locus F_{IS} values were all positive in 2018 and had greater variance as compared to 2019 when most values were closer to 0 (Table 3; Figure 3). Pareto β was >2 in both 2018 and 2019 (Table 2). Linkage disequilibrium ($\overline{r_d}$) was 0.029 in 2018 and 0.004 during 2019 (Table 2). At Maunalua Bay between 2018 and 2019, transition of genotypic frequency inferred rate of clonality (c) fell within a credible interval superior to 98% that includes $c = [0.41, 0.68]$, with a maximum a posteriori probability estimated at $c = 0.55$ (Figure S1a in the Supporting Information).

At ʻEwa Beach, Lagoon East in 2018, 2019, and 2021, genotypic richness (R) and evenness (D^*) were high ($R > 0.848$; $D^* > 0.982$), although both were <1 in 2018 and 2019 (Table 2). Observed heterozygosity (H_O) varied between 0.305 and 0.482 in 2018, between 0.296 and 0.510 in 2019, and between 0.339 and 0.694 in 2021 (Table 3). Expected heterozygosity (H_E) varied between 0.361 and 0.784 in 2018, between 0.374 and 0.781 in 2019, and between 0.389 and 0.790 in 2021

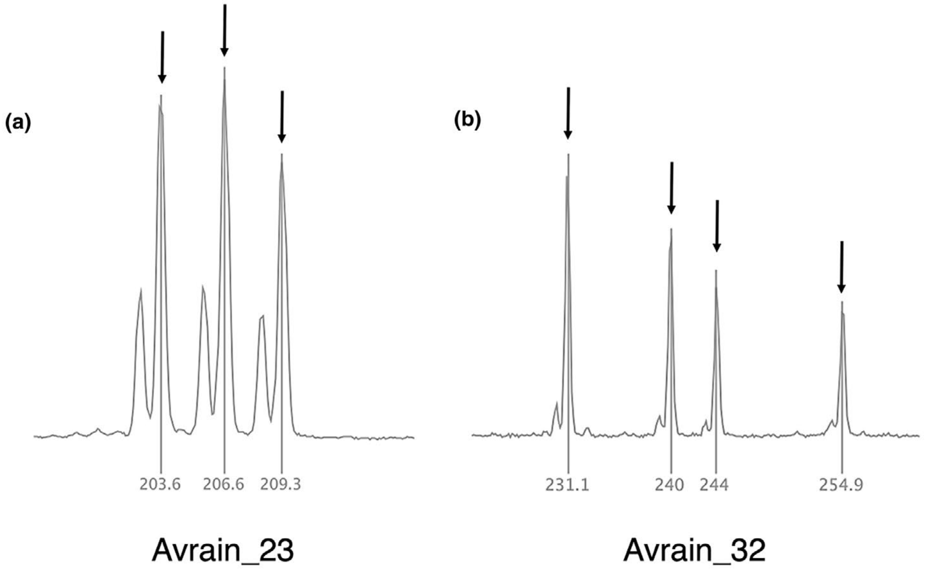

FIGURE 2 Fragment analysis traces for two microsatellite loci developed in *Avrainvillea lacerata*. Each arrow is pointing to a single allele. The architecture of these peaks are typical for microsatellites in which there is a short hitch before the larger peak. (a) Three different alleles are shown at locus Avrain_23. (b) Four different alleles are shown at locus Avrain_32.

TABLE 1 Number of MLGs per mound ($n = 5$ blades) and across all mounds ($N = 40$ blades total) sampled from ‘Ewa Beach, Lagoon East during 2022 before and after allele dosage estimation using GenoDive and allelic relative fluorescence units (RFUs).

Mound #	Number of MLGs based on raw alleles	Number of MLGs after allele dosage using maximum likelihood (Miermans, 2020)	Number of MLGs after allele dosage using RFU
1	5	5	5
2	1	5	1
3	3	5	3
4	2	5	3
5	3	5	4
6	5	5	5
7	3	5	4
8	3	5	5
All mounds	25	40	30

TABLE 2 Genotypic richness (R), genotypic evenness (D^*), pareto β, and linkage disequilibrium ($\overline{r}_d$) at the three sites in which we sampled *Avrainvillea lacerata*.

	Maunalua Bay		‘Ewa Beach’, Lagoon East			‘Ewa Beach’, Kaloi
	2018	2019	2018	2019	2021	2021
	$N = 49$	$N = 49$	$N = 47$	$N = 49$	$N = 30$	$N = 30$
R	1.000	1.000	0.848	0.875	1.000	0.931
D^*	1.000	1.000	0.985	0.982	1.000	0.995
Pareto β	4.644	4.644	1.322	1.000	3.954	2.907
$\overline{r}_d$	0.029	0.004	0.204	0.168	0.028	0.130

(Table 3). For all three time points, all single locus F_{IS} were positive with the greatest variance in 2018 (Table 3; Figure 3). In 2018 and 2019, pareto β was >>0.7 but <2, whereas it was >2 in 2021 (Table 2). Lower levels of linkage disequilibrium ($\overline{r}_d$) were observed at Lagoon East in 2021 ($\overline{r}_d = 0.028$) as compared to 2018 and 2019 ($\overline{r}_d = 0.204$ and $\overline{r}_d = 0.168$, respectively; Table 2). At Lagoon East, transition of genotypic frequencies

TABLE 3 Single locus, multilocus, and variance for observed heterozygosity (H_O), unbiased expected heterozygosity (H_E), and the unbiased inbreeding coefficient (F_{IS}) at the three sites in which we sampled *Avrainvillea lacerata*.

	Maunalua Bay						'Ewa Beach', Lagoon East									'Ewa Beach', Kaloi		
	2018			2019			2018			2019			2021			2021		
	$N=49$			$N=49$			$N=47$			$N=49$			$N=30$			$N=30$		
Locus	H_O	H_E	F_{IS}	H_O	H_E	F_{IS}	H_O	H_E	F_{IS}	H_O	H_E	F_{IS}	H_O	H_E	F_{IS}	H_O	H_E	F_{IS}
Av_24	0.415	0.530	0.217	0.473	0.480	0.016	0.316	0.361	0.126	0.466	0.530	0.121	0.422	0.539	0.217	0.389	0.574	0.323
Av_23	0.507	0.513	0.012	0.602	0.571	−0.055	0.305	0.429	0.289	0.296	0.374	0.209	0.339	0.389	0.129	0.450	0.553	0.187
Av_06	0.432	0.499	0.135	0.503	0.501	−0.005	0.340	0.669	0.491	0.418	0.646	0.353	0.478	0.587	0.186	0.500	0.644	0.223
Av_39	0.517	0.569	0.092	0.537	0.554	0.030	0.404	0.688	0.412	0.415	0.680	0.390	0.544	0.642	0.152	0.517	0.676	0.236
Av_32	0.514	0.613	0.162	0.650	0.662	0.019	0.482	0.755	0.361	0.503	0.769	0.345	0.550	0.701	0.216	0.506	0.764	0.338
Av_49	0.405	0.500	0.190	0.548	0.507	−0.081	0.383	0.685	0.441	0.384	0.656	0.414	0.550	0.597	0.078	0.292	0.614	0.520
Av_36	0.643	0.749	0.128	0.755	0.750	−0.007	0.479	0.784	0.389	0.510	0.781	0.346	0.694	0.790	0.121	0.517	0.744	0.306
Multilocus	0.492	0.564	0.134	0.581	0.575	−0.011	0.387	0.624	0.380	0.428	0.634	0.325	0.511	0.607	0.157	0.453	0.653	0.306
Variance	0.044	0.049	0.028	0.056	0.058	0.010	0.032	0.160	0.090	0.033	0.121	0.069	0.076	0.096	0.016	0.042	0.039	0.073

inferred rate of clonality (c) between 2018 and 2019 fell within a credible interval superior to 98% that includes $c=[0.72, 0.86]$ with a maximum a posteriori probability estimated at $c=0.80$, between 2019 and 2021 fell within a credible interval superior to 98% that includes $c=[0.45, 0.70]$ with a maximum a posteriori probability estimated at $c=0.58$ (Figure S1b).

At 'Ewa Beach, Kaloi in 2021, both genotypic richness (R) and evenness (D^*) were high ($R=0.931$; $D^*=0.995$; Table 2). Observed heterozygosity (H_O) varied between 0.292 and 0.517 (Table 3). Expected heterozygosity (H_E) varied between 0.553 and 0.764 (Table 3). Single locus F_{IS} were all positive and displayed greater variance than Lagoon East in 2021 (Table 3; Figure 3). Pareto β was >2 (Table 2). Linkage disequilibrium ($\overline{r_d}$) was 0.130 (Table 2).

Genetic differentiation

In 2018, pairwise F_{ST} was 0.045 and ρ_{ST} was 0.095 between Maunalua Bay and 'Ewa Beach, Lagoon East. In 2019, F_{ST} was 0.025 and ρ_{ST} was 0.065 between Maunalua Bay and 'Ewa Beach, Lagoon East. In 2021, F_{ST} was 0.012 and ρ_{ST} was 0.028 between 'Ewa Beach, Lagoon East and 'Ewa Beach, Kaloi.

Within mound sampling

Across the eight mounds sampled in 'Ewa Beach, Lagoon East in 2022, we observed 30 MLGs out of 40 blades genotyped (Table 1). Mounds 1, 6, and 8 had blades that were all unique MLGs (Table 1). Mounds 3, 4, 5, and 7 had multiple MLGs, with some that we re-encountered more than once within the mound (Table 1). All blades from Mound 2 belonged to the same MLG (Table 1).

Across all mounds sampled in 2023 and for which we determined the holdfast, we did not find any blades belonging to the same MLG (Figure S2 in the Supporting Information). We observed multiple MLGs from the same putative holdfast (Figure S2). In some cases, MLGs were shared between blades sampled from different holdfasts within the same mound (e.g., Mound 2, MLG 235; Figure S2). In other cases, MLGs were shared among different mounds (e.g., Mounds 2 & 3, MLG 234; Figure S2).

DISCUSSION

We observed evidence of high clonal rates, between 0.55 to 0.80 depending on the years and sites, in *Avrainvillea lacerata*, supporting previous observations of vegetative mound growth (Peyton, 2009; Smith et al., 2002). This provides compelling genetic evidence that vegetative reproduction leads to the persistence and spread of *A. lacerata* at these two sites and likely elsewhere in the Hawaiian Archipelago (Figure 4). In contrast, the pareto β values, low linkage disequilibrium, and positive F_{IS} values hint at the occurrence of sexual reproduction. There is likely significant inbreeding, despite gametes not being observed to date. When conducting within-mound analyses, we observed evidence of what Olsen-Stojkovich (1979) referred to as "grafting." Below, we discuss the ecological and evolutionary consequences of these findings and how they might contribute to the invasive potential of *A. lacerata* in Hawaiʻi.

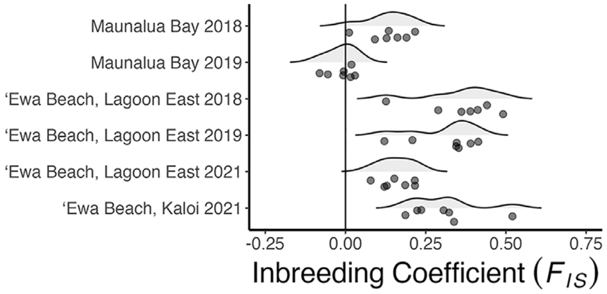

FIGURE 3 Density plots of single-locus values of the inbreeding coefficient (F_{IS}) for each site from which *Avrainvillea lacerata* was sampled in 2018, 2019, and 2021. The line at $F_{IS}=0$ shows Hardy–Weinberg proportions. Single locus F_{IS} estimates are shown as points under the respective density plots ($N=7$ loci).

The reproductive system of *Avrainvillea lacerata*

Asexual reproduction has been assumed for nuisance red algal species in the Hawaiian Archipelago, including for *Acanthophora spicifera* (Smith et al., 2002), *Gracilaria salicornia* (Smith et al., 2002, 2004), *Hypnea musciformis* (Smith et al., 2002), and *Kappaphycus alvarezii* (Conklin & Smith, 2005; Smith et al., 2002), but has only been confirmed with population genetic data in *Chondria tumulosa* (Williams et al., 2024). Here, we show vegetative reproduction has played an important role in the spread of the green alga *Avrainvillea lacerata* in Hawaiʻi. Overall, our predictions were supported, such that vegetative reproduction was greater at ʻEwa Beach Lagoon East as compared to the site in Manaulua Bay. It is unclear whether clonal rates are greater in Hawaiʻi than in the native range (given that the native range is unknown) as has been documented in other macroalgae (e.g., *Caulerpa taxifolia*, Arnaud-Haond et al., 2017; *Gracilaria vermiculophylla*, Krueger-Hadfield et al., 2016). Future sampling throughout the extant range of *A. lacerata* is required to determine whether the prevailing reproductive mode has shifted following the invasion of the Hawaiian Archipelago.

Our population genetic data also hint that sexual reproduction is occurring (Figure 4). To our knowledge, no reproductive *Avrainvillea lacerata* blades have been observed in Hawaiʻi. Sexual reproduction is thought to be

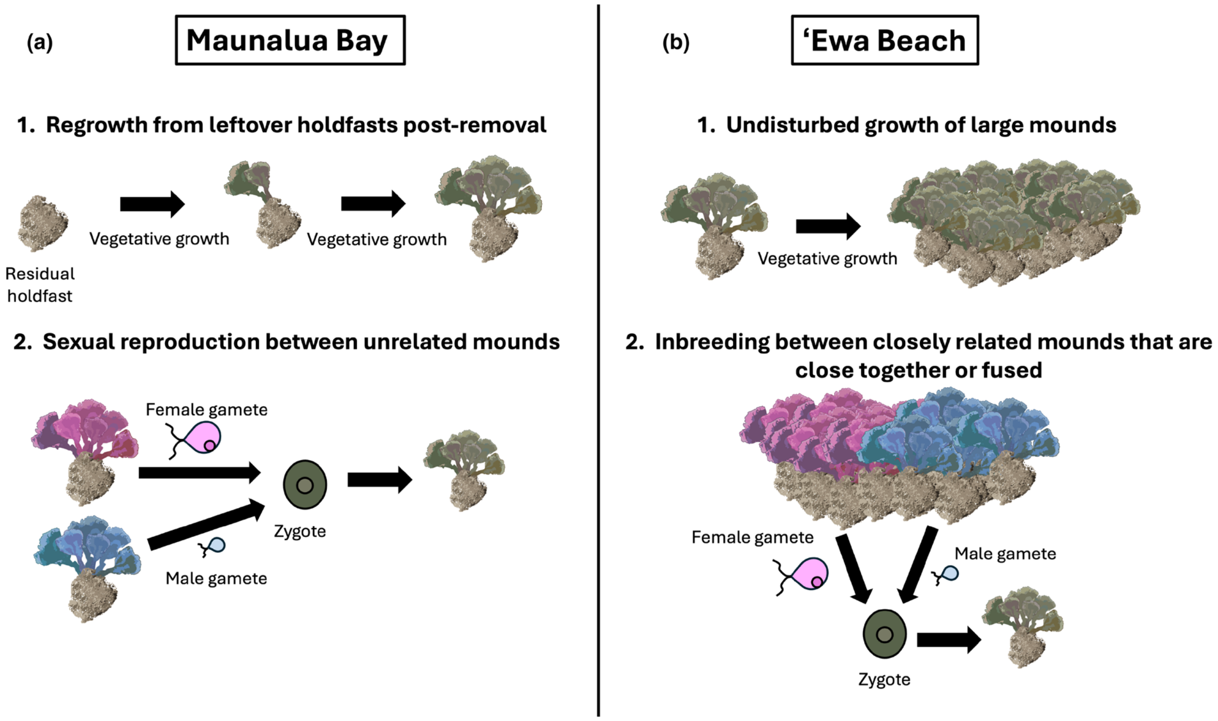

FIGURE 4 Hypothesized reproductive system of *Avrainvillea lacerata* at (a) Maunalua Bay and (b) ʻEwa Beach based on population genetic summary statistics. Pink blades represent female thalli. Blue blades represent male thalli. The sex of green blades is not specified. Drawing of gametes are based on published descriptions of gametes in siphonous green algae (see Clifton, 2013; Clifton & Clifton, 1999).

holocarpic (Clifton, 1997, 2013; Clifton & Clifton, 1999; Hillis-Colinvaux, 1984; Olsen-Stojkovich, 1985), rendering observations challenging, particularly in an uncalcified alga where there is no remnant features following gamete release. Furthermore, gamete production and release in holocarpic taxa can occur rapidly. For instance, in *Halimeda* spp., there is about a 36-h timeframe from when a thallus becomes reproductive to when it releases gametes (Clifton & Clifton, 1999). If *A. lacerata* is holocarpic, reproduction could easily be missed if not observed during the narrow timeframe in which it may occur.

The positive single locus F_{IS} values hint that inbreeding may be common when gametes are exchanged, particularly at the ʻEwa Beach sites (Figure 4b). In the Bryopsidales, gametes are negatively buoyant (Clifton, 2013). Therefore, fertilization likely occurs close to the substrate or via settlement onto other nearby *Avrainvillea lacerata* mounds. This phenomenon likely favors mating among nearby *A. lacerata* blade limiting gene dispersal, such as observed in some corals (see as an example, Combosch & Vollmer, 2011). Inbreeding likely plays a larger role at the ʻEwa Beach sites than at Maunalua Bay. This may be explained by the regular manual removal of *A. lacerata* in Maunalua Bay by volunteer groups (Mālama Maunalua, https://www.malamamaunalua.org/). Thus, the distribution of *A. lacerata* clumps is smaller and patchier at the Maunalua Bay site than at the ʻEwa Beach sites, possibly providing less opportunity for inbreeding (Figure 4a). Future population genetic studies on *A. lacerata* should include greater sampling density within and among sites as well as across the genome (e.g., single nucleotide polymorphisms, SNPs). These data should be paired with phenology studies to determine reproductive patterns (see as an example Krueger-Hadfield et al., 2023).

The role of polyploidy influencing patterns of genetic and genotypic diversity

Genetic differentiation as measured by allele frequencies (F_{ST}) was greater between the Maunalua Bay site and ʻEwa Beach sites than between *Avrainvillea lacerata* from the two sites at ʻEwa Beach. This also held true for ρ_{ST}, which also measures genetic differentiation but is independent of ploidy and the variable inheritance patterns across the loci of polyploid organisms (Meirmans & Liu, 2018; Ronfort et al., 1998). Therefore, it is possible that either repeated introductions or introduction from a genetically diverse source population may have played a role in establishing these patterns of genetic differentiation among sites in which *A. lacerata* is now located in Hawaiʻi. Alternatively, *Avrainvillea* is not thought to have long- distance dispersal strategies (Littler & Littler, 1992), and there could be a lack of gene flow between Maunalua Bay and ʻEwa Beach that further leads to genetic differentiation. It is curious that genetic differentiation between *A. lacerata* at Maunalua Bay and ʻEwa Beach seemed to decrease from 2018 to 2019. This might suggest that gene flow is occurring between these sites. However, Maunalua Bay and ʻEwa Beach are the only intertidal sites for which we have genetic data currently. Additionally, *A. lacerata* also has a substantial presence in the mesophotic regions around Oʻahu (Spalding, 2012), and gene flow could be occurring between the mesophotic and intertidal zones. Therefore, more work needs to be done including a much broader sampling scheme in Hawaiʻi in order to more confidently assess genetic differentiation among *A. lacertata* in Hawaiʻi.

Genetic diversity (H_E) was comparable between the site at Maunalua Bay and ʻEwa Beach, Lagoon East in 2018 and 2019. However, differences in H_E could be minimal, as estimates of H_E are independent of the number of alleles and less sensitive to samples sizes from collection protocols or bottlenecks (Allendorf & Luikart, 2007). In contrast, genotypic diversity is more strongly influenced by sample size and population bottlenecks, which could cause abrupt changes in allele frequencies (Allendorf & Luikart, 2007). This might be why differences in genotypic richness were more pronounced between the Maunalua Bay and ʻEwa Beach sites in which there was greater genotypic richness and pareto β at Maunalua Bay. Greater genotypic richness at the Maunalua site perhaps is not surprising given that the clonal rate was lower than at ʻEwa Beach, Lagoon East. Additionally, it is possible that manual removal at Maunalua Bay provided space for new recruits to settle as adult thalli from nearby sites or zygotes produced from sexual reproduction.

Surprisingly, we observed greater genotypic richness than would be expected if asexual reproduction were dominant at a site. For example, in the red algal genus *Gracilaria* (reviewed in Krueger-Hadfield et al., 2021), genotypic richness was low in many sites dominated by asexual reproduction in the form of thallus fragmentation. We analyzed our data assuming *Avrainvillea lacerata* is tetraploid. However, it is possible that some blades could be diploid or triploid because 141 blades out of 321 (~44%) had a maximum of two alleles and 125 blades (~39%) had a maximum of three alleles across all loci, respectively. Yet, the likelihood of the distributions of allele frequencies at each site suggest at least tetraploidy. Ploidy levels should be assessed using complementary methods, such as micro spectrophotometry or flow cytometry, as well as methods that would enable the determination of the number of copies of an allele (e.g., SNP genotyping).

We attempted to confirm ploidy levels using flow cytometry and fluorescent microscopy (see Appendix S1, Figures S3, S4 in the Supporting Information); however, we were limited in our ability to adapt a working protocol due to the lack of fresh blades, which are preferred for DNA content analysis (Doležel et al., 2007). With the limited amount of fresh blades at our disposal, we were unable to isolate nuclei for flow cytometry using the buffers tested in this study. We had the most success in isolating nuclei and using fluorescence microscopy and DAPI using blades preserved in silica gel (Figure S4), although the images were in poor resolution due to damage to the nuclei likely caused by silica gel preservation. Other studies have had similar difficulties with bryopsidalean algae (see Varela-Álvarez et al., 2012). With access to fresh blades, DAPI staining may be the best method for DNA content estimation in *Avrainvillea lacerata*. However, when nuclei are successfully isolated, a second problem arises, as we do not have a reference of known ploidy, such as gametes. DAPI fluorescence intensity values must be compared to a reference such as this for accurate determination of ploidy (Doležel et al., 2007). Unfortunately, we are unable to do this with *A. lacerata* in Hawaiʻi, as reproductive blades have yet to be documented (Smith et al., 2002).

It would not be surprising for *Avrainvillea lacerata* to be polyploid because polyploidy is common across the green algae, including within the Bryopsidales (Kapraun, 1994, 2005; Kapraun & Nguyen, 1994; Kapraun & Shipley, 1990; Varela-Álvarez et al., 2012) as well as in some reds (e.g., *Porphyra*, Varela-Álvarez et al., 2018) and browns (e.g., *Fucus*, Preston et al., 2022). Polyploidy could be an important factor in the success of *A. lacerata* because it is often correlated with increased genotypic diversity (Parisod et al., 2010; Soltis & Soltis, 1999; te Beest et al., 2012). For example, in studies of mixed ploidy populations of angiosperms, tetraploid plants had increased levels of genotypic diversity relative to diploid plants of the same species (Baldwin & Husband, 2013; Brown & Young, 2000). This has been observed in macroalgae as well; high genotypic diversity was detected in invasive polyploid populations of *Asparagopsis taxiformis* (Andreakis et al., 2009). Polyploidy itself may incur greater genotypic diversity as the larger number of independent alleles at each locus allows for larger effective population sizes than a similar sized population of diploids (Luttikhuizen et al., 2007; Meirmans & Van Tienderen, 2013). Therefore, polyploids can harbor a greater amount of heterozygosity that is lost at a slower rate than in diploids (Moody et al., 1993), and high genotypic diversity is maintained, despite high clonal rates (e.g., Quarin et al., 2001). Moreover, additional genomic copies increase the masking of deleterious mutations (Comai, 2005; Parisod et al., 2010) and lower inbreeding depression relative to diploid populations, which could be beneficial when populations might be forced to undergo inbreeding after a bottleneck event, such as range expansion (Ronfort et al., 1998). Therefore, polyploids may possess increased genomic flexibility, allowing them to adapt and persist within new environments (Parisod et al., 2010).

What is an “individual”?

Our data provide strong evidence for polyploidy in *Avrainvillea lacerata*, but currently, there are still data missing with which to provide evidence on what processes generate polyploidy. For the purposes of our study, we considered autopolyploidization (full genome duplication) as the most parsimonious explanation of the allele distributions we observed at all the loci we analyzed. Our results could have originated from gene duplication, but in this case, only a few of the markers seem to match a tetraploid distribution. Though we hypothesize that *A. lacerata* is tetraploid, we cannot fully disentangle this from the possibility that the seemingly polyploid genotypes could have resulted from coalescence or grafting between multiple diploid individuals.

Coalescence, or the fusion between two or more individuals generating one entity composed of multiple genotypes (e.g., a chimera), has mostly been described in red algae, where some authors have suggested it is widespread and occurs naturally in situ (Santelices et al., 1999, 2003). It seems to incur numerous ecological benefits, such as increased survival (Santelices et al., 1999, 2003). Coalescence was described in another bryopsidalean green alga, *Codium* sp., when González and Santelices (2008) observed that separate, but neighboring crusts often fused together. This was supported after screening thalli with a chloroplast gene (Trn-Gly) when single crusts were shown to include multiple haplotypes (González & Santelices, 2008).

Olsen-Stojkovich (1979) described “grafting” in *Avrainvillea* spp. in which juveniles may grow fused together. This is perhaps not surprising given the lateral spread of subterranean holdfasts (Littler et al., 2004, 2005; Peyton, 2009). Grafting is a term often used in horticultural practices to describe when two different parts of a plant are joined together to fuse and continue their growth in such a way that intercellular connections form (Melnyk & Meyerowitz, 2015). These techniques were inspired by the processes of natural grafting when stems or roots of plants attach and fuse together (Mudge et al., 2009). Grafting is used to adjust the size of plants, improve stress tolerance, or allow plants to grow in different environments (Melnyk & Meyerowitz, 2015). This definition of grafting differs from the coalescence described in *Codium* and other red algae. Rather, coalesced filaments of separate *Codium* crusts grew intermixed without establishing intercellular connections with one another (González & Santelices, 2008). Likewise, *Chondrus crispus* forms large holdfasts that can have female gametophytes, male gametophytes, and tetrasporophytes, but to date no chimeric fronds have been genotyped (see Krueger-Hadfield, 2011; Krueger-Hadfield et al., 2013). Instead, holdfasts may coalesce, enhancing survival, without exchanging genetic material (Krueger-Hadfield, 2011).

Currently, it is unclear whether fusions between *Avrainvillea* thalli are composed of intercellular connections or intermixed filaments, but we cannot discount the possibility of either.

We observed multiple genotypes within single mounds, including prior to estimating allele dosage (i.e., from raw alleles in fragment analysis traces), in *Avrainvillea lacerata*. These results suggest *A. lacerata* might be a mosaic structural unit (see discussion in Clarke, 2012). Moreover, we observed distinct genotypes when blades clearly shared the same holdfast. Until we have an independent measure of ploidy, we cannot fully discount the possibility that some blades are chimeras following the "grafting" described by Olsen-Stojkovich (1979) and the subsequent coalescence of siphons. However, the distribution of allelic RFUs and allele frequencies corresponded to the multinomial sampling of a standard tetraploid. This would have not been the case in chimeras that would have shown all the possible distributions of RFUs depending on the relative importance of each different genetic line. This suggests that most thalli are at least tetraploid, if not a higher ploidy level. Ploidy for *A. lacerata* needs to be verified with alternative methods to support this hypothesis. Additionally, future studies need to use field experimentation, population genetics, and histological studies to confirm the coalescence of siphons in *Avrainvillea*.

Alternatively, genotypic variation within a mound does not necessarily mean that the mound is composed of multiple genets. One major misconception in distinguishing individuals lies in the assumption that all parts that make up the individual genet, including ramets and modules of modular organisms, are genetically homogenous (Scrosati, 2002). In clonal organisms that undergo indeterminate growth and can have relatively long lifespans, such as macroalgae, somatic mutations occur during vegetative growth resulting in a mosaic of genotypes within a single genet (Gill et al., 1995). This may be especially relevant in microsatellites, which generally have higher mutation rates (Anmarkrud et al., 2008). Ramets produced from the same genet could display genetic variation, generating multiple levels upon which natural selection could potentially act (Clarke, 2012; Tuomi & Vuorisalo, 1989). If we define a genet as a distinct MLG, it is possible that we might erroneously assume two *Avrainvillea lacerata* blades with different MLGs as being from separate genets. Instead, it is possible that they could have come from the same zygote and that somatic mutations have generated a genetic mosaic over time. Thus, it is possible that the genotypic heterogeneity within *Avrainvillea* mounds might not be the result of coalescence of different "individuals" but rather an accumulation of somatic mutations within a single genet during vegetative growth of a mound.

Past removal efforts and critical next steps for the management of *Avrainvillea lacerata*

Due to its rapid spread (Smith et al., 2002; Veazey et al., 2019; Wade, 2019) and negative effects on native seagrass beds (Peyton, 2009) and intertidal benches (Foster et al., 2019), finding a way to effectively combat the spread of *Avrainvillea lacerata* is a high priority for local management agencies. Understanding the basic biology of *A. lacerata* is essential; from it, it will be possible to develop management strategies, including an understanding of the spread of this alga (Zamora et al., 1989). *Avrainvillea lacerata* primarily spreads via vegetative growth of mounds, which suggests the total removal of biomass would be critical for effective management. Non-profit organizations, such as Mālama Maunalua, continue to recruit volunteers for manual removal of *A. lacerata* in Maunalua Bay (https://www.malamamaunalua.org/). In 2010, the U.S. government provided a large award that allowed a massive restoration project at Maunalua Bay (Kittinger et al., 2016). During this removal effort, 1.32 million kg of non-native macroalgae, including *A. lacerata*, were removed by hand and transported to a composting site (Kittinger et al., 2016). This removal effort did appear to have some short-term success, as the community composition of cleared areas seemed to resemble those of native plots (Longenecker et al., 2011). Large-scale manual removal efforts can substantially reduce the standing biomass, but holdfasts left behind can result in regrowth via vegetative spread. If manual removal is used, any fragments left behind need to be small enough to hinder lateral spread of holdfasts and blade production (see discussion in Bonnett et al., 2014). Smith et al. (2002) showed that fragments of *A. lacerata* holdfasts that were at least three centimeters showed the highest success for regrowth. Therefore, future experiments should examine whether fragments smaller than three centimeters are able to regrow in the field in order to confirm the maximum fragment size that would hinder regrowth of *A. lacerata*. This is especially critical as it is likely impossible to remove all fragments of *A. lacerata*. Our data from Manaulua Bay suggest the regrowth of some genotypes that were left behind as well as the potential of new recruitment from juveniles or adults.

Large-scale chemical treatment could potentially overcome the issue of leaving behind thalli by causing mortality of entire *Avrainvillea lacerata* mounds. Van De Verg and Smith (2022) showed that injecting *A. lacerata* mounds with small amounts hydrogen peroxide decreased photosynthetic efficiency, potentially leading to a decrease in biomass. Although the immediate effects of injection seem promising, the long-term effects of this form of control remain to be tested, and large-scale application appears impractical. For instance, chemical treatments aiming to control invasive *Caulerpa taxifolia* have been shown to be harmful to animal life within the treatment area (Williams & Schroeder, 2004). This is particularly pertinent given the faunal community observed within *A. lacerata* holdfasts, which is composed of native species (Magalhães & Bailey-Brock, 2014). It is also unclear whether this treatment can be applied to other sites where *A. lacerata* is present. Experimentation of this method has taken place in the more protected, soft sediment habitat of Maunalua Bay (Van De Verg & Smith, 2022), but the logistics of effectively applying this across the diverse habitats *A. lacerata* inhabits, including mesophotic regions (Spalding et al., 2019) and more wave exposed rocky shorelines (Foster et al., 2019) where *A. lacerata* is located, will likely be limited.

Prior to this study, sexual reproduction in *Avrainvillea lacerata* in Hawai'i was not considered based on the anecdotal absence of observable gametangia. Additional research is needed to understand sexual reproduction in this alga. For example, phenology studies are required to examine when and how often sexual reproduction occurs and the environmental triggers for gametogenesis. Phenology studies of bryopsidalean macroalgae have occurred in Caribbean coral reefs (Clifton & Clifton, 1999), but these studies did not include members of the genus *Avrainvillea*. However, for the genera that were studied (i.e., *Caulerpa*, *Halimeda*, *Penicillus*, *Rhipocephalus*, and *Udotea*) several instances of sexual reproduction occurred throughout the seasonal peak for reproductive activity (Clifton & Clifton, 1999). Light levels and water temperature seemed to have some influence on the timing of reproductive activity, as the seasonal peak of reproduction was shown to correspond with the shift from the dry season to the wet season, a period of increased solar radiation (Clifton & Clifton, 1999). If the timeframe and environmental conditions of sexual reproduction for *A. lacerata* are determined, then biomass removal efforts could be prioritized before thalli become reproductive. This is similar to the logic behind suggestions for management in controlling the spread of invasive angiosperms where sexual reproduction and seed dispersal are considered to be a mechanism for spread (Bonnett et al., 2014). The dispersal of sexual propagules, including gametes and zygotes, could be one of the mechanisms that has allowed *A. lacerata* to spread across the southern shores of O'ahu. If this is the case, then sexual reproduction at nearby sites could result in the dispersal and recruitment of new individuals into sites that have been cleared.

Understanding the dispersal of *Avrainvillea lacerata* in Hawai'i is crucial for minimizing or preventing future movement of invasive species (Wilson et al., 2009). If *A. lacerata* can disperse across localities, removal efforts will need to expand beyond Maunalua Bay. By understanding the connectivity of *A. lacerata* at sites across the Hawaiian Archipelago, we can aid in the development of better management strategies. For example, angiosperms with long-distance dispersal mechanisms (i.e., wind-based dispersal) display less genetic differentiation, and therefore greater connectivity, among sites than plants with shorter dispersal distances (Govindaraju, 1988). We assessed connectivity between Maunalua Bay and 'Ewa Beach by estimating pairwise genetic differentiation based on allele identity (F_{ST}) and allele size (ρ_{ST}). Based on both measurements of genetic differentiation, the level of divergence was greater between Maunalua Bay and 'Ewa Beach, Lagoon East than it was between the two sites at 'Ewa Beach. This suggests some isolation by distance, but more sites should be sampled to sufficiently test for a correlation between genetic and geographical distances. It is important to emphasize that this differentiation may also be due to separate colonization events if *A. lacerata* is indeed non-native to Hawai'i. The connectivity of *A. lacerata* among sites in Hawai'i should be explored further by including broader sampling along the shores of O'ahu, at the neighboring islands of Maui and Kauai, and across its broad depth gradient (intertidal to mesophotic depths) Then, the combined understanding of reproduction and connectivity can aid managers in developing the most effective management plans for a given site and more widely throughout the Hawaiian Archipelago.

Finally, the origin of *Avrainvillea lacerata* in Hawai'i is unresolved. The alga may have been introduced from Japan based on the close phylogenetic grouping of *A. lacerata* from Hawai'i with specimens from Japan (Wade, 2019). Alternatively, due to its high abundance in the mesophotic as well as the absence of mesophotic studies prior to the initial collection of *A. lacerata*, it is possible that *A. lacerata* is native in Hawai'i and existed in the mesophotic prior to invading the intertidal (Spalding, 2012). It is also possible that *A. lacerata* was introduced from Guam. A different Hawaiian invader, *Acanthophora spicifera*, was introduced to Hawai'i through increased shipping traffic between Honolulu and Guam during World War II (Doty, 1961). It is plausible that *A. lacerata* could have been introduced in a similar manner and, perhaps, invaded the mesophotic prior to invading the intertidal in the 1980s. Future work needs to include sampling *A. lacerata* broadly across the Indo-Pacific and the Hawaiian Archipelago to determine the source populations of *A. lacerata* in Hawai'i (see, as example, Krueger-Hadfield et al., 2017). If the source of the *A. lacerata* invasion can be determined, comparisons can then be made to understand how the niche of *A. lacerata* may have shifted in the invasion of the Hawaiian Islands. Invasion studies often compare the non-native range to the entirety of the native range (see Petitpierre et al., 2012). However, not all native sites contribute equally to an invasion, thereby leading to an underestimation of adaptation, inaccurate inferences of the direction of evolutionary change, or both. For example, when comparing the niche of non-native *Gracilaria vermiculophylla* thalli to the entirety of the native range, it seemed that non-native thalli experienced niche expansion into colder temperatures (Sotka et al., 2018). However, when compared to the source populations, non-native thalli expanded into warmer habitats (Sotka et al., 2018). Therefore, identifying the source populations will improve the ability to detect the evolutionary shifts that may have allowed *A. lacerata* to proliferate in Hawai‘i.

CONCLUSIONS AND FUTURE DIRECTIONS

The microsatellite data from this study highlight the importance of the most basic biological information, such as the ploidy level, to confidently analyze population genetic data. Analyzing DNA content in relation to a reference of known ploidy will be a critical next step for further exploration of the population genetic structure of *Avrainvillea lacerata* in Hawai‘i and throughout its extant range. Recent advances in genotyping methods that exploit increased sequencing depth with low error rates have unlocked this possibility for population genetic analyses of polyploid species (e.g., Hi-Plex genotyping with SNPs; Delord et al., 2018) and may be an avenue to pursue with which to confidently assign allele dosage (see Stoeckel et al., 2024).

The rate of clonality in *Avrainvillea lacerata* in Hawai‘i is high. Asexual reproduction plays an important role in the spread of many nuisance macroalgae in the Hawaiian Archipelago (Conklin & Smith, 2005; Smith et al., 2002, 2004; Williams et al., 2024). Our genetic data and previous ecological observations at Maunalua Bay suggest that removal efforts may not remove all of the holdfast material, although the downstream consequences of this are unclear. The microsatellite loci used in this study will facilitate future studies that are necessary for understanding the invasive history of this alga. More broadly, this work adds to the body of literature for green algae in which we know very little regarding the population genetic patterns (Krueger-Hadfield et al., 2021), despite the work of Otto and Marks (1996) highlighting the role green algae can play in unlocking the secrets of eukaryotic sex.

AUTHOR CONTRIBUTIONS

Brinkley M. Thornton: Data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Heather L. Spalding:** Conceptualization (equal); funding acquisition (equal); investigation (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Solenn Stoeckel:** Formal analysis (equal); funding acquisition (supporting); investigation (equal); methodology (equal); resources (equal); software (lead); writing – review and editing (equal). **Melissa L. Harris:** Investigation (equal); methodology (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Rachael M. Wade:** Investigation (supporting); writing – review and editing (equal). **Stacy A. Krueger-Hadfield:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (lead); investigation (equal); methodology (equal); project administration (lead); resources (lead); software (supporting); supervision (lead); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

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DATA AVAILABILITY STATEMENT

Genetic data and code are provided on Zenodo (10.5281/zenodo.13730344).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1: Methods.

Figure S1. Posterior probabilities of rates of clonality using changes of genotype frequencies between temporal samples (CEMP). (a) CEMP Plot for Maunalua Bay between 2018 and 2019. (b) CEMP Plot for ʻEwa Beach, Lagoon East between 2018 and 2019 (black posterior distribution, dotted line) and 2019–2021 (dark gray posterior distribution, dashed line).

Figure S2. Breakdown of MLGs in mounds sampled from ʻEwa Beach, Lagoon East during 2023. MLG numbers were assigned to individual blades based on their MLG by GenAPoPop (Stoeckel et al., 2024).

Figure S3. Flow cytometry data for *Arabidopsis thaliana* (a) and *Avrainvillea lacerata* (b) lysed using LB01. (a) Left graphs: forward scatter versus propidium iodide (PI) area in log form for the total dissociated tissue population. Center graphs: histograms demonstrating gating strategy for PI-negative (PI-AREA−) and PI-positive (PI-AREA+) staining on log scale (center graphs). Right graphs: histograms of PI-positive staining on linear scale demonstrating the direct relationship between fluorescence intensity and DNA content. Three prominent PI+ DNA peaks are observed in *Arabidopsis thaliana* cell lysis preparations. No distinct PI+ peaks are visible in *Avrainvillea lacerate* cell lysis preparations. Peaks represent nuclei in different stages of the cell cycle. $2C = G_1$; $4C = G_2$; $8C =$ somatic polyploidy.

Figure S4. (a) Brightfield image of *Avrainvillea lacerata* siphon where nuclei were isolated. Scalebar: 100 μm. (b) Fluorescent imaging of same *A. lacerata* siphon showing DAPI stained nuclei (region circled in white). Nuclei are indicated by oval to circular shaped regions of homogenous DAPI fluorescence with some nuclei showing an apparent nucleolus. Scalebar: 100 μm.

Table S1. Microsatellite locus information for *Avrainvillea lacerata* in Hawaiʻi. Locus name, repeat motif, expected size, forward and reverse oligo sequences, agarose gel amplification profile, forward oligo fluorochrome, and fragment analysis (FA) amplification profile. Note fluorochrome and FA profile columns are only for loci tested on the capillary sequencer.

Table S2. *Avrainvillea lacerata* microsatellite loci. Multiplex primer concentrations and fluorochrome.

Table S3. The proportion of more than two alleles at *Avrainvillea lacerata* microsatellite loci across all thalli genotyped ($N=321$).

Table S4. If we consider this blade genotyped 120,124 with a respective RFU $a=28$ and $b=6$. We can now compute the posterior probability following our development above. The multinomial coefficient at this locus in this individual is $\frac{(a+b)!}{a!b!}=1344904$. For this locus in this sample, the maximum posterior probability of 99.92% indicates [120, 120, 120, 124] as the most probable genotype considering the measured RFU = [28, 6].

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